Structure-Function Studies of the Proprotein Convertases: The Pro- and P-domains

By

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Abstract

The proprotein convertases (PCs) are involved in the activation of a wide variety of precursors via limited proteolysis at either single or paired basic residues, a crucial regulatory process in both normal and disease states. Seven members of this family were identified including furin, PC1, PC2, PC4, PACE4, PC5 and PC7. All of which exhibit a signal peptide, a prosegment, a catalytic domain, a P-domain and a specific C-terminal segment. The present work concentrated on the characterization of two structural elements, namely the prosegment and the P-domain, which are critical for enzymatic function and cellular trafficking. We examined the biosynthesis, functional activity and cellular localization of two PACE4 isoforms generated by differential splicing, the full length PACE4-A and the C-terminally truncated PACE4-CS that lacks 11 amino acids at the end of its chaperone-like P-domain. Cellular expression demonstrated that PACE4-A codes for a functional secretable enzyme capable of cleaving pro7B2 into 7B2. However, PACE4-CS is not secreted and remains in the endoplasmic reticulum as an inactive zymogen form, therefore emphasizing the importance of the integrity of the P-domain. The prosegment is presumed to act both as an intramolecular chaperone and an inhibitor of its parent enzyme. We purified recombinant prosegments of furin (pFurin) and PC7 (pPC7) from bacteria. The inhibitory potencies and selectivities of the prosegments were tested *in vitro* and *ex vivo*. The pFurin and pPC7 are potent inhibitors (IC_{50} low nM) of their parent enzymes. Whereas pPC7 is more selective than pFurin. Most of the inhibitory potency seems to reside at the C-terminal region immediately preceding the primary cleavage site of the prosegment with the P1 Arg playing a critical role. Furthermore, overexpression of prosegments in cell lines efficiently blocked precursor processing of the neurotrophins NGF and BDNF. For the first time our results showed that PC prosegments expressed *ex vivo* as independent domains could act *in trans* to inhibit precursor maturation by intracellular PCs. In conclusion, the prosegment and the P-domain of PCs are critical for PC zymogen activation, enzymatic activity and intracellular trafficking. The prosegment study also presents a novel enzyme silencing strategy, which provides a novel approach to the treatment of a variety of pathologies.

Résumé

Les proprotéines convertases (PCs) sont impliquées dans l'activation d'une panoplie de précurseurs via la protéolyse limitée à des résidus basiques ou dibasiques, un processus essentiel à l'homéostasie ainsi que dans le développement de maladies. Sept membres de cette famille ont été identifiés dont furine, PC1, PC2, PC4, PACE4, PC5 et PC7. Tous possèdent un peptide signal, un prosegment, un domaine catalytique, un P-domaine et un segment C-terminal spécifique. Le présent travail se concentre sur la caractérisation du prosegment et du P-domaine des PCs qui sont critiques pour leur activité enzymatique et leur traffic cellulaire. Nous avons examiné la biosynthèse, l'activité fonctionnelle et localisation cellulaire de deux isoformes de PACE4 générés par épissage alternatif, le PACE4 pleine longueur et la forme tronquée en C-terminal PACE4CS, qui manque 11 acides aminés à la fin de son P-domaine. La surexpression cellulaire de PACE4-A produit un enzyme fonctionnelle, sécrétée et capable de cliver le pro7B2 en 7B2. Cependant, PACE4-CS n'est pas sécrétée et reste sous forme de zymogène inactif démontrant l'importance de l'intégrité du P-domaine. Le prosegment agit possiblement comme chaperon intramoléculaire et inhibiteur de l'enzyme parentale. Nous avons purifié les prosegments recombinants de furine (pFurine) et PC7 (pPC7) de bactéries. Le potentiel d'inhibition et la sélectivité des prosegments ont été testés *in vitro* et *ex vivo*. Le pFurine et le pPC7 sont des inhibiteurs efficaces (IC_{50} de l'ordre du nM) de leur enzyme parentale. Le pPC7 montre une plus grande sélectivité que le pFurine. La majorité du potentiel inhibiteur semble résider dans la région C-terminale précédant immédiatement le site de coupure primaire du prosegment avec l'Arg en P1 jouant un rôle critique. De plus, la surexpression de prosegments dans des lignées cellulaires bloquent efficacement la maturation des neurothrophines NGF et BDNF. Pour la première fois, nos résultats démontrent que les prosegments de PCs exprimés comme domaine indépendant, *ex vivo,* peuvent agir en *trans* pour inhiber la maturation effectuée par des PCs intracellulairement. En conclusion, le prosegment et le P-domaine des PCs sont critiques dans l'activation des PCs sous forme de zymogènes, pour leur activité enzymatique et leur traffic intracellulaire. L'expression cellulaire du prosegment

représente une nouvelle approche afin d'inhiber les convertases et pour le traitement d'une variété de pathologie.

Preface

This thesis is submitted to the McGill University Faculty of Graduate studies and Research. This thesis is presented according to manuscript-based thesis format:

> Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis.
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> The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.
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> The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: a table of contents; an abstract in English and French; an introduction which clearly states the rational and objectives of the research; a comprehensive review of the literature (in addition to that covered in the introduction to each paper); a final conclusion and summary.
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The first chapter is a general introduction and literature review of the relevant work of the research project. Chapter 2 and 3 are the experimental results of two published papers, each of which has its own abstract, introduction, materials and methods, results and discussion as well as reference list. Connecting texts are included in each preface of the chapter. Chapter 4 is a general discussion. Chapter 5 presents the claims for original research. Two other published papers, in which I participated as a co-author, are included in appendices I, II. At the end, the whole reference list is attached for the reader's convenience. The papers included are the following:

1. The prosegments of furin and PC7 as potent inhibitors of proprotein convertases: *in vitro* and *ex vivo* assessment of their efficacy and selectivity. By Mei Zhong, Jon Scott Munzer, Ajoy Basak, Suzanne Benjannet, Seyed J. Mowla, Etienne Decroly, Michel Chrétien, and Nabil G. Seidah. In Journal of Biological Chemistry 1999, 276:33913-33920.
2. Functional analysis of human PACE4-A and PACE4-C isoforms: identification of a new PACE4-CS isoform. By Mei Zhong, Suzanne Benjannet, Claude Lazure, Scott Munzer, Nabil G. Seidah. In FEBS letters 1996, 396:31-36.
3. *In vitro* characterization of the novel proprotein convertase PC7. By Jon Scott Munzer, Ajoy Basak, Mei Zhong, Aida Mamarbassi, Josée Hamelin, Diane Savaria, Claude Lazure, Suzanne Benjannet, Michel Chrétien, and Nabil G. Seidah. In Journal of Biological Chemistry 1997, 272:19672-19681.
4. Mammalian subtilisin/kexin isozyme SKI-1: A widely expressed proprotein convertase with a unique cleavage specificity and cellular localization. By Nabil G. Seidah, Seyed J. Mowla, Josée Hamelin, Aida M. Mamarbassi, Suzanne Benjannet, Barry B. Toure, Ajoy Basak, Jon Scott Munzer, Jadwiga Marcinkiewicz, Mei Zhong,

Jean-Christophe Barale, Claude Lazure, Richard A. Murphy, Michel Chrétien and Mieczyslaw Marcinkiewicz. In Proceedings of the National Academy of Sciences of the United States of America 1999, 96:1321-1326.

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The proprotein convertases are involved in a general mechanism of processing precursors at either single or pairs of basic residues to generate bioactive proteins or peptides. This is a crucial regulatory process in both normal and disease states. The main objectives of this present work center on the characterization of the structural elements in the proprotein convertases that are critical for their enzymatic function and cellular trafficking. Most importantly, I wanted to define the structure-function of two domains, the pro- and P-domains which control the folding, zymogen activation, cellular sorting and location as well as the enzymatic activity of the selected proprotein convertases. The major hypotheses are: Structure-function analyses of the PCs will reveal not only a basic unity in their structural organization and cellular sorting motifs, but also in their distinct ability to interact with cellular or extracellular proteins which control their enzymatic activity, trafficking and intracellular localization. The present manuscript concentrates on the characterization of two of these structural domains of PCs: the pro- and the P-domain. The ultimate outcome of these studies is to rationally design specific inhibitors of PCs for biotechnology, pharmacological and clinical applications.

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List of abbreviations

α_1-AT:	alpha 1-Antitrypsin
α-MF:	alpha-mating factor
α_1-PIT:	alpha 1-Antitrypsin Pittsburgh
α_1-PDX:	alpha 1-Antitrypsin Portland
β-MSH:	β-Melanocyte Stimulating Hormone
β/γ-LPH:	β/γ- Lipotropic hormone
aa:	amino acid(s)
ACTH:	adrenal corticotrophin hormone
AMC:	7-amino-4-methylcoumarin
AP-1:	activator protein-1
BDNF:	brain derived neurotrophic factor
bp:	base pair(s)
BTMD:	before transmembrane domain
CPB/E:	carboxypeptidase B/E
CNS:	central nervous system
DMEM:	Dulbecco's modified Eagle's medium
DNA:	deoxyribonucleic acid
EGF:	epidermal growth factor
ER:	endoplasmic reticulum
Fmoc:	*N*-(9-fluorenyl)-methoxycarbonyl
fur:	*fps/fes* upstream region
GP/gp:	glycoprotein
HAs:	hemagglutinins of the influenza
HIV:	human immunodeficiency virus
hnRNA:	heteronuclear RNA
IC_{50}:	50% inhibition value
ISG:	immature secretory granules

IPTG:	isopropyl β-D-thiogalactoside
Ki:	inhibition constant
MALDI-TOF:	matrix assisted laser desorption ionization time of flight
MCA:	methylcoumarinamide
MEM:	minimal essential medium
MM:	molecular mass
MPO:	myeloperoxidase
mRNA:	messenger RNA
NGF:	nerve growth factor
nts:	nucleotides
PACE:	paired basic amino acid cleaving enzyme
PCR:	polymerase chain reaction
PCs:	proprotein (precursor) (prohormone) convertases
pfu:	plaque-forming units
pfurin:	furin prosegment
pI:	isoelectric point
PNS:	peripheral nervous system
POMC:	proopiomelanocortin
pPC7:	PC7 prosegment
ppfurin:	furin preprosegment
ppPC7:	PC7 preprosegment
proPTH-RP:	proparathyroid hormone-related peptide
PVDF:	polyvinylidene fluoride
RNA:	ribonucleic acid
RT-PCR:	reverse transcriptase polymerase chain reaction
SKI-1:	subtilisin-kexin isozyme-1
SREBP:	sterol regulatory element-binding protein
TGN:	*trans*-Golgi network
TIPS:	triisopropyl silane
VV:	vaccinia virus

CHAPTER 1

Introduction and Literature Review

1. Proprotein Convertases

1.1. Identification of proprotein convertases

Simultaneously in the year of 1967, two research groups, using completely different methods, independently proposed the hypothesis that peptide hormones are synthesized intracellularly as large inactive precursors that are posttranslationally converted into smaller biologically active form (Chrétien & Li, 1967; Steiner *et al.*, 1967). Chrétien and Li proposed that precursors are processed at pairs of basic residues based on their comparison of the amino acid sequences of β-lipotropic hormone (β-LPH), γ-LPH and β-melanocyte-stimulating hormone (β-MSH). They suggested that γ-LPH and β-MSH are parts of β-LPH, and basic pairs of Lys-Lys and Lys-Arg are present at the putative cleavage sites. Independently, Steiner *et al.* showed that the two-chain mature form of insulin is produced via the cleavage of a single chain precursor, proinsulin using delicate pulse-chase experiments. Later on, the cleavage sites of proinsulin were revealed to contain Lys-Arg and Arg-Arg determined by sequencing analysis (Chance *et al.*, 1968). Therefore, in both β-LPH and proinsulin, precursor processing involved cleavage at pairs of basic residues. Over the years the concept that a precursor undergoes limited proteolysis by one or more processing enzymes to release the active component(s) was found to be applicable not only to hormones and neuropeptides, but also to many growth factors, receptors, adhesion molecules, plasma proteases, matrix metalloproteinases, cell surface glycoproteins of pathogenic species such as viral-envelope glycoproteins and bacterial exotoxins (see review articles Chrétien *et al.*, 1995; Denault & Leduc, 1996; Mbikay *et al.*, 1993; Nakayama, 1997; Rehemtulla & Kaufman, 1992; Seidah & Chrétien, 1997; Seidah *et al.*, 1998a; 1998b) (Table 1.1). This processing event has been found in bacteria, fungi, yeast, invertebrates and mammals. This precursor cleavage can occur either intracellularly, at the cell surface or within the extracellular milieu.

The precursor theory entailed the existence, in the same cells as the precursors, of endoproteases that could convert them preferentially after paired basic residues. For more than two decades, researchers had attempted to identify the enzymes with such cleavage specificity. However, due to the low cellular expression levels of these enzymes (later called convertases) and the presence of other contaminating enzymes as well as lack of

reliable *in vitro* assay systems, earlier efforts to purify and characterize these convertases were unsuccessful except for a few reports on such candidate enzymatic activities (Balow *et al.*, 1986; Clamagirand *et al.*, 1986; Cromlish *et al.*, 1986; Davidson *et al.*, 1988; Loh *et al.*, 1985). The molecular characterization of the yeast gene *KEX2* and its product kexin by genetic-complementation analyses was the major event that led to the subsequent identification of the mammalian homologues. Alpha-mating factor (α-MF) is a yeast hormone that promotes the mating of the a and α haploid strain into diploid cells. α-MF derives from the proteolytic processing of a larger inactive precursor after paired basic residues Lys-Arg. Mutant yeast were generated incapable of mating because they could not process pro-α-MF into α-MF. These mutants can be complemented with gene fragments *KEX2* from mating-competent yeast cells (Julius *et al.*, 1984). Later, the cellular expression of the *KEX2* gene product kexin can induce the correct processing of a mouse neuroendocrine peptide precursor proopiomelanocortin (POMC) in mammalian cells which are normally devoid of this activity (Thomas *et al.*, 1988). The *in vitro* enzymatic properties of this enzyme suggests that it is a Ca^{2+}-dependent and structurally related to the subtilisin family of serine proteinase, and it exhibits exquisite selectivity of cleavage after pairs of basic residues (Fuller *et al.*, 1989b; Mizuno *et al.*, 1988; 1989). Yeast kexin was the prototype enzyme responsible for the cleavage of precursors after pairs of basic residues.

The ability of kexin to cleave mammalian precursors immediately after dibasic amino acids suggested that yeast and mammalian endoproteinase responsible for such kind of cleavage specificity are related. The first mammalian homologue of *KEX2* was discovered by computer alignment of the active site Ser and the catalytically important Asn (Bryan *et al.*, 1986) of kexin against protein sequences found in data bases (Fuller *et al.*, 1989a). This search revealed a partial sequence of unknown function, a human gene *fur* which lies immediately upstream of the tyrosine kinase *fps/fes* oncogene (Roebroek *et al.*, 1986a; 1986b). The partial cDNA sequence code for a transmembrane domain and a cysteine-rich region. Roebroek *et al*, at the time suggested that *fur* (*fps/fes* upstream region) encode a membrane-associated receptor protein. They also detected its RNA in a variety of tissues, thus revealing its widespread expression. Unknowingly, they had

cloned the first mammalian kexin-like enzyme. It took another three years before Fuller's group first noticed the homology of the two sequences. Further confirmation of the homology between *KEX2* and *fur* product known as furin was obtained from additional genomic (Fuller *et al.*, 1989a) and cDNA (Van den Ouweland *et al.*, 1990) sequence of furin. Functional analysis of furin demonstrated that furin was able to convert different precursor proteins into their mature, active form. Early examples included pro-von Willebrand factor (Wise *et al.*, 1990), pro-nerve growth factor (Bresnahan *et al.*, 1990), proalbumin and complement pro-C3 (Misumi *et al.*, 1991).

The discovery of furin as the first mammalian endoproteinase, which has the cleavage specificity for paired basic amino acid residues, led to an extensive search for the other homologues of this family. Based on the sequence conservation around the active sites of serine proteinases, polymerase chain reaction (PCR) using degenerate oligonucleotide primers generated homologue DNA fragments, the later were then used as probes to retrieve full-length cDNA from various cDNA libraries. Two groups (Seidah *et al.*, 1990; 1991; Smeekens & Steiner, 1990; Smeekens *et al.*, 1991) were able to isolate two additional mammalian homologues PC1 and PC2. Similarly, additional members of the family have been identified namely PACE4 (Kiefer *et al.*, 1991), PC4 (Nakayama *et al.*, 1992a; Seidah *et al.*, 1992b), PC5 (Lusson *et al.*, 1993; Nakagawa *et al.*, 1993a) and PC7 (Bruzzaniti *et al.*, 1996; Constam *et al.*, 1996; Meerabux *et al.*, 1996; Seidah *et al.*, 1996c). They form a family of calcium-dependent serine proteinases of the subtilisin/kexin type. Although these enzymes may diverge in their tissue distribution and cellular location, one common property is their specificity to process protein precursors at selected single basic residues and/or pairs of basic amino acids permitting the release of active peptides. The various names given to theses "Precursor Convertases, Proprotein Convertases or Prohormone Convertases"(PCs) are present in Table 1.2. For simplicity, throughout the chapters we will refer to the seven convertases as furin, PC1, PC2, PC4, PACE4, PC5 and PC7 and accordingly rPCs, hPCs, mPCs and ykexin will refer the rat, human, mouse and yeast species of different PCs. In a combinatorial fashion, these enzymes determine the cell-type and time at which biologically active products are derived from a given inactive precursor protein (Seidah *et al.*, 1998b).

Table 1.1 Functional classification of precursors processed by the proprotein convertases. The critical basic amino acids at P1, P2, P4 and P6 are in bold. (Adapted from Seidah & Chrétien, 1999)

Protein precursor	***Sequence at the cleavage site***								
Pro-enzymes	**P6**	**P5**	**P4**	**P3**	**P2**	**P1**	↓	**P1'**	**P2'**
Proprotein Convertases									
hpro-PC1	**Lys**	Glu	**Arg**	Ser	**Lys**	**Arg**	↓	Ser	Ala
hpro-PC2	Phe	Asp	**Arg**	Lys	**Lys**	**Arg**	↓	Gly	Tyr
hpro-Furin	**Lys**	Arg	**Arg**	Thr	**Lys**	**Arg**	↓	Asp	Val
rpro-PC4	**Arg**	Arg	**Arg**	Val	**Lys**	**Arg**	↓	Ser	Leu
hpro-PACE4	**Lys**	Arg	**Arg**	Val	**Lys**	**Arg**	↓	Gln	Val
hpro-PC5	**Lys**	Lys	**Arg**	Thr	**Lys**	**Arg**	↓	Asp	Tyr
hpro-PC7	Leu	Arg	**Arg**	Ala	**Lys**	**Arg**	↓	Ser	Val
bADAM-10 (Kuz)	Leu	Leu	**Arg**	Lys	**Lys**	**Arg**	↓	Thr	Thr
hADAM-17 (TACE)	Val	His	**Arg**	Val	**Lys**	**Arg**	↓	Arg	Ala
hPro-Renin	Ser	Gln	Pro	Met	**Lys**	**Arg**	↓	Leu	Thr
hStromelysin-3	**Arg**	Asn	**Arg**	Gln	**Lys**	**Arg**	↓	Phe	Val
Pro-Growth-Factors									
Neurotrophins									
hPro-NGF	Thr	His	**Arg**	Ser	**Lys**	**Arg**	↓	Ser	Ser
hPro-BDNF	Ser	Met	**Arg**	Val	**Arg**	**Arg**	↓	His	Ser
hPro-NT3	Thr	Ser	**Arg**	Arg	**Lys**	**Arg**	↓	Tyr	Ala
hPro-NT4/5	Ala	Asn	**Arg**	Ser	**Arg**	**Arg**	↓	Gly	Val
hPro-PDGF-A	Pro	Ile	**Arg**	Arg	**Lys**	**Arg**	↓	Ser	Ile
hPro-PDGF-B	Leu	Ala	**Arg**	Gly	**Arg**	**Arg**	↓	Ser	Leu
hPro-TGFβ	Ser	Ser	**Arg**	His	**Arg**	**Arg**	↓	Ala	Leu
mPro-IGF-I	Pro	Thr	**Lys**	Ala	Ala	**Arg**	↓	Ser	Ile
hPro-IGF-II	Pro	Ala	**Lys**	Ser	Glu	**Arg**	↓	Asp	Val
hPro-IGF-II	Phe	Arg	Glu	Ala	**Lys**	**Arg**	↓	His	Arg
mPro-EGF (N-terminal)	Gly	His	**His**	Leu	Asp	**Arg**	↓	Asn	Ser
mPro-EGF (C-terminal)	**Arg**	Trp	Trp	Glu	Leu	**Arg**	↓	His	Ala
ProLefty gene factor	**Arg**	Ser	**Arg**	Gly	**Lys**	**Arg**	↓	Phe	Ser
Pro-Receptors									
hInsulin Pro-receptor	Pro	Ser	**Arg**	Lys	**Arg**	**Arg**	↓	Ser	Leu
hIGF-I Pro-receptor	Pro	Glu	**Arg**	Lys	**Arg**	**Arg**	↓	Asp	Val
hIntegrin α3	Pro	Gln	**Arg**	Arg	**Arg**	**Arg**	↓	Gln	Leu
hIntegrin α6	Asn	Ser	**Arg**	Lys	**Lys**	**Arg**	↓	Glu	Ile
hIntegrin α7	**Arg**	Asp	**Arg**	Arg	**Arg**	**Arg**	↓	Glu	Leu
hIntegrin αIIb	**His**	Lys	**Arg**	Asp	**Arg**	**Arg**	↓	Gln	Ile
hIntegrin α4	**His**	Val	Ile	Ser	**Lys**	**Arg**	↓	Ser	Thr
hIntegrin α5	**His**	His	Gln	Gln	**Lys**	**Arg**	↓	Glu	Ala
hIntegrin αv	**His**	Leu	Ile	Thr	**Lys**	**Arg**	↓	Asp	Leu

hIntegrin α8	**His**-Tyr-Ile-Arg-**Arg-Arg** ↓ Glu-Val
hLeptin pro-receptor	Gln-Val-**Arg**-Glu-**Lys-Arg** ↓ Leu-Asp
rPro-PTPμ receptor	Glu-Glu-**Arg**-Pro-**Arg-Arg** ↓ Thr-Lys
Pro-LRP	Ser-Asn-**Arg**-His-**Arg-Arg** ↓ Gln-Ile
Pathogenic pro-proteins	**P6 P5 P4 P3 P2 P1 ↓ P1' P2'**
HIV-1 gp 160	Val-Gln-**Arg**-Glu-**Lys-Arg** ↓ Ala-Val
HIV-1 (V3 Loop)	Ser-Ile-**Arg**-Ile-Gln-**Arg** ↓ Gly-Pro
Haemagglutinin (HA)	**Lys**-Lys-**Arg**-Glu-**Lys-Arg** ↓ Gly-Leu
CMV gB	**His**-Asn-**Arg**-Thr-**Lys-Arg** ↓ Ser-Thr
Varicella zoster virus gp	Asn-Thr-**Arg**-Ser-**Arg-Arg** ↓ Ser-Val
Mumps virus F protein	Ser-Arg-**Arg**-His-**Lys-Arg** ↓ Phe-Ala
Yellow fever virus prM	Ser-Arg-**Arg**-Ser-**Arg-Arg** ↓ Ala-Ile
Ebola virus.GP	Gly-Arg-**Arg**-Thr-**Arg-Arg** ↓ Glu-Ala
PA from B. Anthracis	Asn-Ser-**Arg**-Lys-**Lys-Arg** ↓ Ser-Thr
Dipthteria toxin	Gly-Asn-**Arg**-Val-**Arg-Arg** ↓ Ser-Val
proAerolysin (Hemolysin)	**Lys**-Ala-Ala-Gln-Leu-**Arg** ↓ Ser-Ala
Pro-transcription factors	
	P6 P5 P4 P3 P2 P1 ↓ P1' P2'
mNotch-1-receptor	Gly-Gly-**Arg**-Gln-**Arg-Arg** ↓ Glu-Leu
Pro-hormones	**P6 P5 P4 P3 P2 P1 ↓ P1' P2'**
mPOMC (JP/ACTH)	Pro-Arg-Glu-Gly-**Lys-Arg** ↓ Ser-Tyr
(ACTH/βLPH)	Pro-Leu-Glu-Phe-**Lys-Arg** ↓ Glu-Leu
(αMSH/CLIP)	Pro-Val-Gly-Lys-**Lys-Arg** ↓ Arg-Pro
hPro-Insulin (B/C chain)	Thr-Pro-**Lys**-Thr-**Arg-Arg** ↓ Glu-Ala
(C/A chain)	Gly-Ser-Leu-Gln-**Lys-Arg** ↓ Gly-Ile
hPro-PTH	**Lys**-Ser-Val-Lys-**Lys-Arg** ↓ Ser-Val
Pro-pancreatic polypeptide	Pro-Arg-Tyr-Gly-**Lys-Arg** ↓ His-Lys
rPro-Gastrin-I	Ala-Asp-Leu-Ser-**Lys-Lys** ↓ Gln-Arg
hPro-Gastrin	Ala-Ser-**His**-His-**Arg-Arg** ↓ Gln-Leu
	Met-Asp-Phe-Gly-**Arg-Arg** ↓ Ser-Ala
hPro-Glucagon	Met-Asn-Glu-Asp-**Lys-Arg** ↓ His-Ser
rPro-Glucagon	Leu-Met-Asn-Thr-Lys-**Arg** ↓ Asn-Arg
hPro-GLP-1	Asn-Asn-Ile-Asp-**Lys-Arg** ↓ His-Asp
mPro-Fertilin β	Gln-Ser-**Arg**-Met-**Arg-Arg** ↓ Ala-Ala
hPro-ANF	Leu-Leu-Thr-Ala-Pro-**Arg** ↓ Ser-Leu
rPro-MIS	**Arg**-Gly-**Arg**-Ala-Gly-**Arg** ↓ Ser-Lys
Pro-neuropeptides	
mPOMC(βLPH/βEnd)	Pro-Pro-**Lys**-Asp-**Lys-Arg** ↓ Tyr-Gly
hPro-Enkephalin)	Gly-Gly-Phe-Met-**Lys-Lys** ↓ Asp-Ala
	Met-Asp-Tyr-Gln-**Lys-Arg** ↓ Tyr-Gly
	Gly-Gly-Phe-Leu-**Lys-Arg** ↓ Phe-Ala
rPro-Dynorphin	**Arg**-Lys-Gln-Ala-**Lys-Arg** ↓ Tyr-Gly

	Glu-Asp-Leu-Tyr-**Lys-Arg** ↓ Tyr-Gly
	Arg-Lys-Tyr-Pro-**Lys-Arg** ↓ Ser-Ser
Pro-Nociceptin/Orphanin	**Lys**-Gln-Leu-Gln-**Lys-Arg** ↓ Met-Pro
	Leu-Ala-Asn-Gln-**Lys-Arg** ↓ Phe-Ser
hPro-7B2	Glu-Arg-**Arg**-Lys-**Arg-Arg** ↓ Ser-Val
rPro-TRH (178-199)	**Arg**-Ser-Trp-Glu-Glu-**Lys** ↓ Glu-Gly
hPro-LHRH	**Arg**-Pro-Gly-Gly-**Lys-Arg** ↓ Asp-Ala
hPro-Somatostatin (S14)	Ala-Pro-**Arg**-Glu-**Arg-Lys** ↓ Ala-Gly
hPro-Somatost (S-28)	**Arg**-Leu-Glu-Leu-Gln-**Arg** ↓ Ser-Ala
hProGnRH I	**Arg**-Pro-Gly-Gly-**Lys-Arg** ↓ Asp-Ala
hproGnRH II	Ser-Pro-Gly-Gly-**Lys-Arg** ↓ Ala-Leu
hPRL-RP	**Arg**-Phe-Gly-Arg-**Arg-Arg** ↓ Ala-Thr
Pro-Peptide Y	Gln-Arg-Tyr-Gly-**Lys-Arg** ↓ Ser-Ser
Pro-Peptide YY	Gln-Arg-Tyr-Gly-**Lys-Arg** ↓ Glu-Val
Pro-Galanin	Pro-Ala-Lys-Glu-**Lys-Arg** ↓ Gly-Tyr
	Gly-Leu-Thr-Ser-**Lys-Arg** ↓ Glu-Leu
Pro-MCH	Ser-Thr-Gln-Glu-**Lys-Arg** ↓ Glu-Ile
	Phe-Pro-Ile-Gly-**Arg-Arg** ↓ Asp-Phe
bPro-Substance K(Pro SK)	Gln-Leu-Ser-His-**Lys-Arg** ↓ His-Lys
hPACAP	**Arg**-Pro-Ala-Gly-**Arg-Arg** ↓ Asp-Val
	Glu-Pro-Leu-Ser-**Lys-Arg** ↓ His-Ser
	Lys-Asn-**Lys**-Gly-**Arg-Arg** ↓ Ile-Ala
Other Precursor proteins	
hPro-von Willebrand factor	Ser-His-**Arg**-Ser-**Lys-Arg** ↓ Ser-Leu
hPro-Factor IX	Leu-Asn-**Arg**-Pro-**Lys-Arg** ↓ Tyr-Asn
hPro-Factor X	Leu-Glu-**Arg**-Arg-**Lys-Arg** ↓ Ser-Val
gpSeminal Vesical Prot-1	**His**-Ile-**Arg**-Phe-**Lys-Arg** ↓ Gln-Asp
Protein-2	**His**-Leu-**Arg**-Leu-**Lys-Arg** ↓ His-Asp
rNg-CAM	Gly-Glu-**Arg**-Ser-**Arg-Arg** ↓ Gln-Ala
pro-Sortilin	Gly-Gly-**Arg**-Trp-**Arg-Arg** ↓ Ser-Ala
Pro-hCadherin-15	Leu-Ser-**Arg**-Val-**Arg-Arg** ↓ Ala-Trp
Pro-Collagens (C-terminal)	
Human α1(V)	Ala-Ser-**Arg**-Thr-**Arg-Arg** ↓ Asn-Ile
Hamster α1(V)	Ala-Ser-**Arg**-Thr-**Arg-Arg** ↓ Asn-Ile
Human α1(XI)	Ser-Lys-**Lys**-Thr-**Arg-Arg** ↓ His-Thr
Mouse α1(XI)	Pro-Lys-**Lys**-Thr-**Arg-Arg** ↓ His-Thr
Rat α1(XI)	Pro-Lys-**Lys**-Thr-**Arg-Arg** ↓ His-Thr
Human α2(XI)	Pro-Lys-**Lys**-Thr-**Arg-Arg** ↓ Ser-Val
hPro-Albumin	**Arg**-Gly-Val-Phe-**Arg-Arg** ↓ Asp-Ala
hPro-Protein C	**Arg**-Ser-**His**-Leu-**Lys-Arg** ↓ Asp-Thr
hPACAP RP	Ala-Pro-Leu-Thr-**Lys-Arg** ↓ His-Ser
r/mHypocretins	Thr-Leu-Gly-Lys-**Arg-Arg** ↓ Pro-Gly
mPro-SMR1	Gly-Val-**Arg**-Gly-Pro-**Arg** ↓ Arg-Gln
	Arg-Gln-**His**-Asn-Pro-**Arg** ↓ Arg-Gln

Table 1.2 Nomenclature of precursor convertases

Name Adopted	Name Original	References
Furin	Furin	Roebroek *et al.*, 1986a; 1986b
	PACE	Wise *et al.*, 1990
	SPC1	Rouille *et al.*, 1995
PC1	PC1	Seidah *et al.*, 1991
	PC3	Smeekens *et al.*, 1991
	SPC3	Rouille *et al.*, 1995
PC2	PC2	Seidah *et al.*, 1990; Smeekens *et al.*, 1990
	SPC2	Rouille *et al.*, 1995
PC4	PC4	Nakayama *et al.*, 1992a; Seidah *et al.*, 1992b
	SPC4	Rouille *et al.*, 1995
PACE4	PACE4	Kiefer *et al.*, 1991
	SPC5	Rouille *et al.*, 1995
PC5	PC5	Lusson *et al.*, 1993
	PC6	Nakagawa *et al.*, 1993a
	SPC6	Rouille *et al.*, 1995
PC7	PC7	Seidah *et al.*, 1996c
	LPC	Meerabux *et al.*, 1996
	PC8	Bruzzaniti *et al.*, 1996
	SPC7	Constam *et al.*, 1996

1.2. General structure of mammalian PCs

A schematic representation of the protein structures of the seven known mammalian PCs as well as those of yeast kexin and bacterial subtilisin BPN' is shown in Figure 1.1. The unique and conserved structure motifs of the eukaryotic subtilisin protease family members can be divided into five distinct domains as follows: the signal peptide, the prosegment, the catalytic domain, the P-domain, and the carboxyl-terminal region. The newly synthesized enzyme enters the secretory pathway via its signal peptide. The prosegments of about 80-104 amino acids, ending at the cleavage motif R-X-K/R-R↓, are highly basic except in the case of PC7 (Table 1.3). The prosegments of PCs exhibit 30-67% sequence identity (Table 1.4) (Figure 1.2) and an absolute conservation of 8 amino acids (Seidah *et al.*, 1998a). Overall, PC7 exhibits the least conserved prosegment among the mammalian PCs (Table 1.4) (Figure 1.2). By analogy to that of bacterial subtilisins, the prosegment is thought to act as an intramolecular chaperone guiding its folding within the endoplasmic reticulum (ER). Once cleaved by an autocatalytic event, the prosegments will behave as a potent inhibitor regulating the activation of the enzyme (Shinde & Inouye, 1995a; 1995b), so that the later can only function in the appropriate intracellular compartment. Chapter 2, presents a detailed study on the inhibitory property of the prosegments of furin and PC7 (Zhong *et al.*, 1999), addresses one aspect of the prosegments dual functions. The prosegments are autocatalytically processed in the ER except of PC2. This event is a prerequisite for the PC's efficient egress from ER (Creemers *et al.*, 1995). The rate of removal of prosegment could represent a mechanism by which the cell would control the rate of proprotein processing (Benjannet *et al.*, 1992). The initial cleavage of the prosegments of PCs does not result in the immediate activation of the enzyme; rather the prosegment appears to remain tightly associated with the convertase until it reaches its final cellular destination. At this point, the increase in H^+ and/or Ca^{2+} concentrations in the *trans*-Golgi network (TGN) or secretory granules triggers a secondary cleavage(s) and/or C-terminal trimming, resulting in the dissociation of the prosegment complex (Anderson *et al.*, 1997; Boudreault *et al.*, 1998a; Powner & Davey, 1998) and the generation of the active convertase, which can then process other precursors *in trans*. While PC1, PC2, furin,

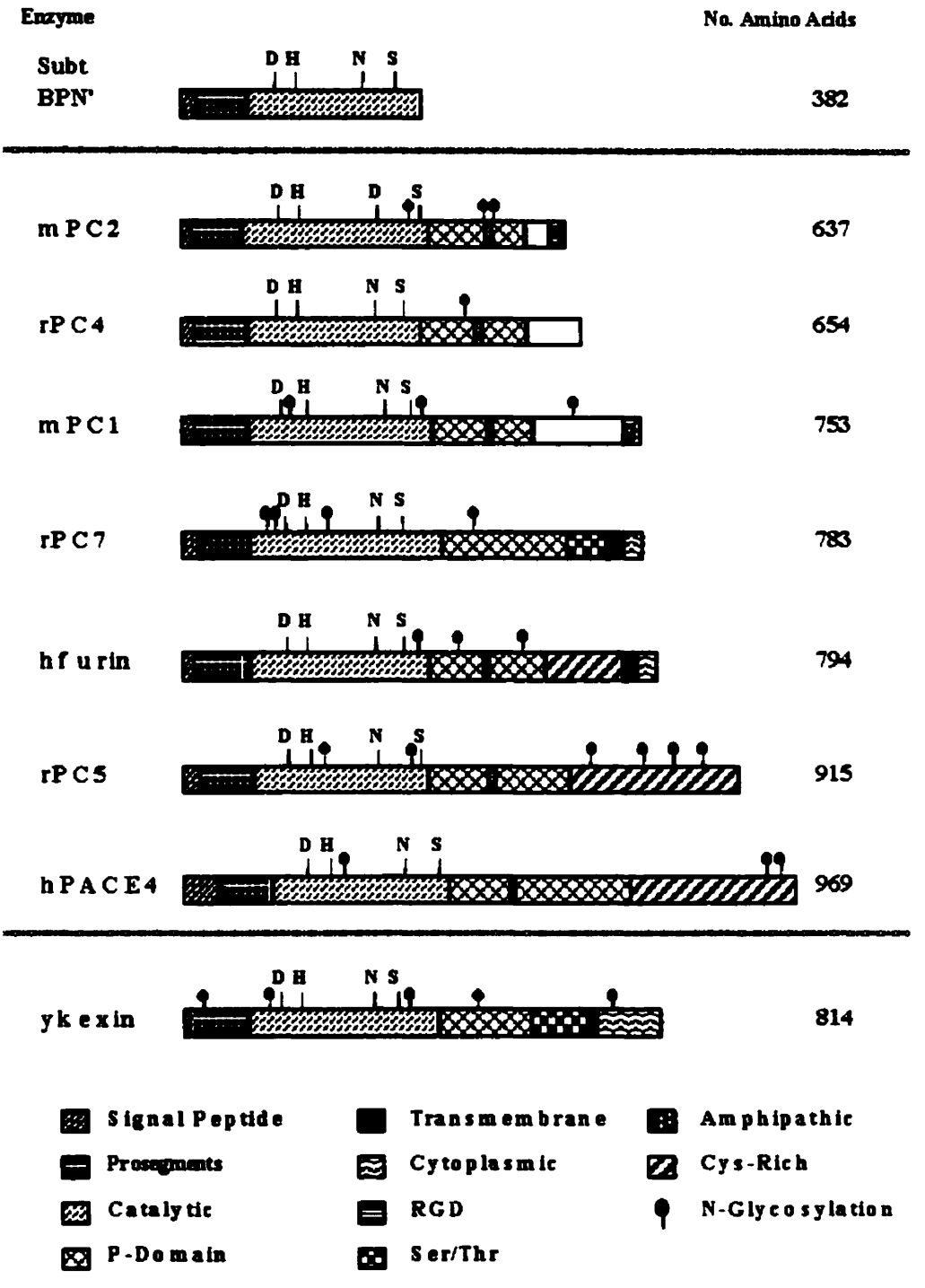


Figure 1.1 Schematic representation of the primary structure of the PCs as compared to those of Subtilisin BPN' and yeast kexin. The various domains are emphasized, together with the active sites Asp, His and Ser and the oxyanion hole Asn (Asp for PC2).

PACE4 and PC5 contain a secondary cleavage site KR within their prosegments, PC4 and PC7 have only an RK site. Thus, it is not yet clear if cleavage at the latter sites is required for the effective activation of these enzymes. In contrast to furin removal of the prosegment in the TGN, PC2 prosegment dissociation occurs within the acidic milieu of the TGN/immature secretory granules (ISG) (Benjannet *et al.*, 1993; Milgram & Mains, 1994) and is highly facilitated by the presence of 7B2 (Seidah *et al.*, 1983; Hsi *et al.*, 1982), a PC2-specific binding protein (Benjannet *et al.*, 1995a; 1995b; Braks & Martens, 1994; Martens *et al.*, 1994; Seidah *et al.*, 1994). Thus, PC2 is unique among the convertases in that it is activated late along the secretory pathway and requires the presence of 7B2 for optimal activation.

Table 1.3 Theoretical isoelectric point (pI) of the prosegment of PCs

prosegments of different PCs	*pI*
hPACE4	10.16
hPC5-A	10.33
hfurin	11.93
rPC4-A	11.52
hPC2	10.68
hPC1	9.10
hPC7(rPC7)	5.26(6.21)

Table 1.4 Sequence identities of the prosegments of PCs

PCs	hPC1	hPC2	rPC4	hfurin	hPACE4	hPC5	hPC7
hPC2	35 %						
rPC4	35 %	31 %					
hfurin	47 %	43 %	53 %				
hPACE4	50 %	41 %	45 %	60 %			
hPC5	41 %	35 %	44 %	58 %	67 %		
hPC7	35 %	31 %	36 %	34 %	35 %	30 %	
ykexin	25 %	29 %	26 %	29 %	26 %	29 %	23 %

Figure 1.2 Alignment of the prosegments of proprotein convertases

```
                  10         20         30         40         50         60
hPACE4       ApppR.pVY TN...HWAVq Vl.GGPa... .....EAdRV AaahGYlNLG QIGnLEdYYH
hPC5A              rVY TN...HWAVk Ia.GGfp... .....EANRI AsKyGFINiG QIGaLkdYYH
hfurin           qkVF TN...tWAVr IP.GGPa... .....vANsV ArKhGFlNLG QIfgd..YYH
rpc4-a         rpp.IY vs...sWAVr Vt.kGyq... .....EAeRl ArKfGFVNLG QIfpdDqYFH
hpc2           eRp.VF TN...HFlVe lhkGGeD... .....kArqV AaehGFg.vr klpfaEglYH
hpc1           kRq..F vN...eWAae IP.GGPE... .....aAsaI AeelGYdlLG QIGsLEnhYl
rpc7        LteAggldtL gagglsWAVh ldslegErke esliqqANaV AqaaGLVNaG rIGeLqghYl
Consensus   ---------- ---------- ---------- ------A--- A---G----- ----------

                  70         80         90        100
hPACE4      FYHsktfKRS tlSS...RGp HtfLrmDPqV kWLqQQevKR RvKR
hPC5A       FYHsrtiKRS viSS...RGt HSfiSmEPkV eWiqQQvvKk RtKR
hfurin      FWHrGvtKRS Lsph...Rpr HSRLqrEPqV qWLEQQvaKR RtKR
rpc4-a      LRHrGvAqqS Ltph...WGH rlRLkkEPkV RWFEQQtlrR RvKR
hpc2        FYHnGlAKak rRrS...lhH rqqLerDPrV RmalQQegfd RkKR
hpc1        FkHknhprRS rRSa...Fhi tkRLSdDdrV iWaEQQyeKe RsKR
rpc7        Fvqpaghgqa Meaeamrqqa eavLakheaV RwhseQrllk RaKR
Consensus   ---------- ---------- ---------V -----Q---- R-KR
```

The subtilisin-like catalytic domain that extends about 350 amino acids is the most conserved among all the PCs (Table 1.5) (Figure 1.3). In particular the active site residues of the Asp, His, Ser catalytic triad and Asn residue (Asp in PC2) which stabilizes the oxyanion hole in the transitional state are present at corresponding positions of all members except PC2 ($^{\bullet}$D, $^{\bullet}$H, $^{\bullet}$S and $^{\bullet}$N in Figure 1.3). The conserved oxyanion hole Asp^{+} in PC2 seems to be important for its interaction with the precursor of 7B2 (Benjannet *et al.*, 1995a). They all present a conserved cysteine, four residues downstream of the histidine residue. This cysteine is presumed to function as a free thiol group, which contributes to the fact that PCs are sensitive to heavy metals such as zinc and mercury. Alignment of the catalytic domain of all the PCs reveals that within the 350 amino acid catalytic domain, PC2 presents 9 substitute residues that are conserved in all the PCs (+ in Figure 1.3), these include 3 conservative and 6 non-conservative substitutions, 4 of which are considered to be significant changes. All these amino acid substitutions occur between the PC2's active site $^{\bullet}D^{166}$ and the oxyanion hole $^{+}D^{309}$ (Figure 1.3). They include: (1) the oxyanion hole $^{+}D^{309}$ itself (as opposed to N), (2) two in the segment $\underline{S}^{81}$-S-N-D-P-$\underline{Y}^{86}$-P-Y-P-R, in which the underlined $\underline{S}^{81}$ and $\underline{Y}^{86}$ are unique as they are $\underline{N}$ and $\underline{D}$ respectively in all other PCs, and finally (3) a $\underline{Q}^{134}$ replacing the usual $\underline{G}$ in all other PCs (Seidah *et al.*, 1998b). The D/N mutant of the oxyanion hole of PC2

Figure 1.3 Alignment of the catalytic subunits of the proprotein convertases. (Adapted from Seidah *et al.*, 1998b)

```
                  10                  30                  50         •
rPACE       ...qaRsdSl YFNDPiWsnm WYMHCaDkNs Rcrs.EMNVq aAWkRGYTGK nVVVTILDDG
rPC5        dYdlsRaqSt YFNDPKWPsm WYMHCsDNth pcqs.DMNIe gAWkRGYTGK nIVVTILDDG
rfurin      ......dVyq eptDPKFPQQ WYL.....sg vtqr.DLNVk eAWaqGFTGr GIVVsILDDG
rpc4-a      .......slV vptDPwFskQ WYM.....Nk eieq.DLNIl kvWnqGLTGr GVVVsILDDG
rpc1        .svprdsaln lFNDPmWnQQ WYLqdTrmta slPkLDLhVi pvWqkGiTGK GVVITVLDDG
rpc2        gYrdineIdI nmNDPlFtkQ WYLfnTgqad gtPgLDLNVa eAWelGYTGK GVtIgIMDDG
rpc7        ........SI hFNDPKYPQQ WhL....NNr RsPgrDiNVt gvWeRnvTGr GVtVvVvDDG
Consensus   ---------- ---DP----- W--------- ---------- --W----TG- -------DDG

                  70            +   + 90          •         110  +
rPACE       IERNHPDLAp NYDsyASYDv NgNDyDPsPR YDasNENKHG TRCAGEVAAs ANNSYCiVGI
rPC5        IERtHPDLmq NYDalASCDv NgNDlDPmPR YDasNENKHG TRCAGEVAAt ANNShCtVGI
rfurin      IEkNHPDLAg NYDPgASFDv NDqDPDPqPR YtqmNDNrHG TRCAGEVAAv ANNgvCGVGV
rpc4-a      IEkdHPDLwa NYDPlASYDF NDyDPDPqPR YtpndENrHG TRCAGEVsAt ANNgFCGaGV
rpc1        lEWNHtDiya NYDPEASYDF NDNDhDPfPR YDptNENKHG TRCAGEIAmq ANNhkCGVGV
rpc2        IDYlHPDLAy NYnsDASYDF ssNDPyPyPR YtddwfNsHG TRCAGEVsAa AsNniCGVGV
rpc7        VEhtvqDiAp NYsPEgSYDL NsNDPDPmPh pDeeNgNhHG TRCAGEIAAv pNNSFCaVGV
Consensus   ------D--- NY----S-D- ---D--P-P- ------N-HG TRCAGE---- --N--C--G-

                 130    +              150                 + 170
rPACE       AYNAKIGGIR MLDGD.VTDV VEAkSLgirP nyIDIYSASW GPDDDGKTVD GPGRLakQAF
rPC5        AFNAKIGGVR MLDGD.VTDm VEAkSvsYNP QHVhIYSASW GPDDDGKTVD GPapLtrQAF
rfurin      AYNArIGGVR MLDGE.VTDa VEArSLgLNP nHIhIYSASW GPEDDGKTVD GPaRLaeeAF
rpc4-a      AFNArIGGVR MLDGa.ITDI VEAqSLsLqP QHIhIYSASW GPEDDGrTVD GPGlLtqeAF
rpc1        AYNsKVGGIR MLDGi.VTDa IEAsSigFNP gHVDIYSASW GPnDDGKTVE GPGRLaqkAF
rpc2        AYNsKVaGIR MLDgpfmTDI IEAsSishmP QlIDIYSASW GPtDnGKTVD GPreLtlQAM
rpc7        AYgsrIaGIR vLDGp.lTDs mEAvaFnkhy QinDIYScSW GPDDDGKTVD GPhqLgkaAL
Consensus   A------G-R -LD----TD- -EA------- ----IYS-SW GP-D-G-TV- GP--L---A-

                 190           +/•     210         +          230
rPACE       EyGIKKGRQG LGSIFVWASG NGGRegDhCs CDGYTNSIYT ISVSStTEnG hkPWYlEECa
rPC5        EnGVrmGRrG LGSVFVWASG NGGRskDhCs CDGYTNSIYT ISISStaEsG kkPWYlEECS
rfurin      frGVsqGRgG LGSIFVWASG NGGRehDsCN CDGYTNSIYT lSISSaTqfG nvPWYsEaCS
rpc4-a      rrGVtKGRQG LGtlFIWASG NGGlhyDnCN CDGYTNSIhT lSVgStTrqG RvPWYsEaCa
rpc1        EyGVKqGRQG kGSIFVWASG NGGRqgDyCd CDGYTdSICT ISISSasqqG lsPWYaEkCS
rpc2        adGVnKGRgG kGSIYVWASG dGG.syDdCN CDGYasSmWT ISInSaindG RtaLYdEsCS
rpc7        qhGVmaGRQG FGSIFVvASG NGGqhnDnCN YDGYaNSIYT VtIgavdEeG RmPFYaEECa
Consensus   --G---GR-G -G-----ASG -GG---D-C- -DGY--S--T ---------G ----Y-E-C-

                 250                 270         + •         290
rPACE       STLATTYSSG afyErk..IV TTDLRQr... .CTDGHTGTS vSAPMvAGII ALALEANnqL
rPC5        STLATTYSSG EsyDkk..II TTDLRQr... .CTDnHTGTS ASAPMAAGII ALALEANPfL
rfurin      STLATTYSSG nqnEkq..IV TTDLRQk... .CTEsHTGTS ASAPLAAGII ALtLEANknL
rpc4-a      STFtTTFSSG vvtDpq..IV TTDLhhq... .CTDkHTGTS ASAPLAAGmI ALALEANPlL
rpc1        STLATsYSSG Dytnqr..It saDLhnd... .CTEtHTGTS ASAPLAAGIf ALALEANPnL
rpc2        STLAsTFSnG rkrnpeagVa TTDLygn... .CTlrHsGTS AaAPeAAGVf ALALEANvdL
rpc7        SmLAvTFSgG Dkmlr..sIV TTDwdlqkgt gCTEGHTGTS AaAPLAAGmI ALmLqvrPcL
Consensus   S------S-G ---------- --D------- -CT--H-GTS --AP--AG-- AL-L-----L

                 310                 330             +     350
rPACE       TWRDvQHLlV kTSRPAhLkA s..DWKvNGA GhKVSHLYGF GLvDAeALVl eA..
rPC5        TWRDvQHvIV RTSRaghLNA N..DWKTNaA GFKVSHLYGF GLMDAeAMVm eAE.
rfurin      TWRDMQHLVV qTSkPAhLNA N..DWaTNGv GrKVSHsYGY GLLDAGAMVa LA..
rpc4-a      TWRDLQHLVV RaSRPAqLqA e..DWriNGv GrqVSHhYGY GLLDAGlLVd LA..
rpc1        TWRDMQHLVV WTSeydpLan N.pgWKkNGA GLmVnsrFGF GLLnAkALVd LADp
rpc2        TWRDMQHLtV lTSkrnqLhd evhqWrrNGv GLefnHLFGY GvLDAGAMVk MA..
rpc7        TWRDvQHiIV FT..atqyed hraDWlTNeA GFshSHqhGF GLLnAwrLVn aA..
Consensus   TWRD-QH--V ---------- ----W--N-- G-------G- G---A---V- -A--
```

abrogates the binding of proPC2 to pro7B2 in the ER but not that of PC2/7B2 in the TGN (Benjannet *et al.*, 1995a). These data suggest that the unique oxyanion hole of proPC2 is important for its binding to pro7B2 in the ER but is not needed for the predicted second binding site of 7B2 (Seidah *et al.*, 1998b). It has been suggested that the substitution of PC2 at the oxyanion hole permit PC2 to acquire a narrower and more discriminate substrate specificity (Brenner *et al.*, 1993; Seidah *et al.*, 1993).

Table 1.5 Sequence identities of the catalytic domains of PCs

PCs	hPC1	hPC2	rPC4	hfurin	hPACE4	hPC5	hPC7	ykexin
hPC2	55 %							
rPC4	59 %	56 %						
hfurin	64 %	58 %	70 %					
hPACE4	63 %	54 %	62 %	68 %				
hPC5	60 %	53 %	62 %	67 %	75 %			
hPC7	55 %	51 %	53 %	54 %	54 %	54 %		
ykexin	50 %	47 %	46 %	49 %	48 %	48 %	48 %	
BPN'	36 %	35 %	34 %	37 %	34 %	34 %	34 %	34 %

In addition, a region of approximately 140 amino acids following the catalytic domain, which has been referred as P-domain (also as Homo B or middle domain) is also well conserved among PCs including yeast kexin but absent in bacterial subtilisin (Table 1.6) (Figure 1.4). The P-domain is essential for zymogen cleavage in kexin (Gluschankof & Fuller, 1994; Powner & Davey, 1997), PACE4 (Zhong *et al.*, 1996) and PC2 (Taylor *et al.*, 1997), as disruption of this segment blocks the normally rapid intramolecular cleavage of the prosegment. The P-domain is also important for catalytic activity (Creemers *et al.*, 1993; Zhong *et al.*, 1996) and the cellular sorting of the enzyme (Creemers *et al.*, 1996). The N-terminus of the P-domain starts at the end of the catalytic domain, marking the end of the homologous subtilisin sequence. Its C-terminal border

Figure 1.4 Alignment of the P-domain of proprotein convertases. (Adapted from Seidah *et al.*, 1998b)

```
                10         20         30         40         50         60
rPC1       RtWrnVPekk eCIIkdnnfE PrAlkangEV iveipTrACE gqEN.aInsL EHVQfeaTIe
rPC2       kdWkTVPerf hCVggsvq.n PekIPPtgkl vlTLqTnACE gkEN.FVrYL EHVQavITVn
rPACE4     RkWTaVPsQh mCVatadK.r PrsIPvvqvl RtTalTnACa DhsdqrVvYL EHVvVRIsIS
rPC5       ekWTTVPqQh vCVestdr.q iKtIrPnsaV RsiykasgCs DnpNhhVnYL EHVvVRITIt
rFurin     qnWTTVapQr KCIIeIla.E PKdIgkrlEV RKT.VTaclg Epnh..IsrL EHVQaRlTlS
rPC4       RvWlptkpQk KCtIrVvh.t PtpIlPrmlV pKn.VTvcCD gsrrrLIrsL EHVQVqlslS
rPC7       kiWTsVPyla sYVspmlK.E nKAVPrsphs levLwnvsrt DlEmsglktL EHVaVtVsIt
Consensus  --W------- ---------- ---------- ---------- ---------L EHV-------

                70         80         90        100        110        120
rPC1       ysRRGDLhVt LTSaaGTstv LLAeReR.Dt SPnGFkNWDF MsVHtWGEnp vGtWTLkVtD
rPC2       atRRGDLnIn MTSPmGTkSi LLsrRPRdDd SkvGFdkWpF MTtHtWGEDA RGtWTLEl.g
rPACE4     hPRRGDLqIh LiSPsGTkSq LLAkRl.LDF SnEGFtNWEF MTVHCWGEkA eGEWTLEVqD
rPC5       hPRRGDLaIy LTSPsGTRSq LLAnRl.FDh SmEGFkNWEF MTIHCWGErA aGDWvLEVyD
rFurin     ynRRGDLaIh LiSPmGTRSt LLAaRP.hDY SaDGFNdWaF MTtHsWdEDp sGEWvLEIEn
rPC4       ysRRGDLeIf LTSPmGTRSt LvAiRP.LDi SgqGYNNWiF MstHYWdEDp qGlWTLglEn
rPC7       hPRRGsLelk LfcPsGmmSl igApRs.MDs dPnGFNdWtF sTVrCWGErA RGvYrLvIrD
Consensus  --RRG-L--- -----G---- ----R---D- ---G---W-F -----W-E-- -G---L----

                130        140
rPC1       msgrMq...N eGrivnWkLI LhGT
rPC2       fvgsap...q kGlLKEWTLm LhGT
rPACE4     ipsQvRnpek qGkLKEWsLI LYGT
rPC5       tpsQLRnfkt pGkLKEWsLV LYGT
rFurin     ts....eanN yGtLtkFTLV LYGT
rPC4       kg....yyyN tGtLyycTLl LYGT
rPC7       vgd...eplq vGiLqqWqLt LYGs
Consensus  ---------- -G------L- L-G-
```

has been defined as that amino acid close to an L-X-(L/F)-X-G sequence (Seidah, 1995b) (Fig. 1.4), beyond which further deletions would irreversibly abolish the enzymatic activity of the PC (Gluschankof & Fuller, 1994; Taylor *et al.*, 1997; Zhong *et al.*, 1996). Interestingly, this functional definition coincides with the endpoint of similarity between the PCs (Gluschankof & Fuller, 1994; Seidah, 1995b). It is noted however, that the P-domain of PC7 extends beyond the L-X-(L/F)-X-G sequence (Munzer *et al.*, 1997) (Appendices). In addition to the above identical sequence at the end of the P-domain of mammalian PCs, this segment exhibits the presence of 20 other identical amino acids, 10 of which are absolutely conserved among all PC orthologues (residues bold and underlined in Fig. 1.4) (Seidah *et al.*, 1998b). Various attempts failed to yield secreted active rPC7 when we introduced stop codon at either 2 or 10 residues following the L-X-L-X-G sequence. When analyzed by Western blots, only proPC7 was detected intracellularly suggesting that inactive PC7 being stuck in the ER. It is possible that this

border is not conserved in all PCs and that the intro/exon junction following the critical Gly is a better indication of this demarcation point. A detailed structure-function study on the integrity of P-domain of PACE4 is presented in Chapter 3 (Zhong *et al.*, 1996).

Table 1.6 Sequence identities of the P-domains of PCs

hPC1	hPC1	hPC2	rPC4	hfurin	hPACE4	hPC5	hPC7
hPC2	43 %						
rPC4	42 %	43 %					
hfurin	44 %	47 %	55 %				
hPACE4	43 %	52 %	43 %	45 %			
hPC5	39 %	52 %	43 %	51 %	61 %		
hPC7	37 %	42 %	38 %	38 %	45 %	43 %	
ykexin	41 %	32 %	33 %	32 %	33 %	38 %	32 %

Within the middle of the P-domain, there is the conserved RRGDL pentapeptide sequence (with RRGSL in PC7). The RGD sequence found in the PCs and in fibronectin was originally suggested that this motif might be important for the interaction of the PCs with cell adhesion integrins (Seidah *et al.*, 1990). Interestingly, three RGD copies exist in *hydra* PC1. The PCs which do not contain an RGD sequence include mammalian (human, rat and mouse) PC7, *drosophila* furin 2, *lymnaea stagnalis* PC2, *aplysia californica* PC1 and PC2 and yeast XPR6 (Seidah, 1995b). The conservation of this motif suggested a structural or functional role, which may be common to most of the PCs. Not much known about the function of the RGD in PCs, except that binding of fibronectin to its integrin receptor indeed depends on the presence of the RGD sequence and the binding was abrogated when RGD was replaced by RGE (Ruoslahti, 1996). The RRGDL motif is critical for the stability of PC1 and for its zymogen activation in the ER, mutagenesis of this sequence leads to a dramatic decrease in the level of zymogen processing in the ER and in missorting of this normally granule-associated convertase (Malide *et al.*, 1995) toward the constitutive secretory pathway (Lusson *et al.*, 1997; Rovere *et al.*, 1999). Future work should define whether these results obtained for PC1 are applicable to other

members of the PC family which undergo zymogen cleavage in the ER, such as furin, PACE4, PC4 and PC5, and whether the integrity of the RRGDL sequence is also critical for the formation of the C-terminally truncated, granule-associated, 65 kDa PC5 (de Bie *et al.*, 1996) and/or 76 kDa furin (Hill *et al.*, 1995). In addition, it will be informative to define whether the variant RRGSL motif found in PC7 also plays a similar role for this convertase (Seidah *et al.*, 1998b).

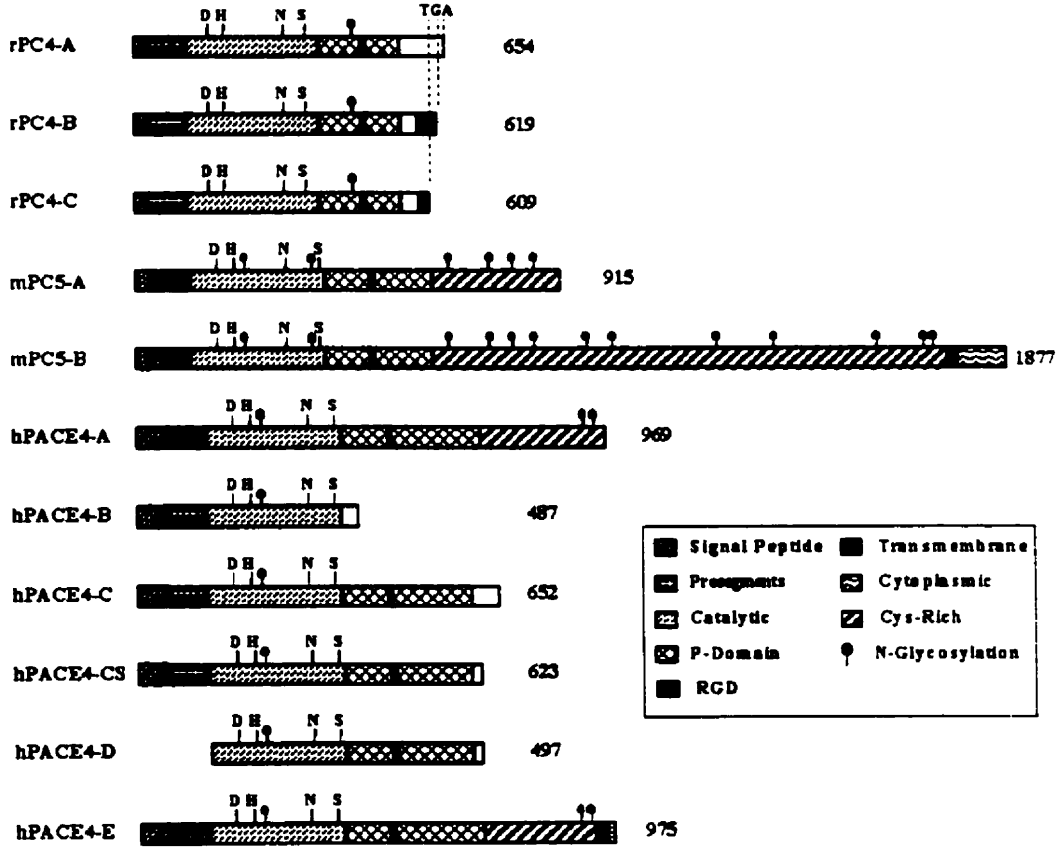
Downstream to the P-domain, each convertase displays a unique C-terminal region, which exhibits the least structure homology among all the members. PC1 and PC2 seem to contain an amphipathic helix, which may interact with the membrane in a pH dependent manner. Furin, PACE4, PC5 contain a Cys-rich domain with a ($C\text{-}X_{9\text{-}16}\text{-}C\text{-}X_2\text{-}C\text{-}X_3\text{-}C\text{-}X_2\text{-}C\text{-}X_{5\text{-}7}\text{-}C\text{-}X_2\text{-}C\text{-}X_{8\text{-}15}\text{-}C\text{-}X_3$) motif repeated 2, 5 or 22 times in furin, PACE4/PC5 and PC5 splice variant PC5-B (Lusson *et al.*, 1993), respectively. This Cys-rich motif was also observed in *D. melanogaster* furin-like convertases (Roebroek *et al.*, 1992). The function of this Cys-rich domain remains unknown. Furin, PC5-B, PACE4-E (an isoform of PACE4 (Akamatsu *et al.*, 1997; Mori *et al.*, 1997)) and PC7 are type-I membrane-associated enzymes which have a transmembrane domain near the C-terminal region. It allows furin to cycle from the cell surface and back to the TGN, in order to efficiently process precursors trafficking in the constitutive secretory pathway, mainly those that are secreted or inserted into the plasma membrane via constitutive vesicles (Sariola *et al.*, 1995), or those interacting with the cell surface. Type I membrane-bound proteins are often recycled from the cell surface to the TGN via a complex retention/retrieval mechanism involving binding of specific cytosolic proteins to conserved motif in the cytosolic tail of the TGN-resident proteins (Luzio & Banting, 1993). Although at steady state, furin is predominantly found in the TGN, it also cycles between the TGN and the cell surface. The cytosolic tail of furin has been analyzed extensively and found to contain several signal regions that direct its intracellular trafficking. These include certain tyrosine-containing sequences, a dileucine-like sequences, as well as an acidic casein kinase II site (Jones *et al.*, 1995; Schafer *et al.*, 1995; Takahashi *et al.*, 1995b). Within the cytoplasmic domain, there are Ser residues, which are sites for phosphorylation by casein kinase II *in vitro*, and a Tyr-containing

sequence, both of which have been shown to be important for the localization of other TGN proteins. Mutation of the Ser residues abrogated the TGN localization. By contrast, mutation of the Tyr residue did not affect the TGN localization but impaired the internalization from the plasma membrane. These observations suggest that distinct cytoplasmic determinants are responsible for the furin retention in the TGN and retrieval from the cell surface (Takahashi *et al.*, 1995b). Thomas and co-worker have proposed a mechanism in which dephosphorylation of the casein kinase II site allows furin to return to the Golgi from the plasma membrane through an endocytotic recycling pathway, while phosphorylation caused it to exit the TGN (Dittie *et al.*, 1997). There is also evidence of the phosphorylation-dependent association of the cytosolic tail of furin with clathrin via AP-1 (activator protein-1) adaptor complex. The role of clathrin coating in immature regulated secretory vesicles in neuroendocrine cells appears to be retrieving furin and mannose-6-phosphate receptors from these vesicles via constitutive-like pathway (Dittie *et al.*, 1997). While the cytosolic sequence of PC5-B exhibits 4 such Tyr-motif, none is found in PC7. Although no such acidic cluster has been seen for PC7, the PC5-B sequence exhibits two acidic clusters and two potential (Ser and Thr) casein kinase II phosphorylation sites. The PC5-B or PC7 signals responsible for their TGN localization are not known. Recent data suggest that the cystine in the cytosolic tail of human PC7 is palmitoylated (Van de Loo *et al.*, 1997). The function of such post-translational modification remains unknown. A systematic approach would be to compare cytosolic binding proteins of each membrane-bound convertase. This should provide a general view of the mechanisms involved in the sorting of each convertase. Another interesting phenomenon is that the prosegment cleavage is accelerated in a truncated form of PACE4 lacking the Cys-rich domain (Mains *et al.*, 1997).

Alternatively spliced mRNA forms have been observed in almost all the PCs (Seidah *et al.*, 1994), but protein isoforms have been reported only in PC4 (Mbikay *et al.*, 1994; Seidah *et al.*, 1992b), PACE4 (Kiefer *et al.*, 1991; Mori *et al.*, 1997; Zhong *et al.*, 1996) and PC5 (Lusson *et al.*, 1993; Nakagawa *et al.*, 1993b) (Figure 1.5). The N-terminal variants of PC4 and PACE4-D lack a signal peptide or prosegment, leading to cytoplasmic localization of likely inactive enzymes. The C-terminally truncated isoform

PACE4-CS, most likely PACE4-C, lacking 11 amino acids of the P-domain, code for inactive enzymes which remain as zymogen form within the ER (Zhong *et al.*, 1996). In the case of PC5, such an alternative splicing event generating the two isoforms PC5-A and PC5-B may lead to functional diversification. Like furin, PC5-B contains a transmembrane domain and a cytosolic tail, is anchored to membranes of the TGN, and along its trafficking to the cell surface it can shed part of its carboxyl-terminal segment. The resulting shed form exits from the cell via the constitutive secretory pathway. In contrast, the soluble PC5-A forms sorted to dense core secretory granules.

Figure 1.5 Schematic representation of the reported isoforms of rat PC4, mouse PC5 and human PACE4. The active sites Asp (D), His (H) and Ser (S) as well as the catalytically important Asn (N) are emphasized.



The unique carboxyl-terminal 38 amino acids of PC5-A seems to be an important signal in this differential sorting of the PC5-A isoforms, since deletion of the 38 amino acids

PC5-A, which lack this carboxyl-terminal segment, is secreted constitutively. These data provide a unique cellular trafficking model in which alternative splicing of the PC5 gene results in two isoforms which are sorted to distinct cellular compartments, likely leading to the processing of different protein precursors (Heidner *et al.*, 1996).

The conservation of the catalytic and P-domains and the variability around these domains suggest that PC genes are evolved from a common ancestral gene through duplications, translocations, insertions, or deletions. Although these enzymes are evolutionarily related, only PC2 requires 7B2 for activation. Moreover, each member of the family has a unique promoter region, which may imply a specific regulation of the expression and transcription of each convertase. Except for closely linked furin and PACE4, all the other PCs genes are dispersed on various chromosomes (Table 1.7)

Table 1.7 Proprotein convertases genes chromosomal localization. Localization was either by cytogenetic (c) methods (including *in situ* hybridization), by analysis of somatic (s) cell hybrids or by linkage (l) analysis in human (h) or mouse (m). (Modified from Seidah *et al.*, 1998b)

PC	Chromosomal localization			
	Human (h)		Mouse (m)	
	Locus Symbol	Chr (c, s, l)	Locus Symbol	Chr (c, s, l)
PC1	*PCSK1*	5q (c)	*Pcsk1*	13 (c, l)
PC2	*PCSK2*	20p (c)	*Pcsk2*	2 (c, l)
Furin	*PCSK3*	15q (c)	*Pcsk3*	7 (c, l)
PC4	*PCSK4*	19 (s)	*Pcsk4*	10 (l)
PC5	*PCSK5*	9q (c, s)	*Pcsk5*	19 (l)
PACE4	*PCSK6*	15q (c, s)	*Pcsk6*	7 (l)
PC7	*PCSK7*	11q (c, s)	*Pcsk7*	9 (l)

(Seidah *et al.*, 1998b). While the mouse PC7 gene (Pcsk7) is located on chromosome 9 (Seidah *et al.*, 1996c), its human counterpart has been assigned to chromosome 11, at a

translocation breakpoint occurring in lymphomas (Meerabux *et al.*, 1996). Both furin and PACE4 are localized on chromosome 7 in mouse (Mbikay *et al.*, 1995), and on chromosome 15 in human (Roebroek *et al.*, 1986b; Barr, 1991; Kiefer *et al.*, 1991). The synteny between mouse and human for all the chromosomal regions carrying PC loci suggests that their multiplication and divergence occurred before the human and murine evolutionary lines branched-about 80 million years ago (Seidah *et al.*, 1998b).

1.3. Cleavage specificity and potential substrates

Most proprotein precursors exhibit multiple potential dibasic cleavage sites. However, not all of them are recognized by convertases or cleaved to generate bioactive peptides. Cellular coexpression of the PCs with proproteins together with site-directed mutagenesis revealed that each enzyme is capable of cleavage at pairs and single basic amino acid in the general consensus sequence K/R-X_n-K/R↓ where n=0, 2, 4, or 6 (X can be any amino acid except Cys and is usually not post-translationally modified (Seidah & Chrétien, 1997). Further *in vitro* (Jean *et al.*, 1995a; 1995b) and phage display data (Matthews *et al.*, 1994) revealed that the X residues and those following the site of cleavage (P′ residues) define the fine specificity of each convertase (see reviews (Chrétien *et al.*, 1995; Denault & Leduc, 1996; Nakayama, 1997)). In general, furin prefers to cleave precursors at the general sequence K/R-X-R-X-K/R-R↓ motif. Since PC7 and furin are very widely expressed in tissues and cells, and mainly reside in the TGN, precursors exhibiting the above motif will be cleaved preferentially by these enzymes. In contrast, PC1 and PC2 like simple pair of basic residues, including most but not all polypeptide hormone precursors. These endocrine and neural processing reactions occur either in the TGN or in immature secretory granules. PC5-A cleaves precursors best at R-X-X-R↓X-K or H-X-X-X-(R/K)-R↓ motifs. Sometimes monobasic, tribasic, tetrabasic or multibasic sites can also be utilized. However, in the case of monobasic cleavages another basic amino acid is usually found at position P4, P6 or P8 preceding the cleavage site. An arginine at P1 is favored over a lysine (Nakayama *et al.*, 1992b). Aromatic residues are usually not encountered in position P2 preceding the cleavage site (Devi, 1991).

The cleavage sites of PCs can be subdivided into four types (Table1.8):

Type I. includes R-X-K/R-R↓ site, normally precursors synthesized in constitutively secreting cells, such as growth factors, neurotrophins, certain receptors, bacterial toxins and viral surface glycoproteins such as gp160. This cleavage usually occurs within the TGN by one or more resident enzymes in cells devoid of dense core secretory granules, the product(s) reach the cell surface by constitutive secretory pathway.

Type II. involves cleavage C-terminal to a pair of basic residues R/K-R/K↓, and often there is a basic amino acid (R, K or H) at four (P4) or six (P6) residues N-terminal of the cleavage site. This group includes most of the polypeptide hormone precursors such as POMC, proinsulin and proenkephalin, as well as proalbumin. This cleavage usually occurs within the immature secretory granules and the cleaved products are stored in dense core granules and exit the cell via the regulated secretory pathway.

Type III. processing at monobasic cleavage residues, usually a single R↓ residue. This cleavage normally requires another basic residue at P4, P6 or P8 (Devi, 1991). Precursors in this class include polypeptide hormones such as prodynorphin and prosomatostatin also the growth factors IGF-I and II and proepidermal growth factor (proEGF). This type of cleavage can happen in both the constitutive and regulated secretory pathways.

Type IV. processing cleavage at specific single or paired basic residues. It differs from the other three by its characteristic presence of either an R or K basic residue C-terminal to the cleavage site (i.e. P2′ see Table 1.8). This includes some hormonal precursors such as promullerian inhibiting substance (proMIS), pancreatic hormones such as proglucagon and propancreatic polypeptide, as well as some growth factors such as proIGF II and membrane associated receptor R-PTPμ (Campan *et al.*, 1996). This type of cleavage can occur in either the constitutive or regulated secretory pathways.

Table 1.8 Precursor classification based on cleavage motifs.

Precursor protein	Cleavage site sequence
Type I precursors [R-X-(K/R)-R]	**P6 P5 P4 P3 P2 P1 P1' P2'**
	[X- X- **R**- X- **K/R**- **R** ↓ X- X]
Pro-βNGF	Thr-His-**Arg**-Ser-**Lys**-**Arg** ↓ Ser-Ser
Leptin pro-receptor	Gln-Val-**Arg**-Glu-**Lys**-**Arg** ↓ Leu-Asp
Integrin α6	Asn-Ser-**Arg**-Lys-**Lys**-**Arg** ↓ Glu-Ile
HIV-1 gp160	Val-Gln-**Arg**-Glu-**Lys**-**Arg** ↓ Ala-Val
Pro-fertilin α	Gln-Ser-**Arg**-Met-**Arg**-**Arg** ↓ Ala-Ala
Pro-7B2	Glu-Arg-**Arg**-Lys-**Arg**-**Arg** ↓ Ser-Val
Type II precursors [(K/R)-(K/R)]	**P6 P5 P4 P3 P2 P1 P1' P2'**
	[X- X- X- X- **K/R**- **R** ↓ X- X]
POMC (αMSH/CLIP)	Pro-Val-Gly-Lys-**Lys**-**Arg** ↓ Arg-Pro
(γLPH/βEnd)	Pro-Pro-**Lys**-Asp-**Lys**-**Arg** ↓ Tyr-Gly
Pro-Insulin (B/C chain)	Thr-Pro-**Lys**-Thr-**Arg**-**Arg** ↓ Glu-Ala
(C/A chain)	Gly-Ser-Leu-Gln-**Lys**-**Arg** ↓ Gly-Ile
Pro-Renin	Ser-Gln-Pro-Met-**Lys**-**Arg** ↓ Leu-Thr
PACAP-RP	Ala-Pro-Leu-Thr-**Lys**-**Arg** ↓ His-Ser
Integrin α4	**His**-Val-Ile-Ser-**Lys**-**Arg** ↓ Ser-Thr
Pro-fertilin β	Ser-Cys-**Lys**-Leu-**Lys**-**Arg** ↓ Arg-Gly
Type III precursors [Single R]	**P8 P7 P6 P5 P4 P3 P2 P1 P1' P2'**
	(B)- X- **(B)**- X- **(B)**- X- X **R** ↓ X - X
Pro-Dynorphin (C-peptide)	**Arg**-Gln-Phe-Lys-Val-Val-Thr-**Arg** ↓ Ser-Gln
Pro-ANF	**Arg**-Ala-Leu-Leu-Thr-Ala-Pro-**Arg** ↓ Ser-Leu
Pro-Somatostatin (SS-28)	Glu-Met-**Arg**-Leu-Glu-Leu-Gln-**Arg** ↓ Ser-Ala
Pro-IGF-I	Leu-Lys-Pro-Thr-**Lys**-Ala-Ala-**Arg** ↓ Ser-Ile
Pro-IGF-II	Ala-Thr-Pro-Ala-**Lys**-Ser-Glu-**Arg** ↓ Asp-Val
Pro-EGF (N-terminal)	Glu-Asp-Gly-His-**His**-Leu-Asp-**Arg** ↓ Asn-Ser
Pro-EGF (C-terminal)	Asp-Leu-**Arg**-Trp-Trp-Glu-Leu-**Arg** ↓ His-Ala
Type IV precursors [P2'= R/K]	**P8 P7 P6 P5 P4 P3 P2 P1 P1' P2'**
	(B)- X- **(B)**- X- **(B)**- X- **(B)**-**R/K** ↓ X -**R/K**
PACAP-RP	Leu-Ala-Ala-Val-Leu-Gly-**Lys**-**Arg** ↓ Tyr-**Lys**
Pro-Mullerian Inhibiting Substance	Glu-Gly-**Arg**-Gly-**Arg**-Ala-Gly-**Arg** ↓ Ser-**Lys**
Pro-Glucagon	Gln-Trp-Leu-Met-Asn-Thr-**Lys**-**Arg** ↓ Asn-**Arg**
Pro-IGF-II	Glu-Ala-Phe-Arg-Glu-Ala-**Lys**-**Arg** ↓ His-**Arg**
Pro-PTP-μ receptor	Val-Glu-Glu-Glu-**Arg**-Pro-**Arg**-**Arg** ↓ Thr-**Lys**

1.4. Tissue expression and subcellular location of PCs

The functional specialization of different PCs correlates very well with their observed cellular and tissue distribution. The expression of PCs was mostly analyzed by Northern-blot and *in situ* hybridization histochemistry. Based on their tissue distribution and intracellular localization, the mammalian subtilisin/kexin-like serine proteinases could be subdivided into four classes (Seidah *et al.*, 1998a) (Table 1.9) where:

1. Furin and the recently discovered PC7 process precursors that reach the cell surface via constitutive secretory pathway;
2. PC1, PC2 and the isoform PC5-A process precursors whose products are stored in secretory granules;
3. PACE4 and PC5, which are expressed in both endocrine and nonendocrine cells, conceivably process precursors in both the constitutive and regulated secretory pathways;
4. PC4 is predominantly synthesized in testicular germ cells.

The class I convertases furin and PC7 are very widely expressed in all mammalian cells. Their homologues in lower vertebrates and invertebrates also exhibit similar widespread expression (Seidah *et al.*, 1998a; Vandeven *et al.*, 1993). These two convertases are localized within the TGN and could cycle to the cell surface and enter endosomal compartments (Sariola *et al.*, 1995; Van de Loo *et al.*, 1997). The *in vitro* characterization of PC7 is presented in the Appendices. The widespread expression of these enzymes suggests that they are of prime importance for the metabolism of the cells. The class II convertases PC1 and PC2 exhibit a restricted distribution primarily expressed in endocrine and neural cells. They traverse the TGN but are ultimately localized within mature secretory granules (Heidner *et al.*, 1996; Malide *et al.*, 1995). The class III PC5 and PACE4 are widely expressed enzymes and are detected early during embryonic development (Zheng *et al.*, 1997). In the adult, PC5 is highly expressed in the gut, endothelial and Sertoli cells as well as the adrenal cortex. Immunoelectron microscopy and biosynthetic analyses revealed that PC5A (class II) could be found in the TGN and/or in the granules, however its isoform PC5-B mostly resides in the TGN and cycles to cell surface processing precursors within the constitutive secretory pathway (Heidner *et al.*, 1996). PACE4 is found at extremely high levels in most cells of the anterior pituitary and

Table 1.9 Tissue and cellular expression of the PCs

Convertase (PC)	Tissue expression	Cellular Localization
Class I		
Furin	**Ubiquitous**, but very rich in a Number of Tissues	TGN, **Endosomes**, Cell Surface
PC7	**Widespread**, very rich in Lymphoid-Associated Tissues	**TGN**
Class II		
PC1	**Neural and Endocrine Cells**, rich in Peripheral tissues and CNS	TGN, **Secretory Granules**
PC2 (7B2)	**Neural and Endocrine Cells**, rich in Peripheral tissues and very rich in the CNS	**Secretory Granules**
Class III		
PC5-A	**Endocrine & Non-Endocrine, Widespread**, very rich in Adrenal Cortex, Endothelial & Sertoli Cells and Digestive System	TGN, **Secretory Granules**
PC5-B	**Restricted**, rich in Digestive System and Adrenal Cortex	**TGN**
PACE4	**Endocrine & Non-Endocrine, Widespread**, rich in Pituitary and Chondrocytes; Important in Bone Morphogenesis	**TGN, Secretory Granules?**
Class IV		
PC4	**Testicular and Ovarian Germ cells**, Important in Fertility	**TGN?**

in cerebellar Purkinje cells. Its mRNA is found in subpopulations of both neuronal and non-neuronal cells. PACE4 expression was very high in some restricted areas of central nervous system (CNS), but many other regions expressed low level PACE4 (Dong *et al.*, 1995). PACE4 is not found at significant levels in a number of non-neuroendocrine tissues such as liver, kidney, spleen, lung, gastrointestinal tract, germ cells and submaxillary glands (Dong *et al.*, 1995; Johnson *et al.*, 1994). It has been suggested that PACE4 may process precursors within the constitutive secretory pathway (Mains *et al.*, 1997). The class IV convertase PC4 exhibits the most restricted distribution of all known mammalian convertases, being detected only in germinal tissues, especially in rat and mouse testis (Nakayama *et al.*, 1992a; Seidah *et al.*, 1993; Torii *et al.*, 1993) and more specifically in spermatocytes and round spermatids. No cell line has so far been demonstrated to express PC4. As the present view of the subcellular localization of the PCs, PC1, PC2 and PC5-A are mostly localized within dense core secretory granules in regulated cells, and furin, PC5-B and PC7 are localized to the TGN in a manner regulated by their cytosolic tail. They can cycle to the cell surface and are retrieved back to the TGN via an endosomal pathway. The exact intracellular localization of PC4 and PACE4 are yet to be defined.

Defining the cellular colocalization of each PC with its cognate substrate(s) is necessary for ascribing the roles of these enzymes in particular precursor processing events (Day *et al.*, 1992; Decroly *et al.*, 1996; Dong *et al.*, 1997; Malide *et al.*, 1995; Marcinkiewicz *et al.*, 1993; Schaner *et al.*, 1997; Wetsel *et al.*, 1995). In addition, dramatic developmental changes in the tissue expression of the PCs have been reported (Marcinkiewicz *et al.*, 1994; Seidah *et al.*, 1998a; Zheng *et al.*, 1994; 1997). In the adult, numerous publications described the cell and tissue distribution of the PCs (Beaubien *et al.*, 1995; Day *et al.*, 1993; Dong *et al.*, 1995; Hendy *et al.*, 1995; Marcinkiewicz *et al.*, 1996; Schafer *et al.*, 1993; Seidah *et al.*, 1993), where unique pattern of each PC is listed (Rouille *et al.*, 1995; Seidah *et al.*, 1994; 1996c; 1998a). For example in the CNS (Day *et al.*, 1993; Dong *et al.*, 1995; 1997; Schafer *et al* 1993), pituitary (Day *et al.*, 1992; Dong *et al.*, 1995; Marcinkiewicz *et al.*, 1993), peripheral nervous system (PNS) (and some peripheral organs including the heart (Beaubien *et al.*, 1995), as well as the thyroid

(Hendy *et al.*, 1995), adrenals (Lusson *et al.*, 1993; Seidah *et al.*, 1998a), gut and gonads (Marcinkiewicz *et al.*, 1996). The study of the co-regulation of PCs and their cognate substrate(s) also provided hints on their physiological role(s) *in vivo*. Alarcon *et al.*, in their study of proinsulin, PC1 and PC2, demonstrated in isolated rat pancreatic islets, the biosynthesis of PC1 was specifically stimulated by glucose relatively parallel to that of proinsulin (Alarcon *et al.*, 1993). In contrast, PC2 biosynthesis was not glucose-regulated. The finding is in agreement with the implication that PC1 is the key endopeptidase in proinsulin to insulin conversion, and is the enzyme that preferentially initiates the process (Rhodes *et al.*, 1992). Other attempts have been made as well (Bloomquist *et al.*, 1991; Day *et al.*, 1992; Dong *et al.*, 1997; Johnson *et al.*, 1994; Marcinkiewicz *et al.*, 1993; Oyarce *et al.*, 1996; Rancourt & Rancourt 1997; Rouille *et al.*, 1995) to address the question of physiological role(s) of PCs *in vivo*.

1.5. Implication in disease states

From the apparent variety of physiologically important substrates that may require proteolytic activation by PCs, it is reasonable to presume that reduced, excessive or ectopic production of one or the other of these enzymes will cause serious pathological conditions in human, manifesting either as generalized physiological imbalances or as localized anomalies. The clinical symptoms that such abnormal expression could induce will probably be very pleotropic due to the relatively broad distribution of many of these enzymes (Chrétien *et al.*, 1995). In 1978, Brennan and Carrell (Brennan & Carrell, 1978) found that a family with a circulating variant of human proalbumin, named proalbumin Christchurch, had a mutation of the P1 Arg residue to Gln. To date many proalbumin variants with a mutation of the Arg residue at P1 or P2 position to a non-basic residue have been reported (Nakayama, 1997). None of the examined proalbumin variants are cleaved by furin *in vitro* (Brennan & Nakayama, 1994). The only known functional difference of these proalbumin variants from mature serum albumin is their decreased affinity for binding Cu^{2+}. There has been one report for a homozygous individual with one of these variants revealing that such mutation is not lethal (Matsuda *et al.*, 1986). A more recent report by the same group on a different thrombin substrate fibrinogen

(Brennan *et al.*, 1995) found that a heterozygous patient with prolonged thrombin time and a mild bleeding tendency has a mutation of Val to Asp near the fibtinopeptide A cleavage site of the fibrinogen A α-chain. This mutation changes the normal RGPRVV sequence into RGPRDV, creating a potential furin cleavage site at RGPR↓DV. This aberrant intracellular cleavage probably by furin gives rise to a three-residue truncated form of the α-chain without cleavage by thrombin.

Proteolytic activation of envelope glycoproteins is necessary for the entry of viruses into host cells and their ability to undergo multiple replication cycles. The cleavability of the envelope glycoproteins has been shown to be important determinant for viral pathogenicity. The hemagglutinins (HAs) of the influenza are susceptible to proteolytic cleavage after R-X-K-R↓ site, which completely fit the consensus sequence for cleavage by furin. *In vitro* experiments using purified furin and co-expression experiments have shown that furin is indeed involved in the cleavage of the glycoprotein precursors of virulent viruses including pathogenic avian influenza viruses, paramyxovirus, human parainfluenza virus, human cytomegalovirus, and human immunodeficiency virus (Garten *et al.*, 1994; Gotoh *et al.*, 1992; Hallenberger *et al.*, 1992; Horimoto *et al.*, 1994; Stieneke-Grober *et al.*, 1992). Furthermore, at least furin has been implicated in the processing of toxins such as those of *aeromonas hydrophila* (pore-forming proaerolysin) (Abrami *et al.*, 1998), anthrax (Klimpel *et al.*, 1992), *pseudomonas* and *diptheria* (Chiron *et al.*, 1994). Cleavage of these toxins at the cell surface of in endosomal compartments is essential for their virulence (Gordon *et al.*, 1995). Experiments in which the activation of these proteins has been prevented through the inhibition of furin and possibly other PCs indicate that there is considerable promise in these novel approaches to treating such pathologies. Among the important substrates for furin are many blood clotting factors and serum proteins that are produced in the liver (where furin is expressed at very high levels). In addition, PCs participates in processing a wide variety of growth factors (Mbikay *et al.*, 1993), which are physiological regulators of normal cell division. After binding to its specific surface receptors, these growth factors initiate a cascade of molecular signaling events which are relayed by the second messengers to the nucleus, generating significant changes in the pattern of gene

expression. The result of this process on cell division could be either stimulatory or inhibitory, depending on the factor, the responsive cell and the tissue context. Proliferative diseases, as consequences of unchecked cell division, often involve the immediate participation of growth factors and, more upstream in the expression pathway, of the PCs responsible for their maturation.

In 1995, the first description of the effects of defects in the gene encoding PC1 was revealed by studies of a patient with severe obesity and hyperproinsulinemia (O'Rahilly *et al.*, 1995). The patient is a compound heterozygote, carrying two different mutations in the alleles of the PC1 gene (Jackson *et al.*, 1997). The first allele carries a $Gly \rightarrow Arg_{483}$ mutation which leads to proPC1 retention in the ER. The second mutation on second allele affects the donor site of intron 5 and causes skipping of exon 5 during transcription, resulting in the loss of 26 amino acids, a frameshift and creation of a premature stop codon within the catalytic domain, thus leading to an inactive PC1. The clinical picture of this patient clearly implicates PC1 in the intricate network of signaling pathways that control body mass and gonadal development, most likely through its processing/activating action on prohormones (proinsulin and POMC) and proneuropeptides. This is the first ever report on naturally occurring mutants of any PCs whatsoever.

Recently, genetic methods of gene disruption including the use of antisense technology (Bloomquist *et al.*, 1991; Rothenberg *et al.*, 1996) and genetic recombination techniques creating PC-null mice have been introduced. Using antisense transgene inhibition Bloomquist *et al* showed that PC1 deficiency in AtT20 cells caused POMC to accumulate and its conversion to ACTH to diminish, supporting a critical role of PC1 in the production ACTH. Similarly, transgene-directed antisense RNA has been used to demonstrate the converting action on several other precursors in various cell types (Seidah *et al.*, 1998b): furin on pro-parathyroid hormone-related peptide (proPTH-RP) monkey kidney COS-7 cells (Liu *et al.*, 1995); PC2 on POMC in rat sommatomammmotrope GH3 (Friedman *et al.*, 1996); PC1 and PC2 on proglucagon in mouse glucagonoma aTC1-6 cells (Rothenberg *et al.*, 1996; Rouille *et al.*, 1994); PC2 on proneuropeptide Y (proNPY) in cultured sympathetic neurons (Paquet *et al.*, 1996); PC2

on proneurotensin (proNT) in rat medullo-thyroid carcinoma rMTC 6-23 cells (Rovere *et al.*, 1996); and PC1 on procholecystokinin (proCCK) in rat insulinoma Rin5F cells (Yoon *et al.*, 1996). A dependence of IGF-R processing on furin may explain the anti-proliferative effect of antisense transgene or oligonucleotide on mouse gastric mucus GDM6 cells (Konda *et al.*, 1997) and on the well differentiated pancreatic beta cell line MIN6 (Kayo *et al.*, 1996).

The production of null mice allowed the assessment of the critical role of convertases *in vivo*. PC4 knockout mice (Mbikay *et al.*, 1997) result in a significant decrease in male fertility, consistent with its highly restricted expression in spermatogenic cells. Spermatogenesis is apparently normal in this mouse, however the spermatozoa are less efficient at fertilizing oocytes, and zygotes derived from them fail to develop to the blastocyst stage. These results suggest a role for PC4 in production of fertile and developmentally competent spermatozoa.

The PC2 null mice have been generated with a mutation in the third exon of the PC2 gene, which leads to the synthesis of a defective proenzyme lacking this exon (Furuta *et al.*, 1997; 1998). Exon 3 encodes a region spanning the carboxyl-terminal end of the prosegment, the beginning of the catalytic domain and the site for the proteolytic autoactivation of the proenzyme to its active form. This deletion leads to the failure of proPC2 to fold normally and the truncated enzyme appears to be degraded in the ER. No evidence for conversion to an active form has been found. The affected mice are still able to reproduce but grow at a slightly subnormal rate. They have elevated proinsulin-like material (insulin and intermediates of proinsulin conversion to insulin) in the circulation with levels reaching approximately 60% of the total immunoreactive insulin. Pancreatic islets in these mice are deficient in their processing of precursors to glucagon, insulin and somatostatin. This deficiency is associated with a secondary hyperplasia of α and δ cells. The mild phenotype resulting from PC2 inactivation was not expected based on its relatively abundant distribution in most neuronal cells and endocrine cells. However, it is likely that the lack of PC2 is partially alleviated by the co-resident PC1. No PC1-null mouse has been described yet.

Furin knock out was recently achieved in mice (Roebroek *et al.*, 1998). Furin transcripts become detectable at early embryonic stages (day 7.5), Embryos lacking Furin fail to undergo axial rotation and die between days 10.5 and 11.5, presumably due to hemodynamic insufficiency associated with severe ventral closure defects and the failure of the heart tube to fuse and undergo looping morphogenesis. The observed dysmorphisms are in agreement with the experimental data derived from cells transfected with inducible furin sense or antisense transgenes, which show that the furin level influences the equilibrium between cell proliferation and cell differentiation (Kayo *et al.*, 1996; Konda *et al.*, 1997).

1.6. Inhibitor of PCs

The accumulated data implicate the PCs in the normal physiology of healthy cells as well as in pathologies such as cancer, viral infections including AIDS, in atherosclerosis, obesity, diabetes and in hypertension (Chrétien *et al.*, 1995). In view of the potential clinical and pharmacological role of the convertase, it was of interest to produce specific PC inhibitors. Several attempts have been made with different strategies involved the development of either peptide-based PC inhibitors, or protein based inhibitors (Seidah *et al* 1998b). These included the use of irreversible chloromethylketone inhibitors (Hallenberger *et al.*, 1992; Jean *et al.*, 1995b) and reversible peptide inhibitors (Angliker, 1995; Jean *et al.*, 1995a). Major limitations of these agents are their cytotoxicity, through interfering with the biosynthesis of many important cellular proteins, and their relatively poor cellular permeability and targeting. A recently identified peptide-based inhibitor which shows promise in terms of selectivity and potency is the C-terminal 31 amino acid of the PC2-specific binding protein 7B2, called CT-peptide (Zhu *et al.*, 1996). This peptide is highly selective for PC2 and requires the presence of the Lys-Lys bond in the CT-peptide of 7B2 for maximal activity. Another very recently identified inhibitory polypeptide is the C-terminal segments of PC1, which is a potent inhibitor of this enzyme, preventing its activity on certain substrates, e.g., prorenin, until the enzyme/substrate complex reaches immature secretory granules (Jutras *et al.*, 1997).

Alternatively, recombinant protein-based inhibitors have been developed. In 1987 Brennan and coworker observed a child with a bleeding disorder having proalbumin circulating which was caused by an unusual alpha 1-antitrypsin mutation (Bathurst *et al.*, 1987). This observation led to the speculation that the presence of this alpha 1-antitrypsin (α_1-AT) mutant, known as α_1-AT Pittsburgh (α_1-PIT), the sequence being ALP**R** instead of ALPM was inhibitory to the convertase furin (Brennan & Peach, 1988). The mutation changes the specificity of this serpin from an inhibitor of elastase (Kurachi *et al.*, 1981) into an inhibitor of thrombin (Owen *et al.*, 1983) and less effectively of furin. This provided an additional base of construct another inhibitor, that contain a furin-like recognition sequence **RLPR** within the inhibitor binding region of either human α_1-antitrypsin (Anderson *et al.*, 1993). This new variant, called α_1-AT-Portland (α_1-PDX) was shown to inhibit furin with a *Ki* of 0.6 nM (Jean *et al.*, 1998). Furthermore, Decroly *et al* demonstrated that, *in vitro*, α_1-PDX is an inhibitor of all tested PCs (Decroly *et al.*, 1996). However, Vollenweider and colleagues showed that in AtT20 cells α_1-PDX only partially inhibits the endogenous processing of gp160 or exogenous processing by furin, PACE4 and PC5-B (Vollenweider *et al.*, 1996). α_1-PDX acts primarily with the constitutive secretory pathway (Benjannet *et al.*, 1997). Biosynthetic and immunocytochemical analyses of AtT20 cells stable transfected with α_1-PDX demonstrated that this 64 kDa serpin is primarily localized within the TGN. A small portion enters secretory granules where it is mostly stored as an inactive 56 kDa product resulting from cleavage of the active 64 kDa form at the engineered RIPR site. Furthermore, expression of α_1-PDX resulted in modified contents of mature secretory granules with increased levels of partially processed products, suggesting a delayed processing. Accordingly, it became apparent that α_1-PDX may not inhibit the processing of all precursors to a similar extent and that processing inhibition occurs primarily within the constitutive secretory pathway (Seidah *et al.*, 1998b). Therefore, α_1-PDX is very useful protein inhibitor to inhibit processing of precursors including endogenous growth factors and viral surface glycoproteins in constitutive cells, and may exhibit a limited toxicity to cells *ex vivo*. On the other hand, strategies based on the expression of proteins like α_2-macroglubulin (Van Rompaey *et al.*, 1997), proteinase-8 (Dahlen *et al.*, 1998), or

the turkey ovomucoid third domain (Lu *et al.*, 1993) have also been used by other groups. Although often reasonably effective, these inhibitors may show little or no discrimination among PC family members. In chapter 2, I present our study on the prosegment of PC7 and furin, which provides a novel enzyme silencing strategy that can be exploited in the treatment of a variety of pathologies (Zhong *et al.*, 1999).

2. Zymogen activation and prosegment inhibition

Proteolytic enzymes are synthesized as inactive zymogens to prevent random protein degradation, and to ensure spatial and temporal regulation of proteolytic activity. Upon sorting to the right compartment. zymogen became active enzyme. This conversion typically involves limited proteolysis and removal of an "activation segment." The sizes of activation segments varies from dipeptide units to independently folding domains comprising more than 100 amino acids. A common form of the activation segment is an N-terminal extension of the mature enzyme, prosegment, that sterically blocks the active site, and thereby prevents enzyme from binding its substrates. In addition to their inhibitory role, prosegments are frequently important for the folding, stability, and/or intracellular sorting of the zymogen. The mechanisms of conversion to active enzymes are diverse in nature, ranging from enzymatic or nonenzymatic cofactors that trigger activation, to a simple change in pH that results in conversion by an autocatalytic mechanism (Khan & James, 1998).

2.1. General activation mechanisms

All known cellular and bacterial proteolytic enzymes are synthesized as inactive zymogens to prevent unwanted protein degradation. Zymogen conversion to the active enzyme generally occurs by limited proteolysis of an inhibitory "activation segments" within a subcellular compartment or the extracellular milieu, in which the particular enzyme exerts its biological function. Conversion may involve accessory molecules, or the process may be autocatalytic, requiring no additional factors other than a drop in pH. Activation segments have also been observed as insertions within the primary sequence of the mature enzyme, between the catalytic residues (Rudenko *et al.*, 1995). However,

the activation segment has never been found at the C-terminus of a zymogen, thus precluding the risk of the active sites gaining activity before the synthesis of the polypeptide is complete. Some activation segments have additional roles in protein folding and/or intracellular sorting like subtilisin (Strausberg *et al.*, 1993a; 1993b). The inhibitory mechanisms utilized by activation segments are diverse, but a common property of zymogens is that the "catalytic machinery" is preformed. Here, the "catalytic machinery" refers to all aspects of the active site (bond-cleavage apparatus, substrate-binding cleft) that are required for productive cleavage of the peptide bonds. For example, the positions and conformations of the catalytic triads of the serine proteinase in zymogens are virtually identical to their corresponding active forms. However the conversion process often involves significant conformational changes in regions that are adjacent to the active site, or within the activation segments that are subsequently removed (Blevins & Tulinsky 1985; Gallagher *et al.*, 1995; Khan & James, 1998).

A recurring theme for many zymogens is the presence of a preformed active site that is rendered sterically inaccessible to substrates by a competitive inhibitory prosegment (Bode & Huber, 1992; Sohl et al., 1997). However, the prosegment-active site interactions are generally loose and poised for disruption, unlike small molecule or peptide inhibitors of the active enzymes that bind tightly and often mimic the cleavage transition state (Khan & James, 1998). Another general feature of the inhibitory interactions is the strategic positioning of prosegments to prevent self-cleavage prior to the appropriate signal. For example, the prosegments of papain-like cystein proteinases extend across the active site in the reverse N→C-terminal orientation that is necessary for productive cleavage. (Cygler *et al.*, 1996; Turk *et al.*, 1996).

The mature segments of zymogens may be subjected to large conformational changes during conversion, or they may remain unchanged, e.g. subtilisin undergoes a major rearrangement of the first ten residues at the mature N-terminus following limited proteolysis at their pro-mature junction (Gallagher *et al.*, 1995). A common feature of zymogens that are activated in low pH compartments is the critical role of salt bridges in maintaining the stability of the prosegment and its interactions with the mature segment. These interactions may be essential for the stability of zymogens in a neutral pH

environment. In the zymogens, like furin, the prosegments are positively charged and form several salt bridges with the mature segment. thus protecting the enzyme during its passage through a cellular environment. Upon transport of zymogens to their appropriate compartments, prosegments are removed by limited proteolysis, often by an autocatalytic mechanism. Autocatalytic conversion is perhaps the most economical mechanism since no other enzymatic cofactors are necessary for activation. In this case, conversion typically requires a conformational change in the prosegment in order to uncover the active site and thereby generate active forms of zymogen. Limited proteolysis and removal of the prosegment may involve unimolecular and/or bimolecular cleavage events. Whether the mechanism is autocatalytic or dependent upon their enzymes, the conversion process frequently proceeds through step-wise proteolytic cleavage to remove the entire prosegment. Following conversion, the discarded prosegments are typically degraded, often by the newly activated enzyme. Digestion of the prosegment of subtilisin is rationalized as a means of recycling the amino acids for subsequent use by the bacterium (Gallagher *et al.*, 1995). It can further be reasoned that the hydrolysis of prosegments ensures that the conversion process is irreversible and that the prosegments do not act as competitive inhibitors of the active enzymes (Khan & James, 1998).

2.2. Prosegment mediates inhibition

The prosegment of bacterial subtilases can act as an intramolecular chaperone assisting the correct folding of the enzyme. Following its autocatalytic cleavage, the prosegment could also behave as a competitive inhibitor keeping the enzyme activity in check until it is destroyed (Eder *et al.*, 1993; Inouye, 1991; Shinde & Inouye, 1993; Winthers *et al.* 1991). In Figure 1.2, we show the results of the alignment of the primary amino acid sequences of the seven mammalian convertases' prosegments. Notice the Q, G and two A residues which are unique to mammalian subtilases. This suggests that these residues (and possibly others) could be critical for the function of the basic prosegment (*pI* Basic, Table 1.3) exhibiting unevenly distributed basic amino acids which are concentrated around the cleavage sites. Two clusters of pairs of basic residues are found in each eukaryotic convertase. The deduced consensus sequence at the last cluster (K/R)-

X-R-X(K/R)-R is that of a type I precursor and, with the exception of PC2, we also notice the presence of a P6 K/R or P8 R for PC7. Cleavage of this cluster results in convertase activation. In contrast, the first basic signal can either be that of a type I or II precursor. Biosynthetic evidence revealed that cleavages at both clusters can occur for PC1 and PC2 with the one producing the active enzyme being the preferred one (Benjannet *et al.*, 1993; Matthews *et al.*, 1994). These results suggest that either these two sites act in competition or are cleaved in succession. So far, little is known about the fate of the prosegment of the convertases. Upon its cleavage does it remain within the ER? Alternatively, in some convertase(s) and similar to subtilases, is it possible that the prosegment acts as a competitive inhibitor non-covalently bound to the enzyme until the latter reaches the TGN where the complex dissociates and the prosegment is concomitantly broken down? In the case of furin it was recently suggested that the latter model is applicable with the prosegment exhibiting *in vitro* an inhibitory activity on furin with a *K*i of 14 nM (Anderson *et al.*, 1997).

Other example of prosegment function involves (Andersson *et al.*, 1998) studying the role of the propeptide for processing and sorting of human myeloperoxidase (MPO). MPO normally stored in azurophil granules of neutrophils, is critical for an optimal oxygen-dependent microbicidal activity of these cells. The propeptide of MPO may have a role in retention and folding of the nascent protein into its tertiary structure or in targeting of pro-MPO for processing and storage in granules. Elimination of the propeptide of pro-MPO blocks the maturation process and abolishes accumulation of the final product in granules suggesting a critical role of the propeptide for late processing of pro-MPO and targeting for storage in granules (Andersson *et al.*, 1998).

2.3. Prosegment swapping between PCs

The prosegments of mammalian PCs exhibit 30-67% sequence identity to each other (Table 1.4, Figure 1.2), with PC7 being the most distant member. The initial cleavage of the prosegment at the R-X-K-R↓ does not result in the immediate activation of the enzyme, but rather until its reaches its final destination or conformation where a secondary cleavage at another dibasic site and pH drop in the TGN or secretory granules

helps the releasing of the prosegment and the fully activation of the PCs (Anderson *et al.*, 1997). As PC7 and PC4 do not have a secondary KR sites in their sequences, the above mechanism may not apply.

Since the prosegment helps the folding of the enzyme and also appears to be a potent inhibitor to its parent enzyme, the question arises whether one prosegment of a certain PC can be replaced by another? PC1 chimeric protein with its prosegment substituted by that of furin was cleaved after its prosegment, producing active PC1 enzyme. A similar furin/PC2 fusion protein underwent prosegment cleavage with low efficiency. However, when the prosegments of PC1 and PC2 were exchanged with one another, both fusion proteins failed to cleave the foreign prosegments, were unable to undergo oligosaccharide maturation, and remained in the ER as zymogen forms. These results further emphasized the uniqueness of PC2, which is the only known convertase whose prosegment is removed later along the secretory pathway (TGN/immature granules) and requires the participation of a specific binding protein 7B2 for its efficient zymogen activation. None of the inactive PC mutants interfered with the processing of endogenous active PCs or with POMC processing even though in theory the mutants could function as dominant negatives (Zhou *et al.*, 1995). A mutant form of furin that lacked the prosegment was nonfunctional, and the addition of PC2 prosegment, did not restore activity. By analogy to the bacterial subtilisin family, the prosegment of PCs may guide the folding of PCs into active enzymes (Rehemtulla *et al.*, 1992a). The prosegment of PACE4 is slightly longer than the prosegments of other PCs, and the signal peptide of PACE4 is much longer than that of other members of this family. Chimeric PACE4 in which the prosegment of PC1 replaced that of PACE4 revealed normal processing and activity of PACE4 (Mains *et al.*, 1997). It appears that the prosegments of these PC proteins are replaceable in a molecule-specific manner, removal of prosegment is essential for sorting the mature enzyme and for endoproteolytic activity. These prosegments swapping data suggested that some of the prosegments are interchangeable and hence could act as inhibitors to more than their parent enzymes. In chapter 2 we present our studies on the prosegments of PC7 and furin as well as a series of peptides derived after these prosegments (Zhong *et al.*, 1999).

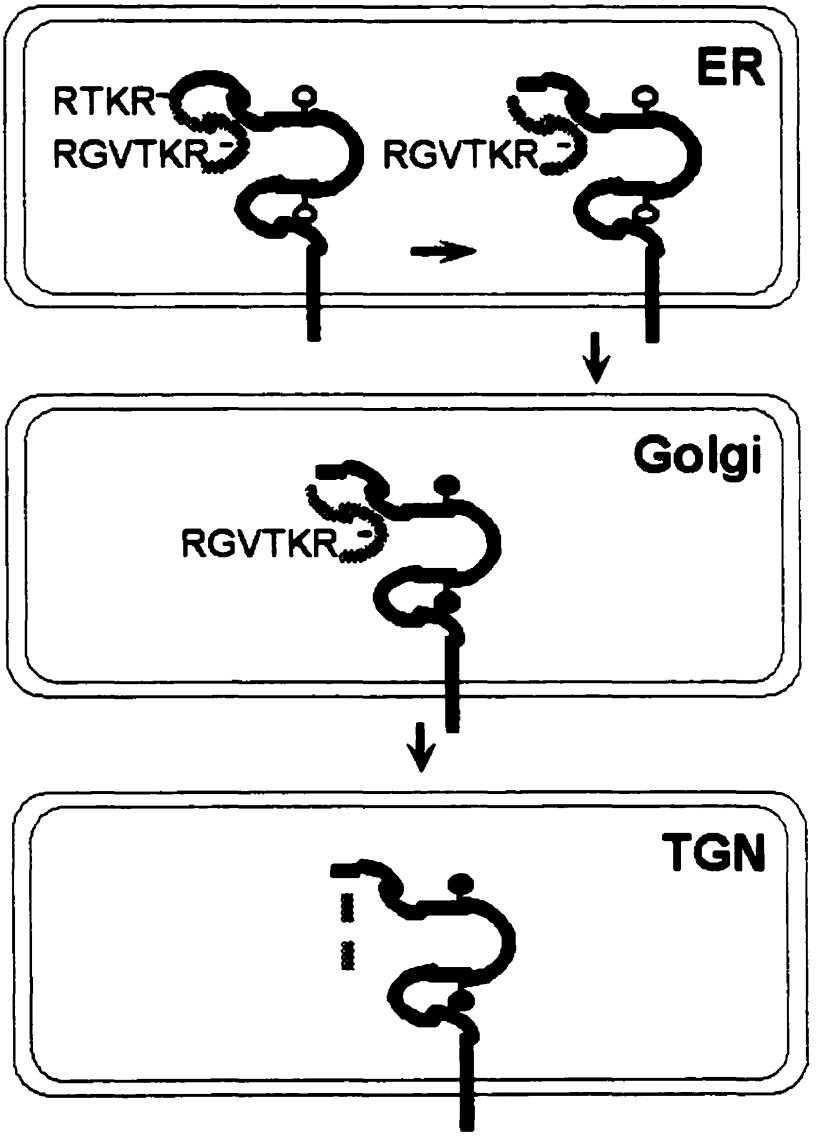
2.4. Regulation of proteolytic activity

The activation of furin has recently been elucidated in elegant detail by Thomas and co-workers (Anderson *et al.*, 1997) and appears to be a useful model for many of the other convertases. In this model (Figure 1.6), by both immunocytochemical and kinetic studies, furin is synthesized as a proenzyme, requires the amino-terminal prosegment for efficient folding in the ER. As soon as a folded state is achieved, autocatalytic cleavage of the prosegment occurs while the enzyme-prosegment complex is still in the ER. This initial cleavage at the carboxyl-terminus R-T-K-R↓ of the prosegment is necessary but not sufficient for full activation of the enzyme. As the prosegment remains attached noncovalently and functions as a potent inhibitor, *in trans* substrates can not be cleaved at this point (Anderson *et al.*, 1997). The cleaved prosegment-enzyme complex then exits the ER and passes through the Golgi to the TGN where the more acidic, calcium-enriched environment facilitates cleavage at a second site in the prosegment R-G-V-T-K-R↓ and assists in the dissociation of the prosegment from its inhibitory attachment to the catalytic domain. Full activation of furin can be achieved by either exposure to an acidic, rich-calcium environment like the TGN or by mild trypsinization at neutral pH (Anderson *et al.*, 1997).

A similar mechanism of activation seems likely for PC1, which is also known to undergo rapid autoactivation early in the secretory pathway. This processing PC1 was blocked when the active site aspartate was changed to asparagine, suggesting that an autocatalytic mechanism was involved. In this system, processing of pro-PC1 was optimal between pH 7.0 and 8.0 and was not dependent on additional calcium. These results are consistent with pro-PC1 maturation occurring at an early stage in the secretory pathway, possibly within the ER, where the pH would be close to neutral and the calcium concentration less than that observed in later compartments (Shennan *et al.*, 1995). In contrast, PC2 has a more complex mechanism of transport and activation. This convertase is unique in that it requires 7B2, a 27 kDa neuroendocrine secretory protein, and acidic conditions of a late post-Golgi compartment for activation. Although it was

first proposed that 7B2 might function as a chaperone protein to assist in proPC2 folding (Braks & Martens, 1994), recent result indicate that proPC2 binds to 7B2 after its folding

Figure 1.6 Schematic representation of the various steps in the maturation pathway of furin.



is completed (Muller *et al.*, 1997). The 7B2 association then facilitates the transport of the proPC2 from the ER. Subsequently, 7B2 is processed at a polybasic site toward the carboxyl-terminus, most likely by furin or related proteases (Benjannet *et al.*, 1995b) to release an inhibitory carboxyl-terminal (CT fragment) (Lindberg *et al.*, 1995; Martens *et al.*, 1994). This fragment contains a lysine-lysine pair which is required for its inhibitory action and which serves as a site of very slow cleavage by PC2. In the presence of carboxypeptidase E (CPE), the inhibitory effect if the CT fragment is relieved by the removal of the two lysine residues (Zhu *et al.*, 1996).

2.5. Integrity of the proteinase domains

2.5.1 Catalytic domain- The catalytic domain of the convertases has the highest structure homology among all the PCs. The resemblance is particularly strong between PC5 and PACE4, which share 75% identity of the catalytic region (Table 1.5). Several reports have been related to the integrity of the proteinase domain. Site-directed mutagenesis of proPC2 showed that a single residue replacement in the catalytic domain, Tyr^{194} into Asp (Y^{86}/D Figure 1.3), prevented proPC2 from binding 7B2 and blocked activation. This residue is present within a loop rich in aromatic amino acids which appears to be on the surface of the molecule as extrapolated from the crystal structure of subtilisin. This loop may represent the primary recognition site for 7B2 within the catalytic domain (Zhu *et al.*, 1998). It has been demonstrated that the PC2 oxyanion hole Asp residue is essential for the binding of the proPC2 to pro7B2 within the ER (Benjannet *et al.*, 1995a) and the carboxyl-terminal domain of PC2 is not involved in 7B2 binding. Furthermore, coexpression of a PC2 mutant in which the Asp was replaced by an Asn residue did not demonstrate differences in kinetics of prosegment cleavage or substrate cleavage, regards to the chosen substrate POMC (Zhou *et al.*, 1995). However, the specific substrate selectivity of the wild type and mutant PC2 were not investigated in this experiment. Several studies have been related to the catalytic domain of furin, where substitution of the Asn by an Ala blocks substrate processing but not prosegment removal, nor the subcellular distribution of this enzyme (Creemers *et al.*, 1993; 1995). In the meantime, substitution of the Asn for Asp in kexin resulted in reduced catalytic activity but

unchanged prosegment cleavage (Brenner *et al.*, 1993). This oxyanion hole-mutated kexin was shed to the same extend as wild type kexin from the cells in which they were expressed, demonstrating that it is not retained in the ER, where it could still undergo prosegment cleavage, but would not have access to its substrates (Brenner *et al.*, 1993). Furin proteins encoded by oxyanion hole mutant N^{295}/A and negative side chain mutant D^{355}/L, which possess autoprocessing activity but lack substrate processing activity, were found in the Golgi and the ER, respectively (Creemers *et al.*, 1995). It seems that the oxyanion hole is not needed for the intramolecular autocatalytic cleavage of the prosegment at the primary site, but is required for *in trans* catalysis of other substrates.

2.5.2 P-domain- Although the catalytic domain of PCs seems to be primarily responsible for their ability to process precursors and defines their catalytic preference, the contribution of the P-domain remains to be established. A three-dimensional structure has not yet been obtained for any of these enzymes, although their catalytic modules have been modeled on the basis of the known structure of subtilisin (Rouille *et al.*, 1995). The role of the P-domain appears to be regulatory; it may influence the marked calcium dependency and mire acidic pH optima of some of the PCs (Zhou *et al.*, 1998). The P-domain is required for the stabilization of PC2 structure and is not exchangeable with the P-domain of PC1 (Zhu *et al.*, 1998). In addition, the P-domain also appears to structurally stabilize the catalytic domain. It may do this by helping to balance surface charge asymmetry in the substrate binding region of the catalytic domain, which is caused by the characteristic multibasic residue specificity of these enzymes (Steiner, 1998). Truncation studies with both kexin and furin demonstrate that this region is essential for enzymatic activity (Fuller *et al.*, 1989b; Hatsuzawa *et al.*, 1992; Wilcox *et al.*, 1991; 1992). Recent studies with the furin-defective LoVo cell line demonstrate that a single point mutation within the P-domain of this convertase can prevent autocatalytic prosegment cleavage, and ultimately result in processing deficiency (Takahashi *et al.*, 1993; 1995a). Deletion of the P-domain in kexin also blocks prosegment cleavage, and prevents transformation of the zymogen into the active enzyme (Gluschankof *et al.*, 1994). In chapter 2, I present our studies on the P-domain of PACE4, using two isoforms generated by alternative splicing in the P-domain. Our results demonstrated that the P-domain is indispensable.

2.6. Overexpression of the PCs and applications

In order to study the structural characteristics of the PCs, an overproduction of soluble forms of the PCs is absolutely necessary. Since mammalian PCs can not be produced in bacteria or in yeast so far, alternative approaches have been exploited. The procedures tested so far include the use of recombinant viruses both vaccinia and baculo virus, stable transfectants and transgenic animals (Seidah & Chrétien, 1997). Vaccinia virus expression in mammalian cell lines yields bioactive enzymes such as PC1 (Jean *et al.*, 1993), soluble furin (Jean *et al.*, 1995a; 1995b) and soluble PC7 (Munzer *et al.*, 1997) at levels of about 0.1-1 mg/l of culture. In contrast, baculovirus infection of high 5 insect cells results in secreted level of 2-20 mg/l of PC1 (Boudreault *et al.*, 1998b), soluble furin (Bravo *et al.*, 1994; Munzer *et al.*, 1997) and soluble PC7 (Munzer *et al.*, 1997). Attaching a hexa-histidine tag to the carboxyl-terminal end of PC7 allowed it to be purified in an active form with a 70% yield on Cobalt affinity columns. To avoid oxidation of the enzyme, the system was layered with argon. Stable transfectants of PC cDNA in dihydrofolate reductase-deficient Chinese hamster ovary cells (Ayoubi *et al.*, 1996; Lamango *et al.*, 1996; Nakayama, 1994; Schlokat *et al.*, 1996; Yi & Lindberg, 1993), in conjunction with methotrexate amplification, led to the production of either soluble furin (Nakayama, 1994; Schlokat *et al.*, 1996), PC1 (Yi & Lindberg, 1993) or PC2 (Lamango *et al.*, 1996) in yields of 0.1-10 mg/l per 24h of culture. Finally the use of transgenic animals overexpressing soluble furin in the mammary gland resulted in its large scale production in levels exceeding 10 mg/l of milk (Velander *et al.*, 1997).

Overproduction of secreted bioactive proteins and peptides is limited by the fact that endogenous expression level of proprotein convertases is relatively low. Thus overexpression of, for example, a growth factor precursor in cell lines does not guarantee its complete and efficient processing to yield the desired bioactive product (Seidah & Chrétien, 1997). One solution would be the coexpression of both precursor and its cognate convertase, which can result in large-scale production of biologically active molecules. Examples include the production in the insect cells of bioactive insulin receptor (Bravo *et al.*, 1994) and in Chinese hamster ovary-cells of bioactive von Willebrand factor (Ayoubi *et al.*, 1996; Schlokat *et al.*, 1996), transforming growth factor

β1 (Ayoubi *et al.*, 1996) as well as factor IX (Wasley *et al.*, 1993). The coagulation factor protein C was produced in yields exceeding 100mg/l milk when coexpressed with furin in the mammary glands of transgenic pigs (Velander *et al.*, 1997).

The specialty of proprotein convertases in recognizing R-X-L-R sequence has been used in making fusion proteins where such a site has been engineered to separate the fused segments. Ghosh and Lowenstein (Ghosh & Lowenstein., 1996) expressed a fusion protein containing a PC cleavage site. After purification they liberated the carboxyl-terminal fusion polypeptide *in vitro* by digestion with yeast kexin. Similarly if the desired segment is placed at the amino-terminus of the fusion protein, followed by a separating motif exclusively composed of four basic residues (e.g. R-R-R/K-R) could then be cleaved *in vitro* with a selected PC, and trimmed out with carboxypeptidase B (Seidah & Chrétien, 1997).

In conclusion, the expression pattern of PCs determines the cascade of events ultimately leading to the tissue-specific production of biologically active peptides and proteins via limited proteolysis of their precursors. This mechanism extends the diversity of products that can be generated from common precursors. The accumulated data implicate the PCs in the normal physiology of healthy cells as well as in pathologies such as cancer, viral infections including AIDS, in atherosclerosis, obesity, diabetes and in hypertension. In biotechnological applications, one can cite the utility of designing cell lines that overexpress one or more PCs together with a chosen precursor following the isolation of milligram amounts of biologically active products, such as coagulation factors, hormones and growth factors. Ultimately, overproducing the PCs should lead to the determination of their crystal structure, enabling the rational design of specific inhibitors and/or activators, which could find wide clinical and pharmacological applications in proliferative, cognitive and metabolic diseases (Seidah & Chrétien, 1997).

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CHAPTER 2

The prosegments of furin and PC7 as potent inhibitors of proprotein convertases: *in vitro* and *ex vivo* assessment of their efficacy and selectivity

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Preface

Furin and PC7 are major convertases of the constitutive secretory pathway, process precursors either within the TGN or at the cell surface. As such, they mediate a wide range of processing events, which in pathological situations may exacerbate a disease state. Examples include the participation of furin, and possibly PC7, in the processing of the viral surface glycoproteins gp160 of HIV and GP of Ebola virus. Furthermore, at least furin has been implicated in the processing of bacterial toxins. Experiments in which the activation of these proteins has been prevented through the inhibition of furin and possibly other PCs indicate that there is considerable promise in these novel approaches to treating such pathologies. The prosegment is an important functional domain of PCs. In bacterial subtilases, it can act as an intramolecular chaperone assisting the correct folding of the enzyme. Following its autocatalytic cleavage, the prosegment could also behave as competitive inhibitor spatially and temporally regulating their enzymatic activities. This chapter describes the characterization of the prosegments of furin and PC7 as inhibitors of these enzymes. Although it has been known that the prosegments can function as inhibitors, this is the first study to test purified prosegment *in vitro*. It is also demonstrated for the first time that proprotein convertase prosegments expressed *ex vivo* as independent domains can act *in trans* to inhibit precursor maturation by intracellular PCs. This study not only allows the usage of prosegments of those convertases as models to design potent and specific convertase inhibitors but also provides the first evidence that these polypeptides can be used to inhibit the cellular processing of precursors *ex vivo*. This technology represents a novel enzyme silencing strategy that will enhance our understanding of the basic cellular functions of these proteinases. It paves the road for future work on improving the performance of these prosegments using site-directed mutagenesis, leading to the design of more selective and powerful convertase inhibitors that may provide novel approaches to the treatment of a variety of pathologies including proliferative, microbial and viral diseases.

Abstract

All proprotein convertases of the subtilisin/kexin family contain an N-terminal prosegment that is presumed to act both as an intramolecular chaperone and an inhibitor of its parent enzyme. In this work, we purified recombinant prosegments of furin (pFurin) and PC7 (pPC7) from BL21 bacterial cells. The inhibitory potencies and selectivities of the prosegments were determined *in vitro* for several PCs using either the fluorogenic peptide pERTKR-MCA or the HIV envelope glycoprotein gp160 as substrates. Both full-length pFurin and pPC7 are potent inhibitors (IC_{50} = 4 and 0.4 nM, respectively) of their parent enzymes. Whereas pPC7 preferentially inhibits PC7, pFurin appears to be less selective, inhibiting PC5 even more strongly than its parent enzyme. Small (10 amino acids) synthetic peptides derived from the C-terminal regions of the prosegments are about 50-fold less inhibitory than the full-length proteins. Most of the inhibitory potency seems to reside in the region immediately preceding the primary cleavage site, with the P1 Arg playing a critical role in these events. The full-length, bacterially produced prosegments were used to generate antibodies against pFurin and pPC7 in order to assess the intracellular metabolic fate of these polypeptides when expressed *ex vivo*. Furthermore, vaccinia virus recombinants and transient transfectants of the preprosegments of furin and PC7 revealed that overexpression in cell lines efficiently inhibited precursor processing of the neurotrophins nerve growth factor and brain-derived neurotrophic factor. Thus, we have demonstrated for the first time that PC prosegments, expressed *ex vivo* as independent domains, can act *in trans* to inhibit precursor maturation by intracellular PCs.

1. Introduction

Limited proteolysis of proproteins is an archetypal mechanism responsible for the generation of diverse bioactive peptides and proteins from inactive precursors (Seidah *et al.*, 1998a; 1998b; Steiner, 1998). Within the secretory pathway, these cleavages involve the processing of precursors at either single or paired basic residues (Seidah *et al.*, 1998a; Steiner, 1998) or at specific hydrophobic and small residues (Seidah *et al.* 1998b). The recently characterized enzymes responsible for many of these intracellular conversions are calcium-dependent subtilisin-like serine proteinases related either to the yeast kexin (Nakayama, 1997; Seidah & Chrétien, 1997; Seidah *et al.*, 1998a; 1998b; Steiner, 1998) or to the pyrolysin (Seidah *et al.*, 1999a) subfamilies of subtilases (Siezen & Leunissen 1997). The mammalian kexin-like proteinases, known as proprotein convertases (PCs), form a family comprising seven members: furin (PACE), PC1 (PC3), PC2, PC4, PACE4, PC5 (PC6) and PC7 (LPC, PC8) (Nakayama, 1997; Seidah & Chrétien, 1997; Seidah *et al.*, 1998a; 1998b; Steiner, 1998). These enzymes cleave precursor polypeptides at specific sites within the general motif **(R/K)**-$(X)_n$-**(K/R)**↓, where n = 0, 2, 4, or 6 and X is any amino acid (aa) except Cys (Nakayama, 1997; Seidah & Chrétien, 1997; Seidah *et al.*, 1998a; 1998b; Steiner, 1998). The only mammalian pyrolysin-like enzyme known to date is SKI-1/S1P, which appears to recognize the motif **R**-X-X-**(L,T)**↓ (Seidah *et al.*, 1999a; Sakai *et al.* 1998).

The synthesis of most proteinases as inactive zymogens provides cells with the means to spatially and temporally regulate their proteolytic activities (Khan & James, 1998), thereby minimizing the occurrence of premature enzymatic activity which could lead to inappropriate protein degradation. The inhibitory mechanism often involves the presence of an inactivating (pro)segment at the N-terminus of the zymogen. In the case of bacterial subtilases, zymogen activation involves the autocatalytic excision of their prosegments, which are thought to act both as intramolecular chaperones (Inouye, 1991) as well as specific inhibitors of the parent proteinase (Anderson *et al.*, 1997; Boudreault, *et al.* 1998a; Inouye, 1991; Seidah 1995b; Shinde & Inouye 1995). The prosegments of mammalian PCs, which exhibit 30-67% sequence identity to each other (Seidah *et al.*,

1998a; 1998b) and an absolute conservation of 8 aa (Seidah *et al.*, 1998a), are autocatalytically processed in the endoplasmic reticulum (ER). With the exception of PC2 (Seidah & Chrétien, 1997; Seidah *et al.*, 1998a; 1998b; Steiner, 1998), this event is a prerequisite for the PC's efficient egress from this compartment (Creemers *et al.*, 1995; Powner & Davey, 1998; Zhong *et al.*, 1996; Zhou *et al.*, 1995). The initial cleavage of the prosegment of PCs does not result in the immediate activation of the enzyme; rather the prosegment appears to remain tightly associated with the convertase until it reaches its final cellular destination. At this point, the increase in H^+ and/or Ca^{2+} concentrations in the TGN or secretory granules triggers a secondary cleavage(s), resulting in the dissociation of the prosegment (Anderson *et al.*, 1997; Boudreault *et al.*, 1998a; Powner & Davey, 1998). While PC1, PC2, furin, PACE4 and PC5 contain a secondary cleavage site KR or RR within their prosegments, PC4 and PC7 have only an RK site. In the latter cases, it is not yet known if cleavage at these sites occurs or is required for the effective activation of these enzymes (Seidah *et al.*, 1998a; 1998b). Moreover, it remains to be determined whether the subsequent trimming of the C-terminal basic residues known to be mediated by specific carboxypeptidases (Lei *et al.*, 1999) is required for full activation of these enzymes.

Furin and PC7, the major convertases of the constitutive secretory pathway (Molloy *et al.*, 1994; Munzer *et al.*, 1997; Nakayama, 1997; Seidah *et al.*, 1996c; Seidah & Chrétien, 1997; Seidah *et al.*, 1998a; 1998b; Steiner, 1998; Van de Loo *et al.*, 1997), process precursors either within the TGN or at the cell surface. As such, they mediate a wide range of processing events, which, in pathological situations, may exacerbate a disease state (Chrétien *et al.*, 1995). Examples include the participation of furin, and possibly PC7, in the processing of the viral surface glycoproteins gp160 of HIV (Decroly *et al.*, 1996; 1997; Hallenberger *et al.*, 1997) and GP of Ebola virus (Volchkov *et al.*, 1998). Furthermore, at least furin has been implicated in the processing of toxins such as those of *aeromonas hydrophila* (pore-forming proaerolysin) (Abrami *et al.*, 1998), anthrax (Klimpel *et al.*, 1992), *pseudomonas* and *diptheria* (Chiron *et al.*, 1994). Experiments in which the activation of these proteins has been prevented through the

inhibition of furin and possibly other PCs indicate that there is considerable promise in these novel approaches to treating such pathologies.

Some of the previous attempts to inhibit the substrate processing activity of PCs *ex vivo* have included the use of irreversible chloromethylketone inhibitors (Hallenberger *et al.*, 1992; Jean *et al.*, 1995b) and reversible peptide inhibitors (Apletalina *et al.*, 1998; Decroly *et al.*, 1994; Jean *et al.*, 1995a). Major limitations of these agents include either their cytotoxicity (through interfering with the biosynthesis of many important cellular proteins) and/or their relatively poor cellular permeability and targeting (Hallenberger *et al.*, 1992; Jean *et al.*, 1995b). Alternatively, recombinant protein-based inhibitors have been developed (Anderson *et al.*, 1993; Benjannet *et al.*, 1997; Dahlen *et al.*, 1998; Jean *et al.*, 1998; Lu *et al.*, 1993; Rompaey *et al.* 1997). These strategies are based on the expression of proteins that contain a furin-like recognition sequence (R-X-X-R) within the inhibitor binding region of either human α_1-antitrypsin (Anderson *et al.*, 1993; Benjannet *et al.*, 1997; Jean *et al.*, 1998), α_2-macroglubulin (Rompaey *et al.* 1997), proteinase-8 (Dahlen *et al.*, 1998), or the turkey ovomucoid third domain (Lu *et al.*, 1993). Although often reasonably effective, the inability of these recombinant proteins to selectively inhibit furin and not other PCs remains problematic (Benjannet *et al.*, 1997; Jean *et al.*, 1998; J.S. Munzer and N.G. Seidah, *unpublished results*).

In response to these challenges, we have begun exploring the possibility that prosegments of PCs can be employed to inhibit specifically and efficiently the processing of cellular substrate proproteins. Here we examine the *in vitro* and *ex vivo* inhibitory characteristics and specificities of the prosegments of furin and PC7. Synthetic peptides derived from these prosegments are used to identify the regions of these molecules that are most important for potent inhibition. We then explore whether cellular overexpression of the preprosegments of furin and PC7 can, acting *in trans* as independent domains, inhibit the PC-mediated maturation of two neurotrophin precursors *ex vivo*.

2. Experimental procedures

2.1 Expression and purification of bacterial recombinant hfurin and rPC7 prosegments

The bacterial expression vector pET24b (+) (*Novagen*) was cut with 5' *Nde*I and 3' *Bam*HI to remove the N-terminal T7 tag. It was then ligated with a linker composed of pre-annealed sense 5' TACACATATG 3' and antisense 5' GATCCATATGTG 3' oligos (the underlined codon represents the initiator Met, which is followed in the recombinant vector by an Asp-Pro sequence). The cDNAs coding for the N-terminal prosegments of human furin [aa 27-107 of hfurin (Van den Ouweland *et al.*, 1990), referred to as pFurin] and rat PC7 [aa 37-140 of rPC7 (Seidah *et al.*, 1996c, referred to as pPC7], were isolated by a 3-step PCR using Elongase (GIBCO-BRL) for 25 cycles; *i.e.* 94°C for 25 sec, 50°C for 50 sec, and 68°C for 50 sec. The hfurin and rPC7 oligonucleotides used were: sense [5' GGATCCGCAGAAGGTCTTCACCAACACGT 3'], [5' GGATCCGCTAACAGAGGCAGGTGGTCTTG 3']; and antisense, which contains a hexa-His anti-coding sequence: [5' CTCGAGTCAGTGGTGGTGGTGGTGGTGCCGTTTAGTCCG 3'], [5' CTCGAGTCAGTGGTGGTGGTGGTGGTGGCGCTTGGCCCT 3'], respectively. These 277 and 346 bp cDNAs were cloned into the PCR 2.1 TA cloning vector for sequencing and then were transferred into the *Bam*HI / *Xho*I sites of the above modified bacterial expression vector pET 24b. These recombinants were transformed into the *E. Coli* strain BL21 (DE3). Protein expression was induced with 1mM isopropyl β-D-thiogalactoside, after which the cultures were grown for 4h at 37°C. The cells were harvested by centrifugation at 4,000g. Since the prosegments were localized to the inclusion bodies, the cell pellets were sonicated on ice in binding buffer (20 mM Tris-HCl, 0.5M NaCl, 5 mM imidazole, 6M guanidine HCl, pH 7.9). The supernatant was applied to a 1 mL column containing a Ni^{2+}-immobilized resin (Novagen), pre-equilibrated at room temperature with binding buffer. The column was washed with binding buffer containing 20 mM imidazole in order to eliminate weakly bound species. Elution was then carried out with the same buffer now containing 1 M imidazole. The eluant dialyzed against 50 mM sodium acetate (pH 5.5) at 4°C overnight. The prosegments were further purified

with a Varian 9050/9010 instrument by reverse-phase high performance liquid chromatography (RP-HPLC) using a 5 μm C4 column (0.94 x 25 cm; Vydac). After binding in an aqueous phase containing 0.1% trifluoroacetic acid (TFA), proteins were eluted at 2 ml/min with a 1%/min linear gradient (10% to 70%) of 0.1% aqueous TFA/ CH_3CN (monitored at 210 nm). The purity, concentrations, and masses of the prosegments were determined by Coomassie staining of 15% tricine SDS-PAGE gels (Fig. 2.1), quantitative amino acid analysis, and Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) mass spectrometry.

2.2 Synthesis of prosegment-derived peptide derivatives

All Fmoc protected amino acid derivatives (L-configuration), the coupling reagents and the solvents were purchased from PE Biosystems Inc. (Framingham, Mass, USA), Calbiochem (San Diego, Ca, USA) and Richelieu Biotechnologies (Montréal, Québec, Canada). The Furin-derived synthetic peptides are **Fur-M15:** ^{62}GDYYHFWHRGVTKRS^{76}, **Fur-C24:** ^{84}HSRLQREPQVQWLEQQVAKRRTKR^{107}, **Fur-C10:** ^{98}QQVAKRRTKR^{107}, and **Fur-C10A:** ^{98}QQVAKRRTK**A**^{107}. The rPC7-derived synthetic peptides are **PC7-N20:** ^{39}EAGGLDTLGAGGLSWAVHLD^{58}, **PC7-M18:** ^{86}GRIGELQGHYLFVQPAGH^{103}, **PC7-C24:** ^{117}EAVLAKHEAVRWHSEQRLLKRAKR^{140}, **PC7-C18:** ^{123}HEAVRWHSEQRLLKRAKR^{140}, **PC7-C10:** ^{131}EQRLLKRAKR^{140}, and **PC7-C18A:** ^{123}HEAVRWHSEQRLLKRAK**A**^{140}. The PC7's N20, M18, C24 and C18 as well as the furin's M15 and C24 peptides were kindly provided by Dr. Feng Ni (the Biotechnology Research Institute, Montréal, Canada), all other peptides were synthesized with the carboxyl-terminal in the amide form ($CONH_2$), on a solid phase automated peptide synthesizer instrument (Pioneer), PE Biosystems, following the HATU (O-hexafluorophospho-[7-azabenzotriazol-1-yl]-N,N,N',N'-tetramethyluronium) / Diisopropyl ethyl amine (DIEA)- mediated Fmoc chemistry. All syntheses were accomplished using an unloaded Poly Amino Linker Poly Ethylene Glycol (PAL-PEG) resin. The following side-chain protecting groups were used: 2, 2, 4, 6, 7-pentamethyldihydrobenzofuran 5-sulphonyl (Pbf) for Arg; t-butyl (But) for Ser, Thr, Asp

and Tyr and trityl (trt) for His, Asn and Gln respectively. The crude peptides were cleaved from the resin and fully deprotected by 3 hours treatment with reagent B (TFA/phenol/water/TIPS = 88:5:5:2). The peptides were purified by RP-HPLC using a semipreparative CSC-Exsil C18 column (0.94 x 25cm, Chromatography Specialty Company), followed by further purification on a ultrasphere C18 analytical column (0.46 x 25cm, Beckman) following the conditions as described above.

2.3 MALDI-TOF mass spectrometry

HPLC fractions were mixed with one of two matrix solutions: 3,5 dimethoxy-4-hydroxycinnamic acid (Aldrich Chemical Co) for prosegments, and α-cyano-4-hydroxycinnamic acid (Aldrich Chemical Co) for synthetic peptides. Spectra were obtained on a Voyageur DE-Pro MALDI-TOF instrument (PE PerSeptive Biosystems).

2.4 *In vitro* inhibition of gp160 processing by furin

HIV-1 [^{35}S]Met gp160 was affinity purified from CV-1 cells overexpressing VV:gp160 as described (Decroly *et al.*, 1994). For the inhibition assay, furin was first preincubated with increasing concentrations of pFurin or pPC7 for 10 min at 25°C according to Decroly *et al.* (Decroly *et al.*, 1994), followed by the addition of 10,000 cpm of [^{35}S]Met gp160 and an overnight incubation at 25°C. The products were analyzed by SDS-PAGE as described (Decroly *et al.*, 1994; 1996).

2.5 Enzymatic activity determination

Media of BSC40 cells infected with vaccinia virus recombinants of soluble PC7 [VV:rPC7-BTMD, (Munzer *et al.*, 1997)] , furin [VV:hfurin-BTMD, (Decroly *et al.*, 1996)] (a generous gift from G. Thomas, Vollum Institute, Portland, Oregon, USA), PC5 [VV:mPC5A], PACE4 [VV:hPACE4A] and the shed form of yeast kexin [VV:ykexin] (Decroly *et al.*, 1996; Munzer *et al.*, 1997) were concentrated 50 fold and kept in 40% glycerol at -20°C. Enzymatic activity was determined by the cleavage of the fluorogenic substrate pERTKR-MCA (Peptides International) (Decroly *et al.*, 1996). For each assay, 5-10 µl of enzyme which will cleave equal amount of substrate was added to a solution

containing 50 mM Tris-HCl (pH 7.0), 2 mM Ca^{2+}, and 100 μM pERTKR-MCA in a final volume of 100 μl. Fluorescence was measured at 0, 30, 60, 90 min using a model LS 50B (Perkin Elmer) spectrofluorimeter (Munzer *et al.*, 1997).

2.6 Inhibition Studies

Stop-time assay: Enzymes were preincubated for 15 min at room temperature with various concentrations of the prosegments (mixed with 0.1% BSA to avoid non-specific binding of the dilute prosegments to the microtiter plate) or synthetic peptides. The fluorogenic pERTKT-MCA substrate was then added and the released AMC measured as above. *Ki* values were derived from Dixon (Dixon 1953) and Cornish-Bowden (Cornish-Bowden 1974) plots using substrate concentrations of 75, 100, and 200 μM for PC7, and 5, 25, and 50 μM for furin. On-line assay: Here we sequentially added to the microtiter plate the prosegment (at various concentrations), 100 μM of pERTKR-MCA, and lastly the buffered enzyme mixture (see above). Fluorescence was continually recorded over a 5 min period to follow the progress of inhibition (Boudreault *et al.*, 1998a).

2.7 Cellular Expression of Preprofurin (ppFurin) and PreproPC7 (ppPC7)

The preprosegments of rPC7 (ppPC7, 450 bp coding for aa 1-142) and furin (ppFurin, 351 bp coding for aa 1-109) were amplified for 25 cycles by PCR (94°C for 25 sec, 50°C for 50 sec, 68°C for 50 sec using Elongase). The sense and antisense pairs of oligos for PC7 and furin which contain 5' *Hind*III and 3' *Bam*HI sites were respectively: [5' AAGCTTGTTGTGATGCCGAAAGGGAG 3'], [5' GGATCCTCATTAGATGCTGCGCTTGGCCCTCTT 3'] and [5' AAGCTTGAAGCCATGGAGCTGAGGCCCTGG 3'], [5' GGATCCTCATTACACGTCCCGTTTAGTCCG 3']. Note that in both sense oligos the initiator methionine codon is underlined and that in both antisense oligos we have introduced two tandem stop codons (underlined). The PCR products were cloned into the PCR 2.1 TA cloning vector for sequencing and then ligated into the *Bam*HI / *Hind*III sites of the pcDNA3 vector (*Invitrogen*) for transient transfection and the PMJ602 vaccinia

virus transfer vector (Davison & Moss, 1990) which led to the isolation of the recombinant VV:ppFurin and VV:ppPC7 virus stocks.

2.8 Prosegment antibodies and vaccinia virus expression

The purified, bacterially-produced prosegments pFurin and pPC7 were treated with carboxypeptidase B (CPB) to remove the C-terminal dibasic and Hexa-His Tag and then used to raise specific antisera in rabbits. For the cellular expression of the preprosegments, 5 x 10^6 BSC40 or AtT20 cells were infected for 2h with 2 plaque forming units (pfu)/cell of either VV:ppFurin or VV:ppPC7. Following overnight incubation in minimal essential medium (MEM) without serum, the cells were extracted in 5M acetic acid, sonicated, and then applied to a SepPak (Waters) C18 cartridge. The retained peptides and proteins were eluted using 60 % CH_3CN/0.1 % TFA. The samples were then resolved by SDS-PAGE on a 14% Tricine gel and the proteins analyzed by Western blots with pFurin (dilution 1:1,000) and pPC7 (dilution 1:2,500) antisera. For inhibition studies, 5 x 10^6 rat Schwann cells (Marcinkiewicz *et al.*, 1998) were infected with 1 pfu/cell of VV:NGF (Seidah *et al.*, 1996b) and 3 pfu/cell of either VV:ppFurin, VV:ppPC7, VV:PDX (kindly supplied by Gary Thomas, Vollum Institute, Portland, OR, USA) or the control VV:POMC. Following overnight incubation, the cells were washed and then pulse-labeled with [^{35}S]Met for 3h. The media were immunoprecipitated with an anti-NGF antibody and analyzed by SDS-PAGE and autoradiography (Seidah *et al.*, 1996b).

2.9 Transient transfection in COS-1 cells

Using lipofectamine (GIBCO-BRL), 60-70% confluent COS-1 cells were co-transfected with pcDNA3 recombinants of proBDNF (Mowla *et al.*, 1999) plus either ppFurin, ppPC7 or an empty pcDNA3 plasmid. After 5h incubation in serum- and antibiotic-free Dulbecco's MEM (DMEM, GIBCO-BRL) media, the cells were incubated for another 48h in DMEM plus 10% fetal calf serum. Concentrated media were run on a 13-22% gradient SDS-PAGE and the separated proteins analyzed by Western blots using a commercial BDNF antibody (Santa Cruz).

3. Results

3.1 Overexpression, Purification and Mass Spectral Analysis of Furin and PC7 Prosegments

In order to define the possible *in vitro* inhibitory character of the prosegments of human furin (pFurin) and rat PC7 (pPC7), we first needed to obtain large quantities of purified proteins. Bacterial expression systems are a logical venue, especially since, based on their amino acid sequences, neither prosegment is expected to undergo post-translational modifications (*e.g.* N-glycosylation or Tyr-sulfation) of its backbone (Seidah *et al.*, 1996c; Van den Ouweland *et al.*, 1990). We therefore expressed these prosegments in BL21 cells, starting from their signal peptide cleavage sites and finishing with their primary autoactivation sites, along with an additional C-terminal hexa-His tag. Therefore the constructs are of the type: MDP...R(T,A)KR-$(H)_6$, where the additional N-terminal MDP and C-terminal hexa-His sequences are common to both proteins. Following a 4h induction with IPTG, both prosegments were purified from cell pellet extracts using a Ni^{2+}-affinity column (Fig. 2.1A). The typical yield observed for each prosegment is 7-8 mg/l of bacterial culture. Note that the 90 aa pFurin construct runs faster on SDS-PAGE than the 113 aa pPC7 product. Further confirmation of the identity of the two polypeptides was obtained from analyses of their MALDI-TOF MS spectra. pFurin and pPC7 have molecular masses of 10,735 Da (*vs.* the calculated value of 10,737) and 12,348 Da (*vs.* the calculated value of 12,356). These values are well within the expected experimental for errors this mass range of $\pm$ 20 Da.

The inhibitory characteristics of the furin and PC7 prosegments towards furin-mediated processing *in vitro* of HIV gp160 into gp120/gp41 (Decroly *et al.*, 1994; 1996; 1997) is shown in Fig. 2.2. At concentrations exceeding 12 nM pFurin, processing of gp160 by the quantity of furin used in this assay is almost completely inhibited (Fig. 2.2A). The low levels of gp77/gp53 (*) that are detected are due to further cleavage of gp120 by furin, as previously reported (Vollenweider *et al.*, 1996). The small amount of gp120 observed in control incubations (C, Fig. 2.2), which is equivalent to that seen with pFurin concentrations $\geq$ 25 nM, is due to its copurification with gp160 on the lentil-lectin affinity column (Decroly *et al.*, 1994; 1996). The prosegment pPC7 (Fig. 2.2B) partially inhibits the furin-mediated cleavage of gp160, but only at concentrations exceeding 200-300 nM, indicating that it is a

much less effective inhibitor of furin than pFurin. We did not test this reaction with PC7, since this enzyme does not cleave gp160 very efficiently *in vitro* (Decroly *et al.*, 1997).

3.2 Specificity of pFurin and pPC7 and their derived peptides: Importance of the C-terminal P1 Arg

In order to assess the extent of prosegment inhibitory selectivities toward their cognate enzymes, we tested the inhibition of pFurin and pPC7 on the processing of the small fluorogenic substrate pERTKR-MCA. Five different soluble convertases were used: human BTMD-furin, human PACE4-A, rat BTMD-PC7, murine PC5-A, and the shed form of yeast kexin (Decroly *et al.*, 1996; Munzer *et al.*, 1997). Starting with equal amounts of pERTKR-MCA-cleavage activity, the data (Fig. 2.3A) reveal that pFurin is a potent inhibitor of these convertases at the nM level. The concentration of pFurin needed to inhibit 50 % of each processing reaction (IC_{50}) is presented in Table 2.1, which depicts the average of five separate experiments. Unexpectedly, the observed order of inhibition for pFurin was: PC5 > furin > PC7 > PACE4 > kexin. pFurin inhibits PC5-A (IC_{50} = 0.4 nM) at 10-fold lower concentrations than furin (IC_{50} = 4 nM). A similar experiment carried out with pPC7 (Fig. 2.3B, Table 2.1) revealed a different rank order of preference: PC7 > PC5 ≈ PACE4 ≈ furin > kexin. PC7 was inhibited at 50-fold lower concentrations of pPC7 than furin and PC5. Neither prosegment was very effective against PACE4 or kexin, which required between 300 and 2500-fold more peptide than the parent enzyme in order to achieve 50% inhibition. Therefore, pPC7 thus seems to be much more selective towards its parent enzyme than pFurin. Finally, from progress curves of on-line assays we deduced that both proteins exhibit slow binding inhibition kinetics of their parent enzymes (*not shown*), in a manner similar to that reported for the prosegment of PC1 (Boudreault *et al.*, 1998a).

To define the regions of the prosegments that are essential for inhibition, a series of peptides was synthesized as indicated in Table 2.2. As also shown in Fig. 2.4, the Fur-M15, PC7-M18 peptides which are derived from the middle portion of the prosegments and the N-terminal PC7-N20 peptide are very poor inhibitors (IC_{50} > 50,000 nM). It is interesting to note that although Fur-M15 encompasses the secondary cleavage site of furin (Anderson *et al.*, 1997), it is a very poor inhibitor (Fig. 2.4A, Table 2.2) and is not a substrate (*not shown*),

possibly because of the absence of extended P' residues. In contrast, the PC7 C-terminally-derived C24, C18 and C10 synthetic peptides (*Ki*'s ≈ 5-7 nM and IC_{50}'s ≈ 20-28 nM) and the furin-derived C24 and C10 peptides (*Ki*'s ≈ 35-40 nM and IC_{50}'s ≈ 150 nM) are relatively good inhibitors of their parent enzymes (Table 2.2). In both cases, peptides as small as 10 aa (C10) can still potently inhibit their cognate enzymes, albeit at ~50-fold higher concentrations than their respective full length prosegments. Kinetic analyses using Dixon plots (Basak *et al.*, 1997; Dixon 1953) revealed that all of the C-terminal synthetic peptides tested act as purely competitive inhibitors of their parent enzyme (*not shown*). Finally, mutation of the C-terminal Arg to Ala in Fur-C10A or PC7-C18A almost completely abolished the inhibitory activity (Fig. 2.4, Table 2.2). This demonstrates the critical importance of the C-terminal P1 Arg for the inhibitory function. In agreement, carboxypeptidase B digestion of the full length prosegments, which removed the C-terminal LysArg-$(H)_6$ sequence as demonstrated by MALDI-TOF spectrometry, also caused a dramatic decrease in the inhibitory activity of these proteins (*not shown*).

In Fig. 2.5, we compare the inhibitory specificities of the C-terminal prosegment peptides PC7-C18 and PC7-C24. Even though these peptides demonstrate an approximately 4-fold selectivity toward their parent enzyme, the rank order of potency is slightly different than that of the full length pPC7 (compare Figs. 3 and 5). Thus, PC7-C24 shows a rank preference order: PC7 > kexin > PC5 > furin >> PACE4, whereas the rank order for PC7-C18 is: PC7 > PC5 > kexin > furin >> PACE4, Therefore, even though kexin was barely inhibited by either full length pFurin or pPC7 (Fig. 2.3), it is relatively well inhibited by PC7-C18 and less so by PC7-C24 (Fig. 2.5). In contrast, while PACE4 was moderately inhibited by pPC7, its activity is almost insensitive to these synthetic peptides.

3.3 Cellular overexpression of the prosegments of furin and PC7 and their *ex vivo* inhibitory properties

BSC40 and AtT20 cells were infected with recombinant vaccinia viruses expressing the preprosegments of furin (VV:ppFurin) or PC7 (VV:ppPC7) which are extended by two amino acids past the primary cleavage sites. The cell lysates were analyzed by Western blots using the specific rabbit polyclonal antisera raised against bacterially produced pFurin and

pPC7 (*see Experimental Procedures*). As shown in Fig. 2.6, Western blot analyses of these prosegments revealed that each antiserum is quite specific for its original antigen such that no cross-reactivity is observed. Although both prosegments are well expressed in both cell lines, ppFurin exhibits two major immunoreactive forms migrating with apparent molecular masses (MM) of 12.5 and 10 kDa (Fig. 2.6A), with the latter migrating slightly faster than the bacterially-produced product which contains an additional 9 aa [MDP and $(H)_6$] (*not shown*). This ~2.5 kDa difference in apparent MM agrees well with the expected theoretical mass difference between ppFurin and pFurin (2,778 Da), suggesting that the 10 kDa protein is pFurin. Thus, we deduced that the upper band could be ppFurin that still contains the N-terminal signal peptide. Accordingly, and in contrast to the full length enzyme, overexpression of the ppFurin domain by itself results in a delayed excision of its signal peptide, especially in BSC40 cells. A similarly delayed signal peptide removal has already been demonstrated using baculovirus overexpression of preproPC1 in Sf9 cells (Boudreault *et al.*, 1998b). Although not shown, when BSC40 cells were pulse-labeled with [^{3}H]Leu for 10 min and then chased in absence of radio-label, our data revealed that it took about 1h to completely transform ppFurin into pFurin (S. Benjannet and N.G. Seidah, *in preparation*). In the case of PC7, only a very low level of an ~15.5 kDa protein can be detected in BSC40 cells, and mostly an ~ 11 kDa product is observed in both cell types (Fig. 2.6B). Again, from the theoretically expected difference of masses between ppPC7 and pPC7 (4,006 Da), we predicted that the ~15.5 and ~11 kDa proteins are ppPC7 and pPC7, respectively. These conclusions were corroborated by N-terminal sequencing (S. Benjannet, C. Lazure and N.G. Seidah, *in preparation*). Finally, although pPC7 is secreted, no pFurin could be detected in the medium (*not shown*).

We next investigated the ability of overexpressed prosegments to inhibit the PC-directed processing of two pro-neurotrophins, namely proNGF (Fig. 2.7) and proBDNF (Fig. 2.8). As seen from Fig. 2.7, the processing of the [^{35}S]Met-labeled 35 kDa proNGF into the 13.5 kDa NGF is apparent when rat Schwann cells are co-infected with VV:NGF and the control vector VV:POMC. This predominantly furin-mediated processing (Seidah *et al.*, 1996b) is inhibited by co-expression of the serpin α1-PDX, as previously reported (Anderson *et al.*, 1993). It is also apparent that overexpression of ppFurin results in a similar inhibition of proNGF to NGF processing and that pPC7 only partially blocks this

reaction. Since PC7 does not effectively process proNGF (S. Benjannet and N.G. Seidah, *unpublished results*), the latter result suggests that overexpressed pPC7 may partially inhibit furin activity.

The differential ability of overexpressed ppFurin and ppPC7 to inhibit the processing of proBDNF was next investigated in COS-1 cells, wherein this neurotrophin precursor is best cleaved by PC1 and furin (Seidah *et al.*, 1996a). In this experiment we further compared the inhibitory potency of ppFurin and ppPC7 by transient transfection. The vector pcDNA3 containing sense (S)-oriented cDNAs, as well as negative controls carrying these cDNAs in an antisense (AS) orientation, were tested. Thus, Western blot analyses of the proteins in conditioned media using one of the commercial BDNF antibodies (Santa Cruz) revealed that only the expression of the sense ppFurin construct results in an effective inhibition of the processing of the 32 kDa proBDNF into the 14 kDa BDNF (Fig. 2.8). Again, ppPC7 does not inhibit this processing, suggesting that its overexpression does not affect endogenous furin. The antisense controls do not exhibit significant inhibition of proBDNF processing.

4. Discussion

The ability of prosegments to inhibit their parent enzymes is a well-established phenomenon (Inouye, 1991; Khan & James, 1998; Shinde & Inouye 1995). Among the best-studied prosegments of serine proteinases are those of the bacterial subtilases, in particular those of subtilisin E (Inouye, 1991; Power *et al.* 1986; Shinde & Inouye 1995) and α-lytic protease (Sohl & Agard 1995). In addition to being essential for the proper folding of these enzymes during synthesis, the prosegments are powerful, specific inhibitors with K_i values typically in the nM range (Shinde & Inouye 1995; Strausberg *et al.*, 1993). These observations appear to hold true also for members of the eukaryotic kexin family of subtilases. Studies of the prosegment of yeast kexin-like krp1 from *Schizosaccharomyces pombe* (Powner & Davey, 1998) confirm that it plays a critical role in the folding of the nascent zymogen during synthesis. Subsequent to an autocatalytic intramolecular cleavage at the prosegment-catalytic domain junction, the prosegment remains noncovalently attached to the enzyme, serving as a potent auto-inhibitor of its activity (Inouye, 1991; Khan & James, 1998; Powner & Davey, 1998; Shinde & Inouye 1995). In eukaryotes, as this complex progresses along the secretory pathway, additional events mediate the degradation and dissociation of the prosegment, leading to a fully activated enzyme (Anderson *et al.*, 1997). For kexin, both prosegment cleavage and enzyme activation occur within the ER (Chaudhuri *et al.*, 1992). Except in the case of PC2, prosegment cleavage of all other members of the mammalian PC family occurs within the ER (Nakayama, 1997; Seidah & Chrétien, 1997; Seidah *et al.*, 1998a; 1998b; Steiner, 1998). Enzyme activation, however, occurs later in the secretory pathway, typically in the TGN or in immature secretory granules (Nakayama, 1997; Seidah & Chrétien, 1997; Seidah *et al.*, 1998a; 1998b; Steiner, 1998).

A detailed study of furin has shed significant light on the mechanism of its prosegment release. Andersen *et al.* (Anderson *et al.*, 1997) demonstrated that furin becomes active only after it reaches the TGN, where the drop in pH (to ~6.2) coupled with an increase in Ca^{2+} concentration (to mM levels) leads to a secondary cleavage and subsequent dissociation of its attached prosegment. Other studies (Khan & James, 1998)

have characterized the nature of prosegment inhibition *in vitro*. With respect to the mammalian PC's, there have been several studies involving prosegment inhibition *in vitro*. Anderson *et al.* (Anderson *et al.*, 1997) showed that a bacterially expressed furin prosegment potently (K_i ~14 nM) inhibited this enzyme *in trans* at an equimolar ratio. A more detailed kinetic study (Boudreault *et al.*, 1998a) confirmed that, similar to subtilisins, an extended prosegment of PC1 purified from Sf9 insect cells acted as a slow, tight-binding inhibitor of both PC1 and furin, but not of PC2. In a preliminary study, Basak *et al.* (Basak *et al.* 1999), using synthetic peptides based the prosegment of PC1, observed differential inhibition of PC1 and furin. Moreover, the authors established that the region most effective for inhibition resides within the C-terminal 34 amino acids of the PC1-prosegment, with the C-terminal dibasic LysArg residues being critical.

The data presented here on the inhibition of PCs by prosegments *in vitro* confirm and extend previous observations. Using full-length prosegments of furin (pFurin) and PC7 (pPC7) purified from bacterial lysates, we demonstrate differential inhibition of the activity (hydrolysis of the synthetic fluorogenic peptide pERTKR-MCA) of five distinct soluble PC preparations (Fig. 2.3). As expected, pPC7 is most inhibitory toward its parent enzyme (Table 2.1). The IC_{50} values of PC5 and PACE4, the next most susceptible PCs tested, were 50-60-fold higher, followed by furin at 100-fold and kexin at 2500-fold higher. These findings suggest that the structural conformation of pPC7 imparts considerable specificity to this polypeptide as a PC inhibitor. As seen in Fig. 2.3, this selectivity is powerful enough to inhibit ~90 % of the activity of PC7 but only 10% of the activity of furin, laying the foundation for the possibility of developing even more specific inhibitors.

Identical experiments using pFurin reveal that it is 5-, ~30-, and ~125-fold less effective against PC7, PACE4, and kexin, respectively, than against its parent enzyme. This selectivity also holds true for a large, biological substrate. Thus, the processing *in vitro* of HIV gp160 by furin (Fig. 2.2) is only partially inhibited by 300 nM pPC7, whereas 25 nM pFurin causes full inhibition (a difference of at least 10-fold). Surprisingly, pFurin inhibits PC5 approximately 10-fold better (IC_{50} value) than furin (Table 2.1). Previous cellular expression studies have demonstrated that both furin and

PC5 can effect similar processing of the precursors of Mullerian inhibiting substance in testicular Sertoli cells (Nachtigal & Ingraham, 1996), the receptor PTPμ in endothelial cells (Campan *et al.*, 1996), and gp160 in LoVo cells (Vollenweider *et al.*, 1996). Also, *in vitro* data regarding the processing of gp160 (Decroly *et al.*, 1996), the α5, αv and α6 integrin chains (Lissistky, J-C. *et al.*, *submitted*), inhibition by the serpin α1-PDX (Jean *et al.*, 1998), and K_m values for the fluorogenic peptide substrate pERTKR-MCA (S. Munzer and N. Seidah, *unpublished results*) all suggest similar and probably overlapping substrate cleavage abilities of furin and PC5. Moreover, homology alignments of the prosegment of furin with that of other PCs shows that it is 58% identical to that of PC5 (Seidah *et al.* 1998b). Taken together, these findings argue considerable functional and probably structural similarity (within the catalytic subsites) between furin and PC5. Since specific active site titrants for PCs are not available, assays were carried out with equal amounts of pERTKR-AMC-hydrolysing activities. Thus, we cannot rule out variations in IC_{50} values caused by differences in the amounts of active enzyme in these experiments. We also point out, however, that the rank order of inhibition using synthetic peptides derived from pPC7 (Fig. 2.5) and pFurin (*not shown*) is different from that of the full-length prosegments (Table 2.1), suggesting that the latter contain additional structural determinants modulating PC inhibition. Experiments are currently underway to compare the inhibitory specificities of the full-length PC5 and furin prosegments on various PCs.

In order to further define where most of the inhibitory potency of pFurin and pPC7 resides, we analyzed a number of prosegment-derived synthetic peptides. Our data reveal that neither the N-terminal nor middle section of these prosegments is essential for the inhibitory function of these polypeptides (Table 2.2). The most important region is clearly located at the C-terminus of the prodomain, in agreement with a previous report employing a PC1 prosegment-derived peptide (Boudreault *et al.*, 1998a). Our peptide truncation series (Fig. 2.5 and Table 2.2) shows clearly that most of the inhibitory potency of pFurin and pPC7 resides in the 10 aa preceding the prosegment primary cleavage site. According to NMR analyses of pPC7 peptides (S. Bhattacharjya *et al.*, *in preparation*) and secondary structure predictions of pFurin (Siezen *et al.* 1995), these residues, with the exception of the last pair of basic residues, are part of an amphiphilic α-helix. This

combination of a common structure and conserved aa within this C-terminal region of the prosegments probably explains their similar nM inhibitory potencies. While these observations may explain minor discrepancies in the selectivity of inhibition, major ones (*e.g.* pPC7 inhibition of kexin) presumably must take into account differences in enzyme structure. Finally, P1 Arg to Ala mutants demonstrated that this C-terminal residue is crucial for these peptides' inhibitory potency (Table 2.2). A similar finding was also confirmed using carboxypeptidase B digested peptides (*not shown*). These effects of carboxypeptidase are reminiscent of the overall decrease in endopeptidase activity in *fat/fat* mice lacking carboxypeptidase E (Fricker *et al.*, 1996), suggesting that the removal of C-terminal basic residues is essential for maximum convertase activity.

Although peptides derived from other regions of pFurin or pPC7 were ineffective as inhibitors, they nonetheless appear to play some role in the inhibitory potency of the full-length prosegment. For example, comparing the IC_{50} values of the full-length prosegments with those of the C-24 peptides reveals a difference of 40-70 fold. Also, removal of the N-terminal 17 aa of pPC7 resulted in a substantial decrease in this polypeptide's inhibitory potency (*not shown*). These findings argue that regions of multiple interfacial contacts between the full-length prosegment and proteinase domains are involved in the inhibitory process. The exact relationships of these interactions remain to be determined. Interestingly, we found no convincing evidence that pFur-M15, the pFurin peptide encompassing the secondary processing site, plays a significant role in inhibition (Table 2.2). Moreover, mixing the furin M15 and C24 peptides did not change the inhibitory nature of the latter (*not shown*). Hence, although cleavage at this secondary site occurs in cells, the exact nature of its pH-dependent interaction with furin (Anderson *et al.*, 1997) remains to be determined. Further experimentation with peptides derived from this region of the prosegment may shed some light on the mechanism of the secondary site cleavage.

Having demonstrated that the prosegments of furin and PC7 are potent and reasonably specific inhibitors of their parent enzymes *in vitro*, we turned our attention to the question of whether these prosegments, expressed as independent domains, can act *in trans* to inhibit intracellular furin and PC7. Successful inhibition would require not only

that these polypeptides enter the secretory pathway, but also that they remain there long enough to interact with the mature target PC (*i.e.* most likely within the TGN). Current evidence suggests that a polypeptide must have a minimum size of at least 50 aa in order to be recognized by the signal recognition particle and threaded through the membrane of the ER (Ibrahimi *et al.* 1986, Okun *et al.* 1990). In agreement with this hypothesis, a 64 aa long prepropeptide derived from frog skin is the smallest known to-date (Hoffmann *et al.* 1983). Including their signal peptides, the preprosegments of furin (ppFurin) and PC7 (ppPC7) contain 80 and 104 aa, respectively (Seidah *et al.*, 1998a). To test whether these prosegments can independently enter the secretory pathway, we produced antisera specific for each of the prosegments (Fig. 2.6). Overexpression of either ppFurin or ppPC7 using vaccinia virus infection of BSC40 or AtT20 cells revealed that the former loses its signal peptide very slowly (Fig. 2.6), and that only pPC7 is secreted into the medium (*not shown*). We interpreted these data to mean that the independently expressed prosegments were reasonably stable within the secretory pathway and that, at least in the case of PC7, were able to pass through it intact. In order to test the *ex vivo* inhibitory function of these polypeptides, we examined the processing of proNGF to NGF in the presence and absence of the vaccinia virus prosegment construct-infected Schwann cells. As seen in Fig. 2.7, ppFurin significantly inhibits the maturation of this neurotrophin, which is known to occur in the TGN and be best carried out by furin (Seidah *et al.*, 1996b). In fact, it is nearly as effective as the serpin α1-PDX, which was included as a positive control, having been shown previously to be a potent inhibitor of the proNGF to NGF conversion (Anderson *et al.*, 1993). In contrast, ppPC7 has only a slight inhibitory effect (Fig. 2.7). Although PC7 is expressed in Schwann cells (Marcinkiewicz *et al.*, 1998), it is a poor effector of proNGF maturation (S. Benjannet and N.G. Seidah, *unpublished observations*). Thus, the mild inhibition seen in this experiment is most likely due to cross-reactivity between pPC7 and furin (see Table 2.1).

The processing of proBDNF to BDNF represents another well characterized neurotrophin maturation event on which to test the inhibitory potency of our preprosegment constructs (Basak *et al.*, 1997). In these experiments, cellular co-expression of ppFurin and proBDNF via transient transfection in COS-1 cells completely

inhibits the production of the 14 kDa BDNF (Fig. 2.8). Transfection with the ppPC7 polypeptide was noticeably less effective, again demonstrating the selective nature of these preprosegments as *ex vivo* inhibitors. Even though PC7 is expressed in COS-1 cells (Seidah *et al.*, 1996c), it is a poor effector of proBDNF maturation in this system (S. Benjannet and N.G. Seidah, *unpublished observations*). Thus, we presume that the inhibition seen in this experiment is also due to cross-reactivity between pPC7 and furin. Interestingly, the antisense preprosegment constructs employed as controls in this experiment were not significantly inhibitory. This is of interest in view of the reported use of antisense full length furin for the down regulation of its expression (Liu *et al.*, 1995). Finally, the expression of ppFurin resulted in the appearance of small amounts of the 28 kDa form of BDNF. This product, which is best detected by immunoprecipitation and not by Western blots (*not shown*), is generated by SKI-1 (Seidah *et al.*, 1999a). This result was further confirmed by metabolic labeling, as described (Seidah *et al.*, 1999a) (*not shown*). We conclude from these data that the PC preprosegments tested here selectively inhibit the maturation of proBDNF by furin-like enzymes but not by SKI-1. These events likely occur in the TGN, where the processing of neurotrophins in constitutively secreting cells has been localized (Seidah *et al.*, 1996a; 1996b).

In conclusion, this work on the inhibitory properties of PC prosegments provides the first evidence that these polypeptides can be used to inhibit the cellular processing of precursors *ex vivo*. This technology represents a novel enzyme silencing strategy that will enhance our understanding of the basic cellular functions of these proteinases. Future work will involve improving the performance of these prosegments using site-directed mutagenesis, leading to the design of more selective and powerful convertase inhibitors that may provide novel approaches to the treatment of a variety of pathologies including proliferative, microbial and viral diseases (Chrétien *et al.*, 1995).

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TABLE 2.1

Inhibition of soluble PCs and kexin by pFurin and pPC7

The IC_{50} values were obtained as described in Experimental Procedures from nonlinear curve fitting (SigmaPlot software) using a general equation for competitive enzyme inhibition.

	Enzyme				
	PC7	furin	PC5	PACE4	kexin
Prosegment	IC_{50} [nM] (Ratio relative to parent enzyme)				
pFurin	20 (5)	4 (1)	0.4 (0.1)	110 (27.5)	500 (125)
pPC7	0.4 (1)	40 (100)	20 (50)	25 (63)	1000 (2500)

Table 2.2 *Inhibition of PC7 and furin by full-length prosegments vs prosegment-derived peptides*

The IC_{50} values were obtained as described in Experimental Procedures from nonlinear curve fitting (SigmaPlot software) using a general equation for competitive enzyme inhibition. The *Ki* values are representative of triplicate Dixon plots.

		Enzyme			
		Furin	PC7	Furin	PC7
Inhibitor	Peptide sequence	IC_{50} (nM)		*Ki* (nM)	
hFur-FL [1]	^{27}QKVFTN.....QQVAKRRTKR^{107}	4	-	- [2]	-
hFur-M15	^{62}GDYYHFWHRGVTKRS^{76}	>50,000	-	-	-
hFur-C10	^{98}QQVAKRRTKR^{107}	~ 150	-	40	~ 500
hFur-C24	^{84}HSRLQREPQVQWLEQQVAKRRTKR^{107}	~ 150	-	35	90
hFur-C10A (R107A)	^{98}QQVAKRRTK**A**^{107}	>100,000	-	-	-
rPC7-FL [1]	^{37}LTEAGG.....EQRLLKRAKR^{140}	-	0.4	-	-
rPC7-N20	^{39}EAGGLDTLGAGGLSWAVHLD^{58}	-	>100,000	-	-
rPC7-M18	^{86}GRIGELQGHYLFVQPAGH^{103}	-	>100,000	-	-
rPC7-C10	^{131}EQRLLKRAKR^{140}	-	28	81	6
rPC7-C18	^{123}HEAVRWHSEQRLLKRAKR^{140}	-	20	88	5
rPC7-C24	^{117}EAVLAKHEAVRWHSEQRLLKRAKR^{140}	-	23	65	7
rPC7-C18A (R107A)	^{123}HEAVRWHSEQRLLKRAK**A**^{140}	-	>100,000	-	-

[1] FL corresponds to "full-length" prosegment constructs which contain additional N-terminal MDP and C-terminal hexa His sequences.

[2] Indicates that this value was not determined.

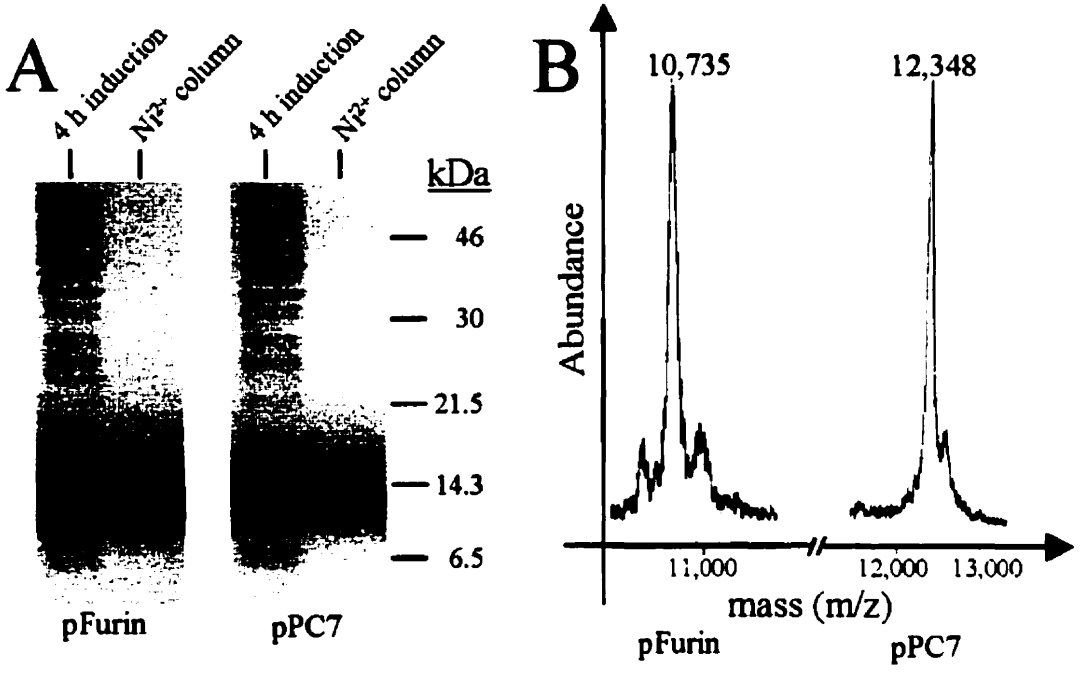

FIG. 2.1. **Expression, purification and characterization of the prosegments of hfurin and rPC7 from BL21 cells.** [**A**] Coomassie staining of proteins resolved by SDS-PAGE on 15% polyacrylamide gels. The pFurin and pPC7 samples represent bacterial extracts following 4h induction with IPTG and Ni^{2+} affinity purification. [**B**] MALDI-TOF analysis of the purified pFurin (MM 10,735) and pPC7 (MM 12,348).

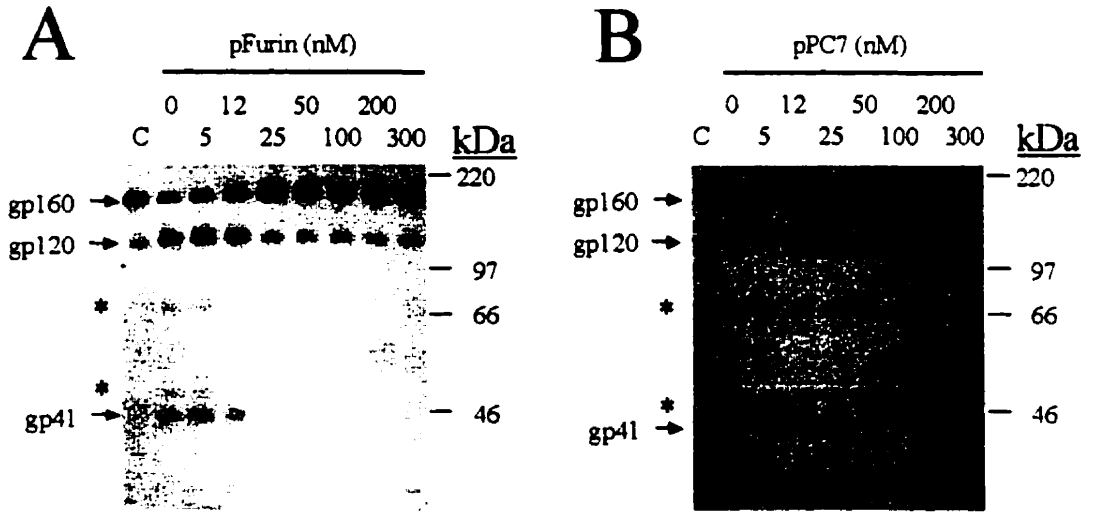

FIG. 2.2. **Inhibition of furin-mediated gp160 digestion by pFurin and pPC7.** SDS-PAGE analysis on 8% polyacrylamide gels of the processing of [^{35}S]Met-labeled gp160 into gp120 and gp41 following incubation for 16h with furin in the absence (control, C) or presence of increasing amounts of [**A**] pFurin or [**B**] pPC7. (*) depicts the position of gp77 and gp53 which are also produced by furin (Vollenweider *et al.*, 1996).

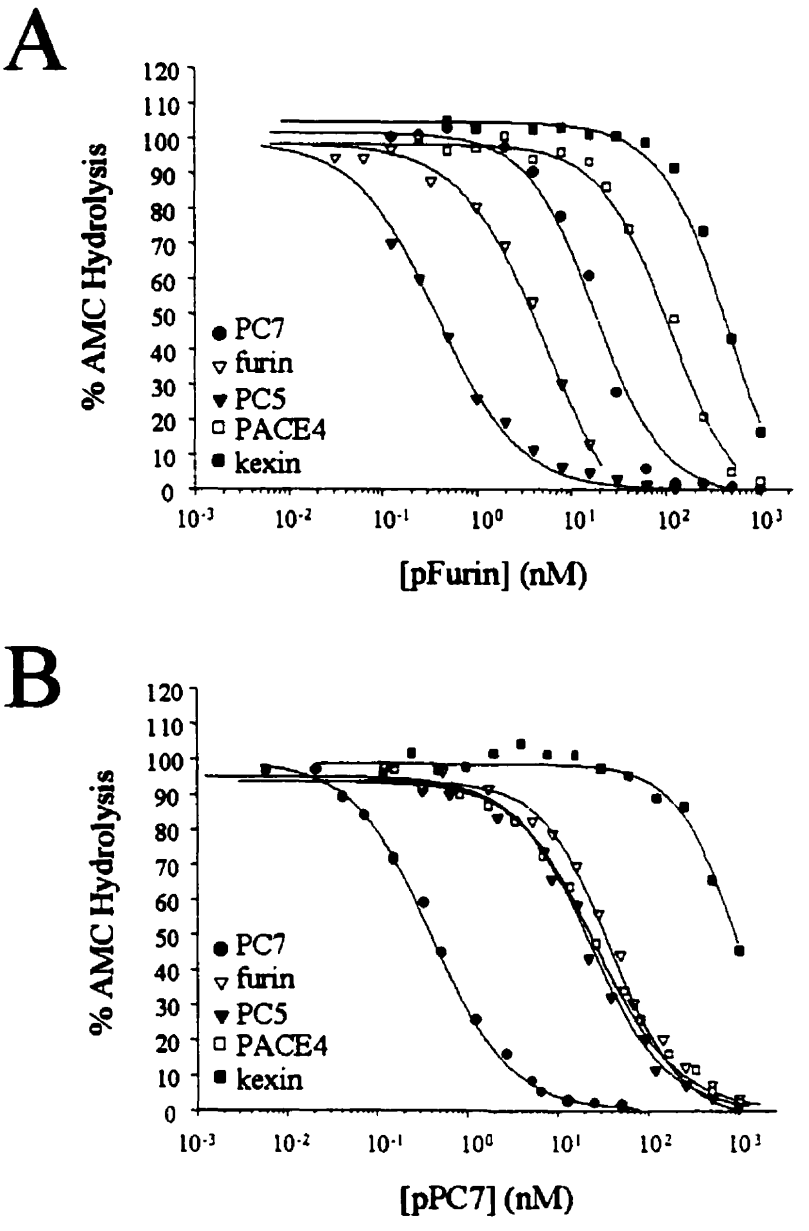

FIG. 2.3. **pFurin and pPC7 inhibition of the pERTKR-MCA processing by PCs and kexin.** The curves represent the progressive inhibition of the furin-, PC7-, PC5-, PACE4- and kexin-mediated cleavage of 100 μM pERTKR-MCA after 1h by increasing amounts of either [**A**] pFurin or [**B**] pPC7. The ordinate represents the % cleavage activity with respect to the control incubation in absence of inhibitor. Nonlinear curve fitting was carried out using SigmaPlot software.

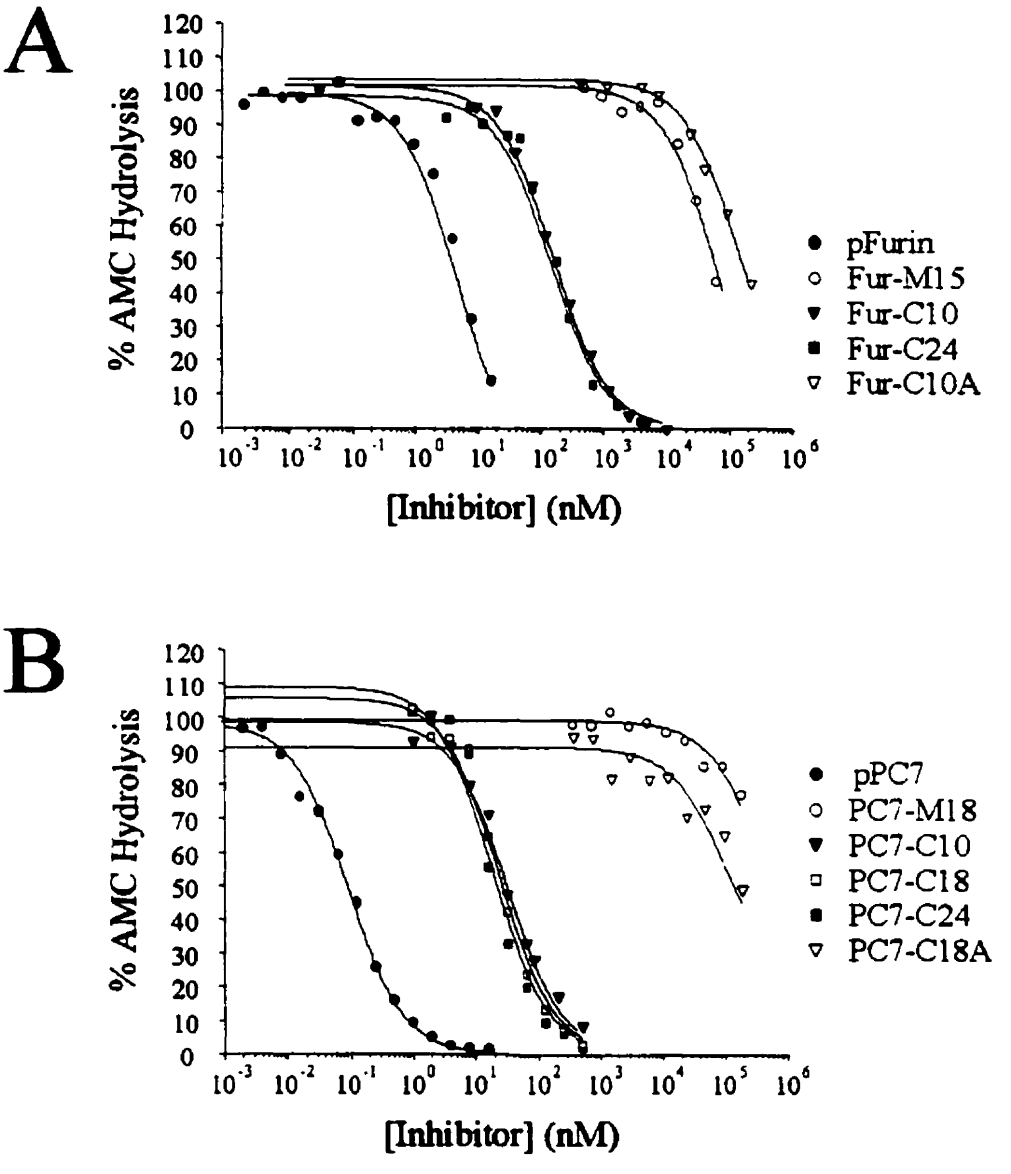

FIG. 2.4. **Comparative inhibitory effects of pFurin, pPC7 and their synthetic peptides on furin and PC7-mediated processing of the pERTKR-MCA.** The curves represent the progressive inhibition of [**A**] furin activity by pFurin, M15, C10, C24 and C10A or **[B]** PC7 activity by pPC7, M18, C10, C24, C18 and C18A.

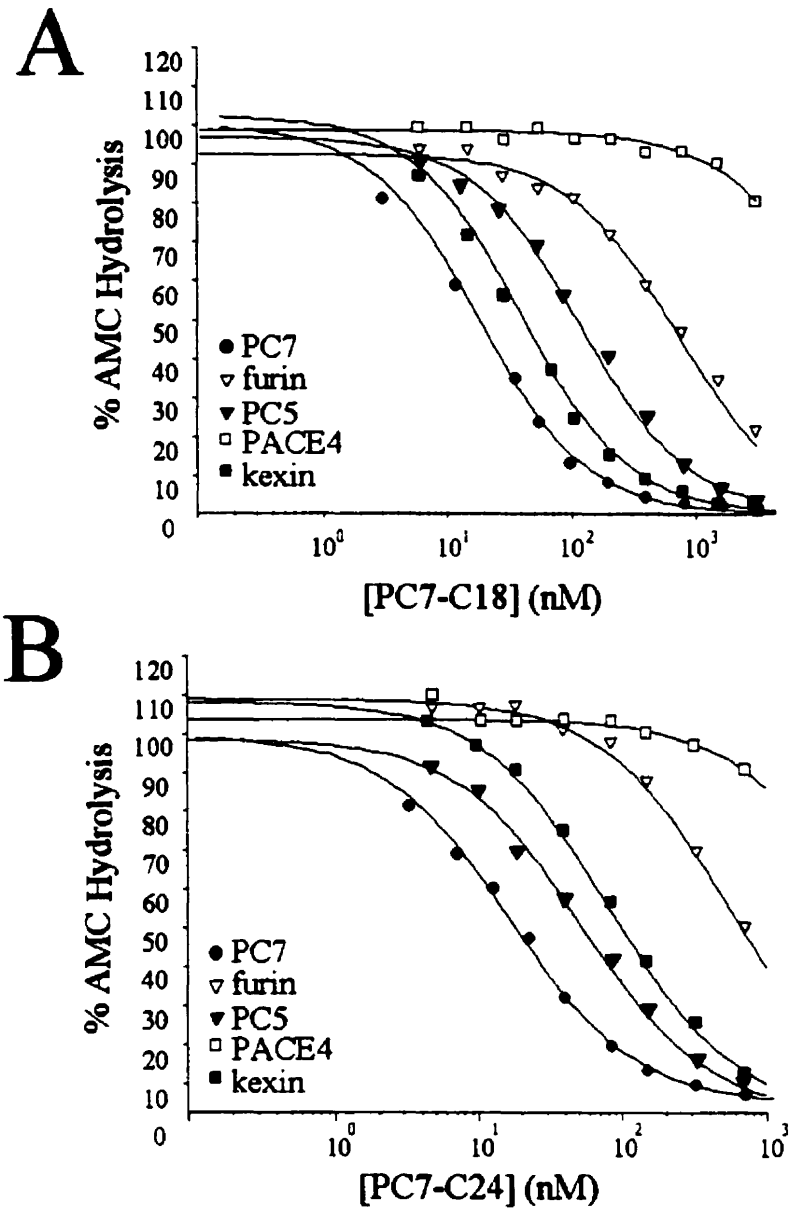

FIG. 2.5. **Inhibition by PC7-C18 and PC7-C24 of the pERTKR-MCA processing by PCs and kexin.** The curves represent the progressive inhibition of the furin-, PC7-, PC5-, PACE4- and kexin-mediated cleavage of 100 μM pERTKR-MCA after 1h by increasing amounts of either [**A**] PC7-C18 or [**B**] PC7-C24.

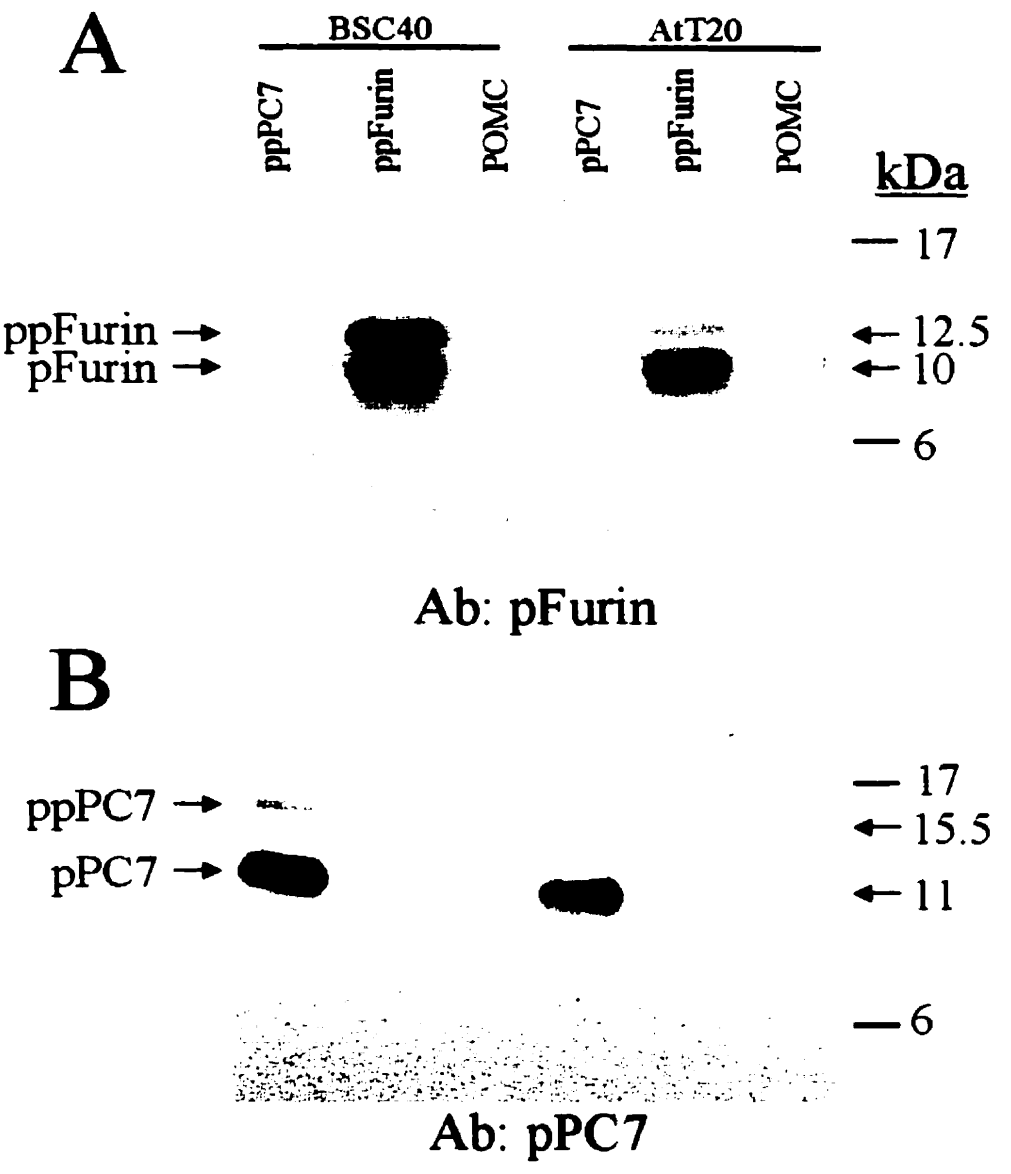

FIG. 2.6 **Cellular expression of ppFurin and ppPC7.** VV:pPC7- and VV:ppFurin-infected BSC40 and AtT20 cells were pulse-labeled with [^{35}S]Met for 2h and the cell lysates immunoprecipitated with [**A**] anti-pFurin or [**B**] anti-pPC7. The immunoprecipitates were then analyzed by SDS-PAGE on 14% polyacrylamide Tricine gels. The estimated MM of preprosegments and prosegments are depicted together with the position of the MM standards.

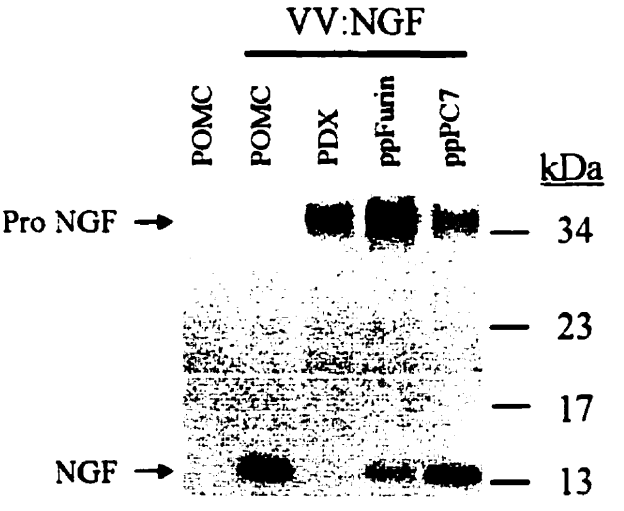

FIG. 2.7. **Inhibition of proNGF processing.** Rat Schwann cells were infected with either VV:POMC (antigen control), or co-infected with VV:NGF and either VV:POMC (control), VV:PDX, VV:ppFruin or VV:ppPC7. The cells were then pulse-labeled with [^{35}S]Met for 4h and the media immunoprecipitated with an NGF antiserum. The migration positions of the 35 kDa proNGF and the 13.5 kDa NGF are shown.

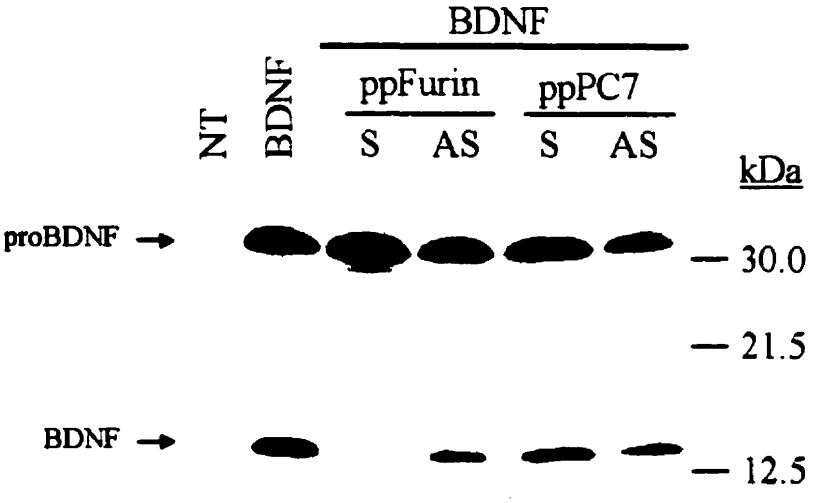

FIG. 2.8. **Inhibition of proBDNF processing.** Western blot analysis of non-transfected (NT) COS-1 or cells transfected with pcDNA3 recombinants of proBDNF as control (BDNF) or together with recombinants expressing sense (S) or antisense (AS) ppPC7 or ppFurin. The secreted products resolved by SDS-PAGE were analyzed with a Santa Cruz BDNF-specific antiserum. Notice the appearance of a 28 kDa proBDNF product in the presence of S-ppFurin.

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CHAPTER 3

Functional analysis of human PACE4-A and PACE4-C isoforms: identification of a new PACE4-CS isoform

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Preface

The paper presented in this chapter describes another structural domain of proprotein convertases, namely the P-domain. Three members of the proprotein convertase family; PACE4, PC4 and PC5, exhibit alternative splicing of their RNAs resulting in the generation of multiple isoforms (Figure 1.5). In this work, we isolated a new isoform of PACE4 called PACE4-CS (the shortened form of PACE4-C that lacks 11 amino acids at the end of its chaperone-like P-domain) which led us to address the question of the integrity of the P-domain. We examined the biosynthesis, functional activity and cellular localization of these isoforms. Cellular expression results demonstrated that PACE4-A (intact P-domain) codes for a functional secretable enzyme capable of precursor cleavage. In contrast, PACE4-CS (disrupted P-domain) is not secreted and remains in the endoplasmic reticulum as an inactive zymogen form, thereby emphasizing the importance of the integrity of the P-domain. One year after the paper was published, another new isoform of PACE4 was cloned from a human cerebellum cDNA library by the same Japanese group who found PACE4-C. They named it PACE4-E (Mori *et al.*, 1997). It codes for a functional enzyme and possesses a hydrophobic cluster in its C-terminus acting as a transmembrane domain. The size of PACE4-E mRNA from adult rat brain was estimated by Northern blotting to be 4.4 kb, just like that of PACE4-A. *In situ* hybridization histochemistry revealed that the highest level of PACE4-E mRNA was expressed in mitral cells of the adult rat olfactory bulb, which is a unique sensory organ in that it has a lifelong regenerating capacity and it affects brain development. They also analyzed the expression of PACE4-E mRNA in the developing olfactory system. On day 13.5 of gestation, PACE4-E mRNA was expressed at high levels in the neuroepithelium of the forebrain vesicle, olfactory epithelium, and cells in the fiber bundles projecting to the forebrain vesicle. As development proceeded, PACE4-E mRNA was expressed in developing mitral cells but decreased in the olfactory epithelium. In the newborn, its expression was confined to the mitral cells in both the main and accessory olfactory bulb and in some periglomerular cells, as shown in adult rats. The spatio-temporal expression of PACE4-E suggests that it plays a role in the establishment and maintenance of the olfactory receptor (Akamatsu *et al.*, 1997).

Abstract

There are seven known subtilisin/kexin-like pro-protein convertases responsible for the processing of numerous precursors at either pairs or specific single basic residues. Three members, PACE4, PC4 and PC5 exhibit alternative splicing of their RNAs resulting in the generation of multiple isoforms differing in their C- or N-terminal segments. In this study we examined the biosynthesis, functional activity and cellular localization of two of these isoforms namely the full length PACE4-A and C-terminally truncated PACE4-C which misses 11 amino acids at the end of its chaperone-like P-domain. We report the existence of a new isoform, termed PACE4-CS, which is a C-terminally shortened version of PACE4-C. Cellular expression results demonstrated that PACE4-A codes for a functional secretable enzyme capable of cleaving pro7B2 into 7B2. In contrast, PACE4-CS is not secreted since it remains in the endoplasmic reticulum as an inactive zymogen form, thereby emphasizing the importance of the integrity of the P-domain. Microsequencing of the intracellular PACE4-CS protein in two cell lines revealed that it is proPACE4-CS with an N-terminal trimming reminiscent of the action of a dipeptidylpeptidase (DPP-IV) recognizing the motifs X-Ala and X-Pro.

1. Introduction

Processing of proproteins and prohormones at either single or pairs of basic residues is a general mechanism to generate bioactive proteins and peptides from inactive precursors (Rouille *et al.*, 1995; Seidah & Chrétien, 1992). The subtilisin/kexin-like proteinases responsible for such intracellular cleavages were recently described and called "proprotein convertases" (PCs). So far, seven members of this family were identified including: furin, PC1, PC2, PC4, PACE4, PC5 and PC7 (for reviews see (Rouille *et al.*, 1995; Seidah & Chrétien, 1992; Seidah *et al.*, 1994; 1996c)). All of these PCs exhibit an N-terminal signal peptide, followed by a pro-segment, a catalytic-domain, a P-domain and an enzyme-specific C-terminal segment (Seidah *et al.* 1995b). Variant cDNA structures, possibly arising from alternative gene splicing, were reported for PC4 (Mbikay *et al.*, 1995; Seidah *et al.*, 1992), PC5 (Nakagawa *et al.*, 1993) and PACE4 (Kiefer *et al.* 1991; Tsuji *et al.*, 1994a). In the case of PC5, the isoform PC5-A is directed to secretory granules while PC5-B localizes to the TGN (de Bie *et al.*, 1996). For human PACE4 four different isoforms were so far reported and named PACE4-A (full length), PACE4-B (also known as PACE4.1), PACE4-C and PACE4-D (Kiefer *et al.* 1991; Tsuji *et al.*, 1994a). Furthermore, as compared to human PACE4-A (Kiefer *et al.* 1991), the rat and mouse homologues (Hosaka *et al.*, 1994; Johnson *et al.*, 1994) exhibit an extra Leu residue close to the predicted signal peptidase cleavage site and additionally show three deletions within the signal peptide, the N-terminal of the catalytic domain and the C-terminal segment which follows the conserved P-domain. From the reported importance of the pro-segment acting as an intramolecular chaperone in subtilisin-like enzymes (Sinde & Inouye 1995), it was suggested that PACE4-D would code for an inactive enzyme (Tsuji *et al.*, 1994a). Similarly, as the integrity of the P-domain has been reported to be critical for the activity of yeast kexin (Gluschankof & Fuller, 1994) and mouse furin (Hatsuzawa *et al.*, 1992), it was also predicted that PACE4-B, which lacks the P-domain, should be inactive (Kiefer *et al.* 1991). In contrast, PACE4-C (652 aa), which is truncated at the C-terminus compared to PACE4-A (969 aa), has a distinct sequence at the end of the P-domain, resulting in a shorter form in which the 32 amino acids following Gly^{620}

are different. While the C-terminal border of the P-domain has been suggested to be close to Gly^{631} (Gluschankof & Fuller, 1994; Hatsuzawa *et al.*, 1992; Seidah *et al.* 1995b), its exact site is not known with certainty and could be convertase-dependant. Therefore, it was suggested that PACE4-C could code for an active convertase (Tsuji *et al.*, 1994a). Furthermore, it was reported that PACE4-C is expressed in a tissue-specific manner, based on RT-PCR (Tsuji *et al.*, 1994b) and immunocytochemical (Nagamune *et al.*, 1995) criteria.

In this work, we isolated a fifth isoform called PACE4-CS (shortened form of PACE4-C) which led us to address the question of the integrity of the P-domain, the tissue distribution of the PACE4 isoforms and to suggest that PACE4-A is the only active proteinase of the isoforms so far known.

2. Materials and Methods

2.1. Cellular Expression of PACE4 isoforms

The mRNA expression of PACE4 isoforms was analyzed by RT-PCR on 5 µg of total RNA (Seidah *et al.* 1995a) isolated from established cell lines including: Caco2 (colon carcinoma, human), LoVo (colon adenocarcinoma, human), HepG2 (hepatocellular carcinoma, human), and SKNM (neuroepithelioma, human). The sense oligonucleotides used were the general PACE4 oligonucleotides: oligo II: TTCATGACTGTCCACTGCTGGGGAG, oligo VI: GGGACCTCAGTCTCTGCCCCCATG; and the PACE4-C/CS-specific oligo IV: CCTGGACTAAAACACGT. The antisense oligonucleotides were either the PACE4-A-specific: oligo I: CAGACAGTACATTTCTCA; or the PACE4-C/CS-specific oligo III: CTACTGGAGATACAGAACCGAC, and the [HindIII]-containing oligo V: [AAGCTT]GCGGGAGCTGAGAGATCCAGCTCTGGAC. The 30 cycle PCR reactions were performed at an annealing temperature of 58°C for PACE4-A-specific reactions (oligo pair II/I) and for PACE4-C/CS-specific PCRs at either 50°C (oligo pair II/III) or 55°C for the pair (VI/V) and for the nested PCR using the pair (IV/III).

2.2. Vaccinia virus expression of PACE4-A and PACE4-CS

The RT-PCR product of a PACE4-C-specific sequence using the pair oligo (VI/V) allowed the isolation of a 770 bp PACE4-C-like fragment. Transfer to the PCR™II vector and sequencing revealed that this segment is an alternatively-spliced product of PACE4-C called PACE4-CS (Fig. 3.2). This allowed us to construct a PACE4-CS cDNA by ligation of the 5' NcoI- and 3' HindIII digested PCR-product (745 bp) into the original PMJ601 VV-transfer vector containing the full length PACE4-A cDNA digested with the same enzymes (Benjannet *et al.*, 1995). The isolation of the recombinant VV was obtained as previously described (Benjannet *et al.*, 1993). The green monkey kidney epithelial BSC40 cells or the mouse corticotroph AtT20 cells were infected with 4 pfu/cell of either VV:hPACE4-A, VV:hPACE4-CS or VV:wild type (VV:WT) (Benjannet *et al.*, 1993). Following overnight growth, the cells were washed and then pulse-labeled with [^{35}S]methionine, [^{3}H]tyrosine or [^{3}H]valine for 4h, as previously described (Benjannet *et al.*, 1993; 1995). The media and cell extracts were then purified on a lentil-lectin column (Pharmacia) and analyzed by autoradiography of an SDS-PAGE separation. The identified PACE4-CS (77 kDa) [^{3}H]Tyr- and [^{3}H]Val-labeled bands were then excised and the underlying protein microsequenced on an Applied Biosystem model 470A sequenator, as previously described (Benjannet *et al.*, 1993; 1995; Vollenweider *et al.*, 1996).

2.3. Bioactivity of PACE4 isoforms

The enzymatic activity of PACE4-A or PACE4-CS was gauged by its capacity to process the neuroendocrine precursor pro7B2 (Benjannet *et al.*, 1995). Accordingly, BSC40 cells infected with 2 pfu/cell of VV:m7B2 were coinfected with 4 pfu/cell of either VV:PACE4-A, VV:PACE4-CS or VV:wild type. Following overnight growth, the cells were pulse-labeled for 30 min (P30) with [^{35}S]methionine and then chased with cold methionine for 60 min (C60). The cell-extracts and media were immunoprecipitated with a 7B2-specific antibody and the precipitates analyzed by SDS-PAGE as described elsewhere (Benjannet *et al.*, 1995).

3. Results

3.1. Cellular expression of PACE4 isoforms

Previous results using a general PACE4 probe recognizing all isoforms demonstrated the widespread tissue and cellular mRNA expression of PACE4 (Dong *et al.*, 1995; Hosaka *et al.*, 1994; Johnson *et al.*, 1994; Kiefer *et al.* 1991; Seidah *et al.*, 1994; Tsuji *et al.*, 1994b). Aside from the major 4.4 kb transcript, a 3.9 kb mRNA was also detected in some rat tissues such as the jejunum, duodenum and kidney (Seidah *et al.*, 1994), suggesting the presence of alternatively spliced mRNA forms of PACE4. Recent reports of Tsuji *et al.* (Tsuji *et al.*, 1994a; 1994b) suggest that an alternatively spliced human PACE4-C isoform can be isolated from human placenta. However, none of these isoforms could be detected by screening mouse liver, kidney or brain cDNA libraries (Hosaka *et al.*, 1994). In order to probe for the specific expression of the PACE4-A and PACE4-C isoforms, we first attempted to perform Northern blots on cell lines and rat tissues with human PACE4-A and PACE4-C-specific probes obtained by PCR using the oligo pairs (II/I) and (IV/III) respectively (Fig. 3.1A). Although similar results to those obtained in (Seidah *et al.*, 1994) were found for PACE4-A expression, we could not detect PACE4-C-specific mRNAs in the tissues or cells analyzed in (Seidah *et al.*, 1994) (*not shown*). Therefore, we opted for the use of RT-PCR as an alternative and more sensitive approach for the detection of PACE4-C-specific mRNA. Accordingly, two consecutive PCR amplifications were needed in order to detect PACE4-C-like transcripts. In the first PCR, no visible amplification product was obtained with the oligo pair (II/III). However, nested PCR of an aliquot of this first amplification using the oligo pair (IV/III) allowed the detection of PACE4-C-like expression in the human cell lines Caco2, LoVo, HepG2 but not in SKNM cells (Fig. 3.1A,B). In contrast, PACE4-A expression was easily detected by RT-PCR using the single oligo pair (II/I) (Fig. 3.1A,C). This result suggests that in the cells analyzed, PACE4-A transcripts are much more abundant than those of PACE4-C.

In order to obtain a construction of the full length PACE4-C, it was necessary to amplify the variant 3' end using the oligo pair (VI/V) starting from either HepG2 or

Caco2 cells (Fig. 3.1A,D). With this pair it was possible to detect the expected PACE4-C-like product of 770 bp on the first amplification reaction by using 35 cycles of amplification instead of the usual 30 (Fig. 3.1D). In contrast, the same RT-PCR done on human pituitary RNA did not yield any product (Fig. 3.1D). To our surprise, the DNA sequence of this PCR product was itself a spliced form of PACE4-C, where the gt/ag donor/acceptor splice sites were derived from an exon of PACE4-C (Fig. 3.2). Accordingly, this new variant, called PACE4-CS, is a 3' shortened version of PACE4-C with a different C-terminal sequence. Thus, both PACE4-C and PACE4-CS diverge from PACE4-A at Gly^{620} within the predicted P-domain (Fig. 3.2). However, PACE4-C and PACE4-CS are C-terminally extended by 32 and 3 amino acids to give proteins composed of either 652 or 623 residues, respectively. Analysis of 11 different clonal inserts in PCR^{TM}II (see methods) gave the same DNA sequence and did not reveal the presence of an authentic PACE4-C product. This 770 bp product was then digested with appropriate restriction enzymes (see methods) and ligated to the equivalently digested PACE4-A cDNA, resulting in a full length PACE4-CS construct which was used to obtain a VV:PACE4-CS recombinant.

3.2. Biosynthetic analysis of PACE4-A and PACE4-CS

Cellular expression of PACE4-A and PACE4-CS was analyzed after infection of the constitutively secreting cell line BSC40 with either VV:PACE4-A or VV:PACE4-CS and followed by a 4h pulse-labeling with [^{35}S]methionine. The labeled products could not be immunoprecipitated as PACE4-specific antibodies have not yet been possible to obtain. As an alternative, since PACE4 is predicted to be N-glycosylated (Kiefer *et al.* 1991), we used a lentil lectin column (Decroly *et al.*, 1994) to concentrate N-glycosylated proteins from cell extracts and media. As shown in Fig. 3.3, this technique revealed that PACE4-CS encodes a non-secretable protein (even after very long exposure of the gel, no protein is detected in the medium) migrating with an apparent Mr of 77 kDa on SDS-PAGE. In contrast, VV:PACE4-A expression results in the intracellular labeling of a number of bands migrating with Mr between 160-180 kDa but releasing only the 150 kDa product. Therefore, although PACE4-A is a secretable protein, PACE4-CS is not

detectable in the medium. Furthermore, digestion of the 77 kDa PACE4-CS with either endoglycosidase H or N-glycanase F (Seidah *et al.*, 1996c) revealed that this form is sensitive to both enzymes (*not shown*). In contrast, the secreted 150 kDa PACE4-A is only digested by N-glycanase F with only about a 5 kDa decrease in apparent molecular mass *(not shown)*. Therefore, both PACE4-CS and PACE4-A migrate with apparent molecular masses much higher than predicted from their amino acid sequences and that this is not primarily due to carbohydrate attachment, but could in part be due to the Cys-rich domain in PACE4-A, as observed for *Drosophila* furins (de Bie *et al.*, 1995). These data suggest that PACE4-CS is localized within a cellular compartment where trimming of carbohydrate chains does not proceed to completion, most likely the endoplasmic reticulum itself, whereas PACE4-A can exit the cell through the constitutive secretory pathway of BSC40 cells.

In order to unequivocally define the nature of the 77 kDa PACE4-CS product, we microsequenced the [^{3}H]-Val- and [^{3}H]-Tyr-labeled proteins obtained from both AtT20 and BSC40 cells. The N-terminal sequence of the intracellular 77 kDa PACE4-CS product reveals a Tyr^7 in AtT20 cells (Fig. 3.4A) and a $Tyr^{6,7}$ in BSC40 cells (Fig. 3.4B). This surprising result, led us to examine the sequence of valine labeled PACE4-CS from BSC40 cells. The data obtained suggest the presence of two chains differing by one amino acid, i.e., of sequence $Val^{5,12,14}$ and $Val^{6,13,15}$ (Fig. 3.4C). This interpretation is based on the much lower carry-over of radioactivity from cycle to cycle obtained for labeled proteins which were sequenced before and after PACE4-CS (*not shown*). From the predicted protein structure of PACE4 (Kiefer *et al.* 1991), these results suggest that the 77 kDa sequence starts at either Pro^{64} or Pro^{65} in BSC40 cells and at Pro^{64} in AtT20 cells. Furthermore, the data demonstrate that proPACE4-CS is not processed into PACE4-CS.

3.3. Processing of pro7B2 by PACE4-A and PACE4-CS

In order to demonstrate enzymatic activity of the PACE4 isoforms, the media of BSC40 cells overexpressing PACE4-A or PACE4-CS was partially purified on a DEAE column as reported for PC1 (Jean *et al.*, 1993). Accordingly, although appreciable activity

towards the fluorogenic substrate pGluArgThrLysArg-MCA was observed for PACE4-A (Munzer, S. *et al.*, *in preparation*), none was obtained for PACE4-CS (*not shown*). This result is in agreement with the biosynthetic data (Fig. 3.3) which revealed that only PACE4-A could be detected in the medium. In order to further probe the intracellular enzymatic activity of either PACE4-A or PACE4-CS, we co-expressed these isoforms with pro7B2, a PC2-specific binding protein (Benjannet *et al.*, 1995; Braks & Martens, 1994; Martens *et al.*, 1994) known to be processed in the TGN by furin-like enzymes (Benjannet *et al.*, 1995; Paquet *et al.*, 1994). As shown in Fig. 3.5, only PACE4-A was able to increase the intracellular processing of pro7B2 (30 kDa) into 7B2 (23 kDa), which is then secreted. No increased processing over background control (pro7B2/wild type virus) was observed when PACE4-CS was co-expressed with pro7B2 (Fig. 3.5). In conclusion, our data demonstrate that PACE4-CS does not exhibit intracellular (*ex vivo*) or extracellular (*in vitro*) enzymatic activity. Accordingly, we suggest that only PACE4-A is an enzymatically active convertase.

4. Discussion

Tissue-specific processing of inactive precursors into active polypeptides is a general mechanism to generate and regulate the level of biological diversity achieved with a given proprotein. The role of the PCs in the generation of such diversity is now well accepted and is the subject of intense studies aimed at defining the specific role of each of the seven known convertases in this process (Rouille *et al.*, 1995; Seidah & Chrétien, 1992; Seidah *et al.*, 1994; 1995; 1996). Another level of diversity can also be achieved by alternative splicing of either the proprotein substrate or its cognate convertase(s). For example, the differential intracellular localization of the two PC5-A and PC5-B isoforms is expected to affect the fate of different sets of precursors since PC5-B would cleave constitutively secreted proteins, whereas PC5-A could also process proteins sorted to dense-core secretory granules (de Bie *et al.*, 1996). In a similar fashion, it was thought that the isoforms of PACE4 could also lead to different subcellular localizations of the resultant enzymes. Indeed, our results clearly show that whereas PACE4-A is secreted and active, PACE4-CS is retained intracellularly (likely in the ER)

as an inactive proPACE4-CS zymogen. Therefore, analogous to pro-furin (Molloy *et al.*, 1994) and proPC1 (Benjannet *et al.*, 1993) which remain in the endoplasmic reticulum and are not secreted, it is likely that the 77 kDa proPACE4-CS undergoes a similar fate. What then is the function of a seemingly inactive convertase in the ER? One possibility is that it may act as a dominant negative and affect the level of active PACE4-A, e.g., by interfering with its transport through the cellular secretory pathway. However, cellular co-expression of PACE4-A and PACE4-CS together with pro7B2 did not affect its processing to 7B2 by PACE4-A (S. Benjannet and N.G. Seidah, *unpublished results*). Therefore, although we still do not know the role of the PACE4 isoforms, it is possible that it may be an accident of genetic noise as a result of evolutionary pressures.

The P-domain found at the C-terminus of the catalytic segment in the PCs is not found in the ancestral bacterial subtilisins and has been acquired by the eukaryotic PC genes. The function of this domain is not well known, but recent data suggest that it may play an important chaperone-like role in the folding of the zymogen, hence allowing the autocatalytic processing of the proPCs (Gluschankof & Fuller, 1994; Hatsuzawa *et al.*, 1992; Sinde & Inouye 1995).The integrity of this domain is obviously affected in the PACE4-C/CS isoforms as it misses at least 11 C-terminal amino acids which are replaced by either 36 or 3 aa of different composition, respectively. This may explain why proPACE4-CS is not processed to PACE4-CS (Fig. 3.3), rendering the enzyme functionally inactive (Fig. 3.5). By analogy, we believe that PACE4-C should also code for an inactive enzyme.

In certain rat tissues such as the jejunum, duodenum and kidney, aside from the major 4.4 kb transcript of PACE4-A, a 3.9 kb mRNA was also detected (Seidah *et al.*, 1994). However, northern blot analyses of those same tissues with a PACE4-C/CS-specific probe did not reveal any labeling of specific transcripts (*not shown*), demonstrating that the 3.9 kb form is not PACE4-C/CS. In agreement with the mouse data of Hosaka *et al.* (Hosaka *et al.*, 1994), the mRNA level of PACE4-C/CS seems to be low compared to PACE4-A in all the cell lines and rat tissues examined (Fig. 3.1). Intriguingly, a recent report suggested that PACE4-C immunoreactivity could be detected in rat pancreatic β-cells (Nagamune *et al.*, 1995) which are also known to express PC1

and PC2 (Malide *et al.*, 1995; Rouille *et al.*, 1995). Furthermore, the authors claim that PACE4-A immunoreactivity was undetectable in pancreatic islets (Nagamune *et al.*, 1995). The peptide antigen used to obtain this PACE4-C-specific antibody (Nagamune *et al.*, 1995) would not recognize PACE4-CS as these two isoforms differ at their C-terminus (Fig. 3.2). It remains to be seen whether PACE4-CS is also expressed in β-cells, as we did not study the expression of PACE4 in the pancreas (Seidah *et al.*, 1994). However, we believe that the PACE4-C/CS isoforms would be functionally inactive and hence would not directly participate in the processing of proproteins in this tissue.

Using the Von Heijne criteria (Von Heijne 1990), we estimated the best predicted signal peptidase cleavage site of human proPACE4 as ACS↓APPPRPVYTNHWAVQV and of rat/mouse PACE4 as ACSA↓LPPPRPVYTNHWAVQV (Hosaka *et al.*, 1994; Johnson *et al.*, 1994). The second best predicted site occurs at the AC↓SAPPP sequence. Interestingly, if this site would be recognized by the signal peptidase, the N-terminal sequence of human PACE4-CS would reveal the absence of either the dipeptide AlaPro ($Val^{5.12.14}$ and Tyr^{6} in BSC40 cells) or SerAla ($Val^{6.13.15}$ in BSC40 cells and Tyr^{7} in AtT20 cells) (Fig. 3.4), suggesting an aminodipeptidase activity endogenous to BSC40 and AtT20 cells which recognizes these sequences. It is a matter of speculation whether rat/mouse proPACE4 would also lose its N-terminal LeuPro dipeptide. It has been reported that the secretory pathway of honeybee, amphibian skin and yeast (Kreul 1990) express a dipeptidylaminopeptidase with an X-Pro > X-Ala > X-Gly specificity, resembling the mammalian DPP-IV enzyme. However the restrictions on the nature of the X-residue are not yet clear. It would be informative to pursue this observation in the future and identify the putative mammalian homologue which could be related to the enzyme responsible for the reported stepwise processing of canine gastrin releasing peptide at the N-terminal ValPro-LeuPro- sequence (Kreul 1990).

In conclusion, our data demonstrate that PACE4-A is likely to be the only active form of the so far known PACE4 isoforms. This does not exclude the possibility that other C-terminal variant forms which retain the integrity of the P-domain may turn out to be active enzymes. Thus far, differential splicing of convertase transcripts has resulted in multiple active forms only for PC5 where PC5-A and PC5-B were demonstrated to be

functionally active convertases derived from a single *PC5* gene (Benjannet *et al.*, 1995; de Bie *et al.*, 1996; Vollenweider *et al.*, 1996).

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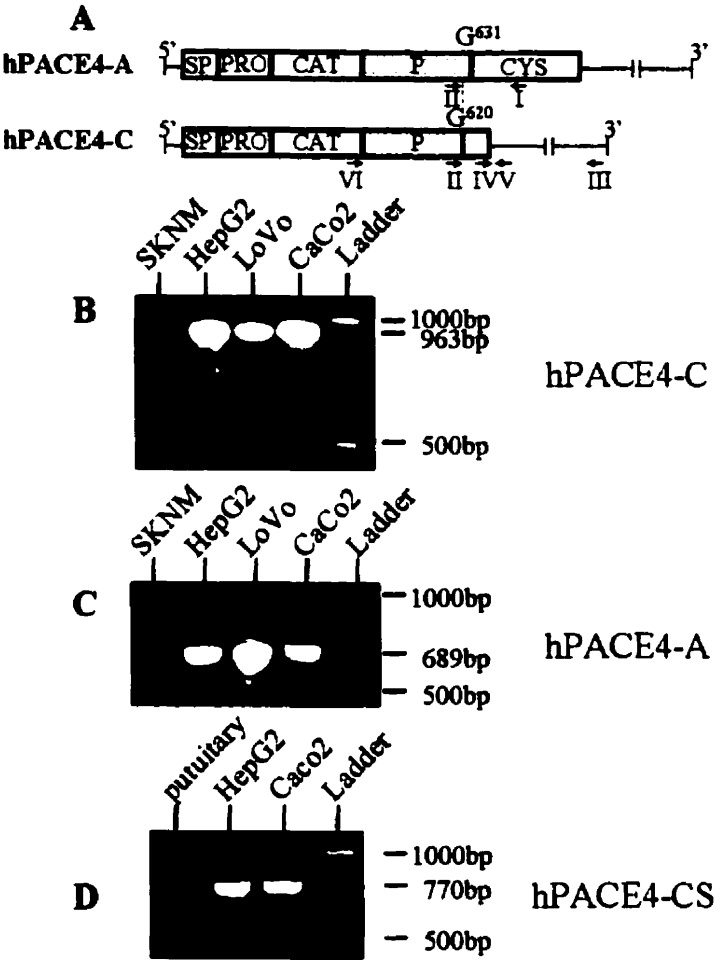

Fig. 3.1. *Tissue and cell expression of PACE4.* [**A**] Diagrammatic representation of human hPACE4-A and hPACE4-C DNA structures including the signal peptide (SP), pro-segment (PRO), catalytic domain (CAT), P-domain (P) and C-terminal Cys-rich domain (CYS). The 5' and 3' non-coding segments are represented by a horizontal line. The positions of the oligonucleotides used in the RT-PCR amplifications are shown. The putative Gly^{631} at the C-terminal end of the P-domain as well as the Gly^{620} where splicing caused the generation of PACE4-C and PACE4-CS, are emphasized. [**B**] RT-PCR of PACE4-C in the human cell lines SKNM, HepG2, LoVo and Caco2. The first RT-PCR done with the oligo pair (II/III) (30 cycles) was followed by a nested PCR with the pair (IV/III) (30 cycles). [**C**] RT-PCR of PACE4-A done with the oligo pair (II/I) (30 cycles). [**D**] RT-PCR of PACE4-CS done on cell lines and human pituitary RNA with the oligo pair (VI/V) (35 cycles).

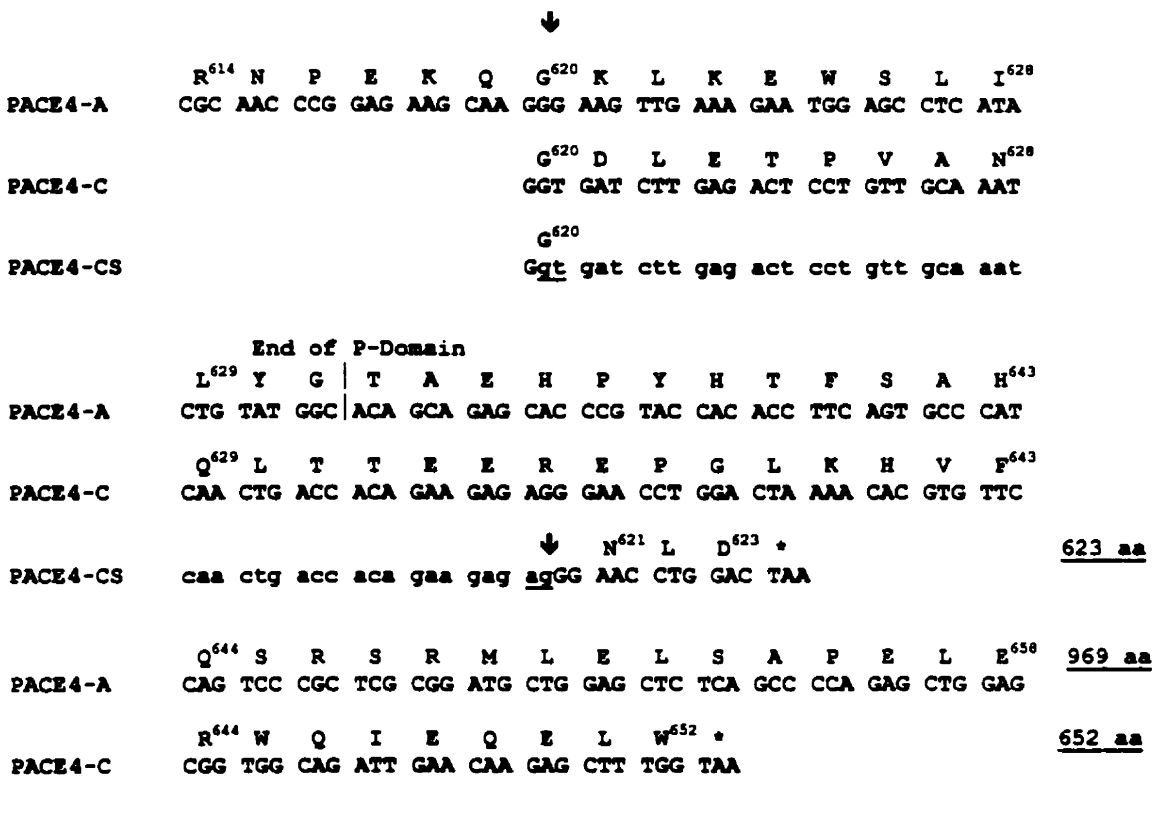

Fig. 3.2. *Generation of PACE4-A, PACE4-C and PACE4-CS isoforms by alternative splicing of the human PACE4 gene.* The positions of the intron-exon junction (↓) and the end of the P-domain (|) are shown. The small letter notations in PACE4-CS emphasize the splicing of this segment at the canonical gt / ag sites resulting in the production of the PACE4-CS isoform.

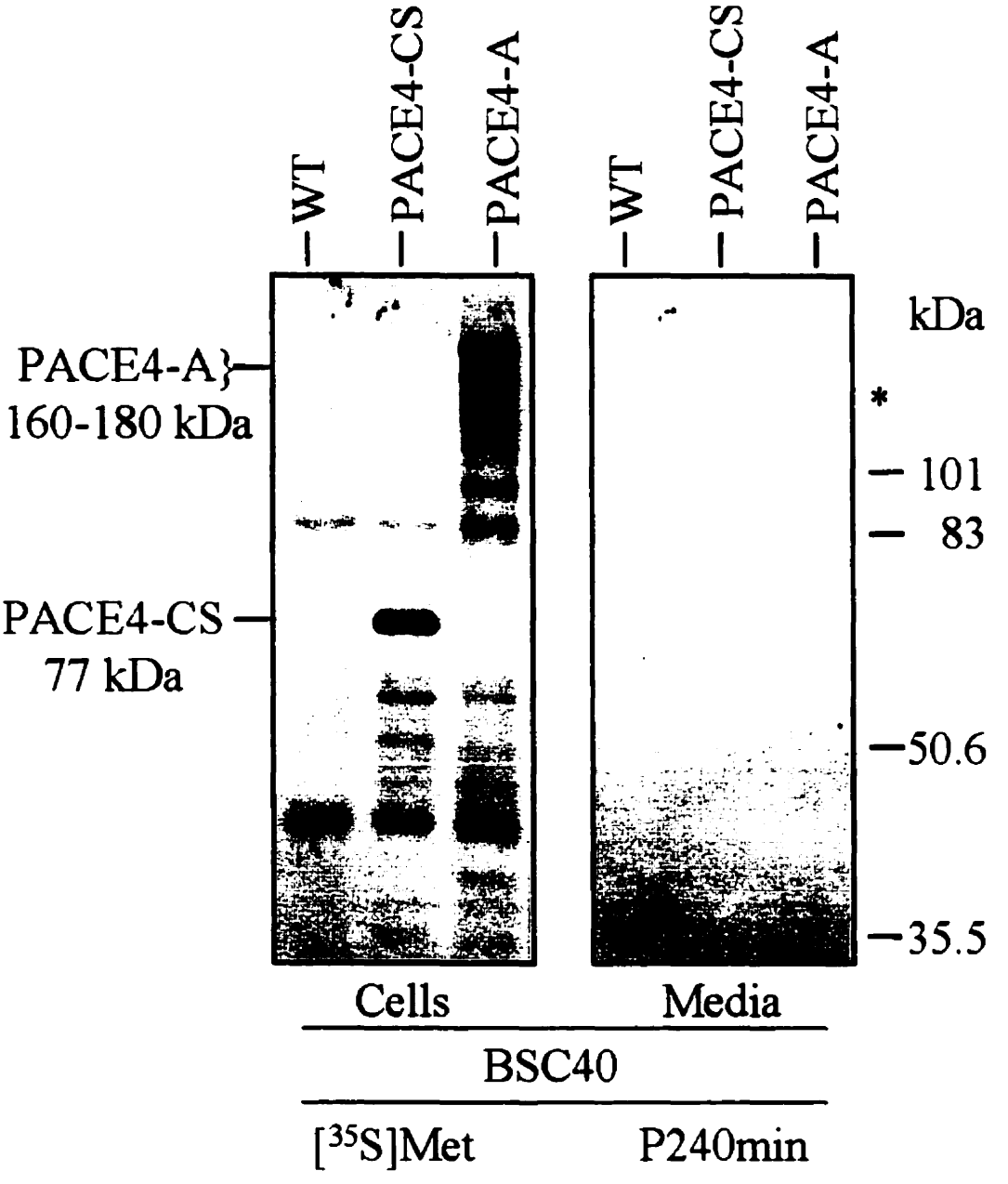

Fig. 3.3. *SDS-PAGE analysis of the biosynthesis of PACE4-A and PACE4-CS*: BSC40 cells were infected with 4 pfu/cell of either VV:WT, VV:PACE4-CS or VV:PACE4-A and then pulse-labeled for 240 min. (P240) with [^{35}S]methionine. The media and cell extracts were then purified on a lentil-lectin column and analyzed by SDS-PAGE (8% T, 2.7% C). Based on the migration of the molecular standards the molecular weights of PACE4-CS (77 kDa) and PACE4-A (160-180 kDa) were estimated.

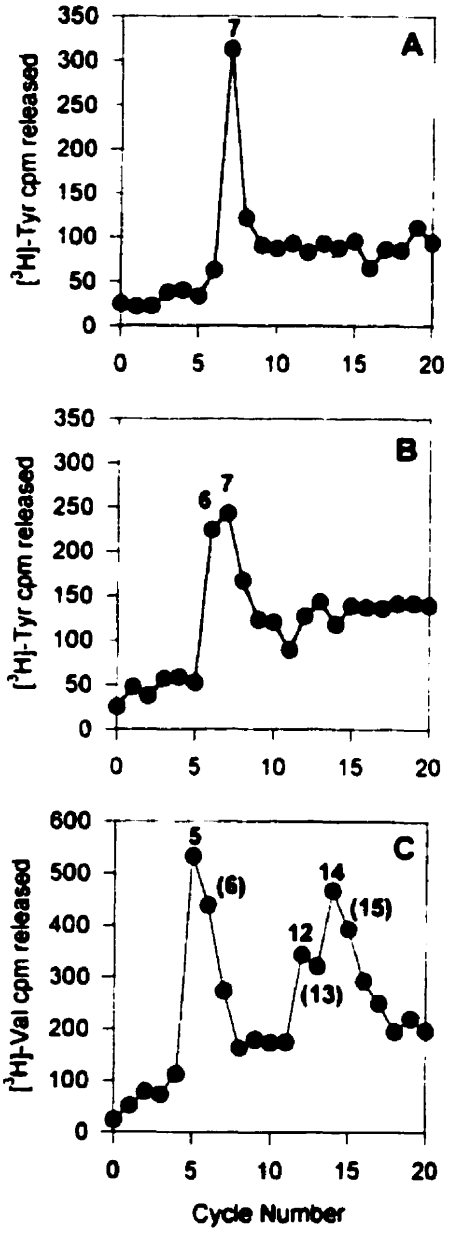

Fig. 3.4. *Microsequence analysis of PACE4-CS:* Radiolabeled 77 kDa PACE4-CS was obtained from the cell extracts of AtT20 [A] and BSC40 [B,C] cells pulsed for 4h with either [^{3}H]tyrosine [A,B] or [^{3}H]valine [C]. The labeled proteins were separated by SDS-PAGE (8% T, 2.7 % C), and the gel was then sliced (1 mm) and the eluted 77 kDa proteins were sequenced for 20 cycles. Cycle 0 corresponds to a full sequencing cycle performed in the absence of the coupling reagent phenylisothiocyanate. The numbers above the peaks represent the deduced sequence positions.

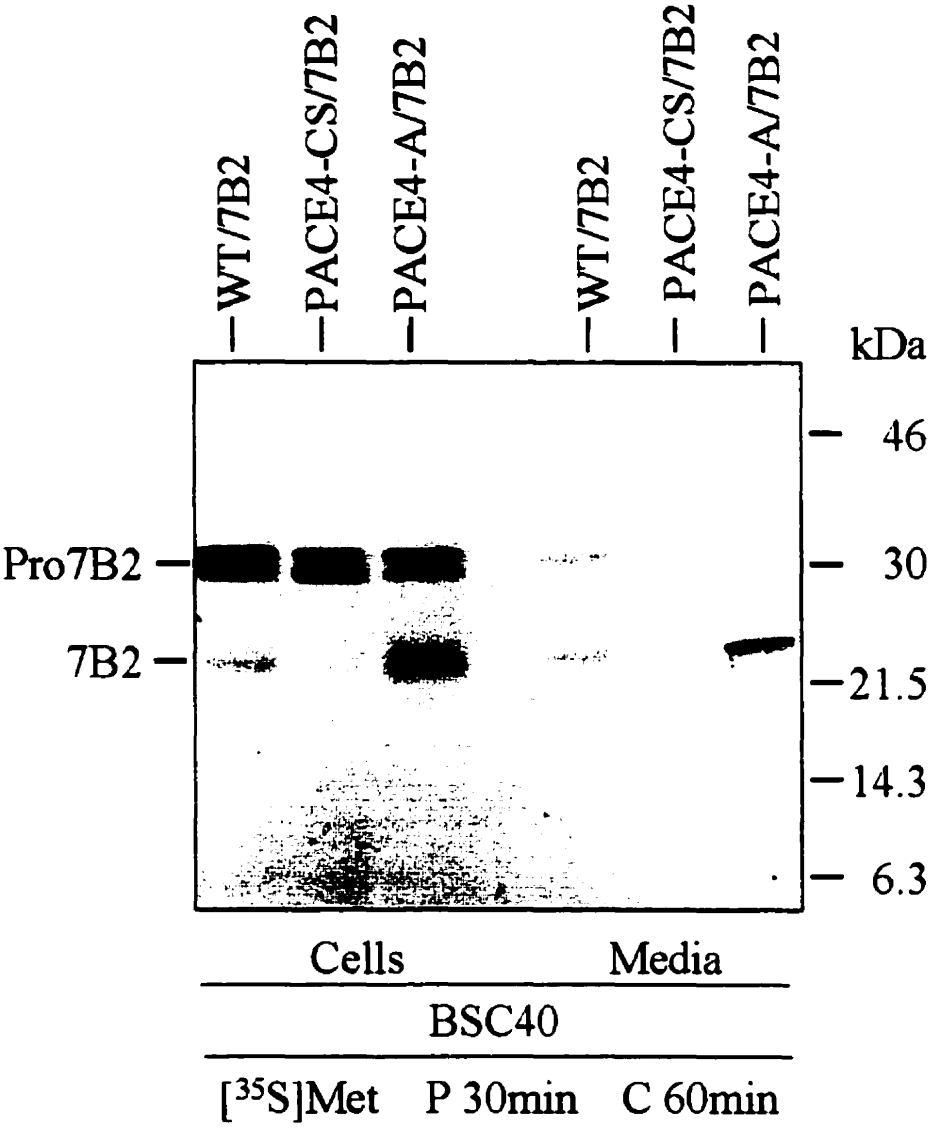

Fig. 3.5. *Comparative processing of pro7B2 by PACE4-CS and PACE4-A:* BSC40 cells were co-infected with VV:7B2 (2 pfu/cell) and with 4 pfu/cell of either VV:WT, VV:PACE4-CS or VV:PACE4-A and then pulse-labeled for 30 min (P30) with [^{35}S]methionine, followed by a chase of 60 min (C60) in the presence of cold methionine. The media and cell extracts were then immunoprecipitated with a 7B2 antibody (Benjannet *et al.*, 1995) and analyzed by SDS-PAGE (15% T, 1.3% C). The positions of pro7B2 (30 kDa) and 7B2 (23 kDa) are emphasized. The band above pro7B2 was observed previously (Benjannet *et al.*, 1995) and is a non-specific protein which is not secreted.

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CHAPTER 4

General Discussion

1. General discussion

The discovery of a novel family of mammalian subtilisin/kexin like proprotein convertases has led to a more detailed understanding of the molecular basis of maturation of biologically active peptides and proteins. The presented work concentrates on characterization of the roles of two structural domains of the PCs: the pro- and the P-domain, and most importantly defines the structure-function of these domains in controlling the folding, zymogen activation, cellular sorting and location as well as the enzymatic activity of the proprotein convertases. The hypotheses are: structure-function analyses of the PCs will reveal not only a basic unity in their structural organization and cellular sorting motifs, but also in their distinct ability to interact with cellular or extracellular proteins which control their enzymatic activity, trafficking and intracellular localization. The approaches chosen use techniques involving biochemistry, molecular and cell biology and enzyme kinetics. Our studies provided a firm basis for understanding the functions of the various PC domains and their regulatory role in terms of zymogen activation, cleavage specificity and cellular sorting of each PCs. The ultimate outcome of these studies is to rationally design specific inhibitors of PCs for biotechnology, pharmacological and clinical applications. The implications of proprotein convertases in both health and disease states is now evident but many questions remain to be answered and many problems remain to be solved.

1.1. Bacterial chimeric prosegments

The critical technical steps of this work in complement of what is written in the material and methods are as followings: We cloned the prosegments of furin and PC7 into a bacterial expression vector, taking the advantage that no post-translational modification occurs in the prosegments. After transforming the recombinant constructs into *E. coli* BL21 (DE3) cells, 1 mM IPTG was used to induced protein expression. Aliquots were taken in a time course study from both media and cell pellet to find the optimum expression condition for each prosegment construct. All our prosegment constructs express their product inside the cells stored in inclusion bodies and are not secretable into the media. Normally it takes 3-4 hours to give the maximal expression levels, which lasts

for about 2 hours and then the expression level starts to decrease, possibly due to the toxicity of the heterologous protein. In one of the experiments (data not shown), we tried to make a mutation where the primary cleavage site of furin prosegment R-T-K-R was replaced by R-T-K-A. The expression of this construct in BL21 cells is quite different from the wild-type furin prosegment. The expression of the mutated furin prosegment starts at 2 hours after IPTG induction and at 3 hours the expression is stopped. This narrow expression window might due to its toxicity to the cells or unstable folding of this mutation. Another observation regarding the expression of the bacterial prosegments is that all the expressed prosegments still retain the N-terminal initiator methionine, as confirmed by MALDI-TOF mass spectrometry. This is not consistent with the similar prosegment constructs grows in ^{12}C and/or ^{13}N added media (unpublished results). The present or absence of the initiator methionine in the prosegments seems related to the kind of media provided. The bacterial expression system allows large quantity production of these bacteria made proteins and which laying the foundation for further purification by column chromatography providing milligram quantity of pure prosegments for generating specific antisera and for *in vitro* kinetic studies.

1.2. Problems associated with overexpression

The question of which one of these seven PCs are responsible for individual precursor cleavage in tissues is difficult to answer. PC's enzymatic activity and specificity are normally evaluated by both *in vitro* and *ex vivo* assays. *In vitro* experiments rely on the use of either the shed form of PCs or of a truncated soluble form of PCs to measure the cleavage of various substrates such as small fluorogenic or non-fluorescent peptides, internally quenched fluorogenic peptides or relatively big protein molecules (e.g. gp160 in chapter 2). The *ex vivo* assays rely on the coexpression of various proprotein precursors with each PC. It has been observed that characterization of such enzymatic activity must take into account the level of actual enzyme produced which, in overexpression systems, tends to lead to an elevated, non-physiologically relevant enzyme to substrate ratio that normally ends up with a broadened enzyme specificity. Complementation of *ex vivo*

studies with *in vitro* enzymatic digestions using purified enzymes and defined substrates is necessary to compensate for such discrepancies (Denault & Leduc, 1996).

In addition, the delineation of certain PC enzymatic specificity using *ex vivo* assays is further complicated by the cell-type dependent presence of unknown endogenous convertases other than the enzyme interest which might exhibit similar cleavage specificity to a particular substrate. The choice of a suitable cell line thus becomes very crucial. As a consequence, a few cellular models have been found and/or defined to eliminate such endogenous participation of various enzymes. Examples involve LoVo cells, a human colon carcinoma cell line, and RPE.40, a mutant cell line derived from Chinese-hamster ovary (CHO) cells. Cloning of furin cDNAs revealed that LoVo cells have two mutant alleles of the *fur* gene; one is a point mutation of a conserved Trp residue to Arg at the position 547 of the P-domain, the other is a frame-shift mutation on the P-domain (Takahashi *et al.*, 1993; 1995a). The combination of both accounts for the recessive nature of the processing incompetence of LoVo cells. Immunofluorescence analysis revealed that the endogenous furin neither underwent the autocatalytic activation nor existed the endoplasmic reticulum. These data again indicate that both P-domain and the catalytic domain are required for autocatalytic activation of furin. Moehring and colleagues isolated RPE.40 as a mutant strain of Chinese hamster ovary (CHO-K1) cells, which differ from wild-type in their increased resistance to *Pseudomonas* exotoxin and diphtheria toxin and in their inability to process the insulin proreceptor and certain viral envelope proproteins. Since bacterial toxins and viral envelope glycoproteins have a consensus furin cleavage site, they transfected RPE.40 cells with cDNA for mouse furin causing them to lose all resistance and to become as sensitive as wild-type cells to toxins and viruses (Moehring *et al.*, 1993). The mechanistic basis for the endoprotease-deficient phenotype of RPE.40 cells has been revealed. RPE.40 cells were diploid at the *fur* locus, and had a Cys (TGC) to Tyr (TAC) mutation at position 303 on one allele near the oxyanion hole Asn residue and a point mutation in an intron transcribed from the second allele causing defective splicing (Spence *et al.*, 1995). The mutant furin exhibited no evidence of autocatalysis, consistent with the lack of activity versus the test substrates, and its glycosylation pattern suggested that it remained in the endoplasmic reticulum

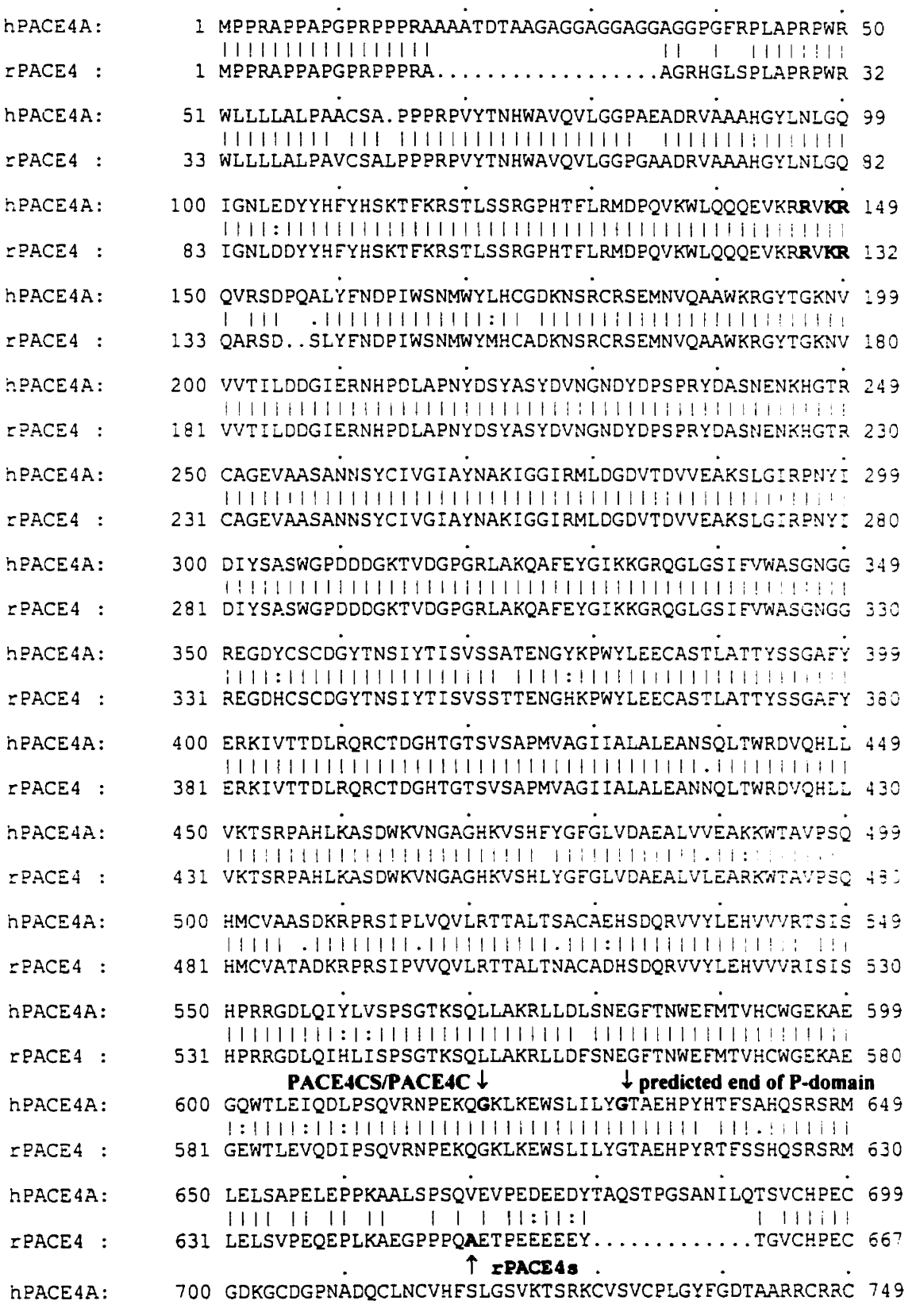
(Sucic *et al.*, 1998). Another furin-deficient cell strain, 7.P15, selected from the monkey kidney cell line COS-7 has also been reported (Inocencio *et al.*, 1997). These furin-deficient cell lines can be used as host for exogenous expression of furin together with candidate precursor proteins to identify furin substrates. We should always bear in mind the possibility that there is considerable redundancy in these PCs activities and that different PCs could act on the same precursor in different tissues. This redundancy in PC's actions on precursors may be adopted by cells as backup systems to ensure proper processing under certain physiological conditions.

1.3. Structure function studies of the P-domain

The highly conserved sequence of the P-domain located immediately downstream of the catalytic domain is a unique feature of eukaryotic subtilisin-like PCs, which is not found in their ancestral bacterial subtilisins. The function of this domain is not well known, but recent data suggest that it may play an important chaperone-like role in the folding of the zymogen, hence allowing the autocatalytic processing of enzymatically active PCs (Gluschankof & Fuller, 1994; Hatsuzawa *et al.*, 1992; Sinde & Inouye, 1995). Chapter 3 presents a study on the PACE4 P-domain. Taking the advantage of different isoforms of PACE4 generated by alternative splicing in the P-domain, we examined the biosynthesis, functional activity and cellular localization of two of these isoforms, namely the full length PACE4-A (Kiefer *et al.*, 1991) and C-terminally truncated PACE4-CS which lacks 11 amino acids at the end of its chaperone-like P-domain (Zhong *et al.*, 1996). Consistent with the studies of yeast kexin and mouse furin, our results demonstrated that the P-domain is indispensable for proprotein convertases maturation. C-terminal truncation PACE4-CS isoform with a shortened P-domain result in inactive enzyme, probably by preventing the processing of its prosegment in the ER (Zhong *et al.*, 1996). According to the deletion and truncation studies on the C-terminal region of furin and kexin (Gluschankof & Fuller, 1994; Hatsuzawa *et al.*, 1992), the boundary of the P-domain has been set to a critical glycine, which marks the end of the structural homology between kexin and the mammalian PCs (Seidah *et al.*, 1995b). Since the identified PACE4-CS isoform is shorted by at least 11 C-terminal amino acids and are replaced by 3

different amino acids, the integrity of this domain is obviously affected. This may explain why proPACE4-CS is not processed to PACE4-CS (Fig. 3.3), rendering the enzyme functionally inactive (Fig. 3.5). In the same vein, the PACE4-C isoform with the same splice site has 36 aa of different sequence replacing the 11 conserved C-terminal amino acids of the P-domain. By analogy, we believe that PACE4-C should also code for an inactive enzyme. Taken together with the recently discovered PACE4-E isoform (Mori *et al.*, 1997), there are so far only two bioactive PACE4 isoforms, namely PACE4-A and PACE4-E. In contrast, a report on rat PACE4 provides a new candidate active C-terminal truncated PACE4 just following the P-domain (Figure 4.1) (Mains *et al.*, 1997). This group studied the biosynthesis and secretion of two forms of rat PACE4, namely the full length 937 aa form rPACE4 (Johnson *et al.*, 1994) and a C-terminally truncated 650 aa form rPACE4s (Figure 4.1). The latter lacks the entire Cys-rich region (residues 663-937 of rPACE4, Figure 4.1). Even though the authors did not study the enzymatic activity of the truncated form, biosynthesis data showed that rPACE4 underwent intracellular cleavage from proPACE4 about 106 kDa to a secreted form about 97 kDa. Similarly, rPACE4s is also converted from a 72 kDa proform into a 66 kDa mature form. As a matter of fact, the authors claim that the rate of PACE4 conversion from the promolecule into the mature product was accelerated in rPACE4s, possibly due to the deletion of the entire C-terminal Cys-rich region. Judging from the biosynthetic processing of rPACE4s into a smaller size molecule, it is reasonable to think that the proform underwent prosegment removal. The rate of the prosegment cleavage is partially affected by the C-terminal Cys-rich domain of rPACE4, as rPACE4s was processed and secreted more rapidly than full-length rPACE4 (Mains *et al.*, 1997). Interestingly, the efficiency of secretion of this truncated rPACE4s was also much higher than that of rPACE4, much of which was degraded in the cells and never released by AtT20 cells. The C-terminal portions of PC1 and kexin also transit retard the transportation of the enzymes through the cell (Gluschankof & Fuller, 1994; Zhou *et al.*, 1995), the enzymatic properties of PC1 are also changed by the presence or absence of the C-terminal region (Jutras *et al.*, 1997).

Figure 4.1 Alignment of human PACE4A and rat PACE4



```
rPACE4 :     668 GDKGCDGPSADQCLNCVHFSLGNSKTNRKCVSECPLGYFGDTAARRCRRC 717
hPACE4A:     750 HKGCETCSSRAATQCLSCRRGFYHHQEMNTCVTLCPAGFYADESQKNCLK 799
rPACE4 :     718 HKGCETCTGRSPTQCLSCRRGFYHHQETNTCVTLCPAGLYADESQRLCLR 767
hPACE4A:     800 CHPSCKKCVDEPEKCTVCKEGFSLARGSCIPDCEPGTYFDSELIRCGECH 849
rPACE4 :     768 CHPSCQKCVDEPEKSTVCKEGFSLARGSCIPDCEPGTYFDSELIRCGECH 817
hPACE4A:     850 HTCGTCVGPGREECIHCAKNFHFHDWKCVPACGEGFYPEEMPGLPHKVCR 899
rPACE4 :     818 HTCRTCVGPSREECIHCAKSFHFQDWKCVPACGEGFYPEEMPGLPHKVCR 867
hPACE4A:     900 RCDENCLSCAGSSRNCSRCKTGFTQLGTSCITNHTCSNADETFCEMVKSN 949
rPACE4 :     868 RCDENCLSCEGSSRNCSRCKAGFTQLGTSCITNHTCSNADETFCEMVKSN 917
hPACE4A:     950 RLCERKLFIQFCCRTCLLAG     969 aa
rPACE4 :     918 RLCERKLFIQFCCRTCLLAG     937 aa
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Recently, more studies on the P-domains of PCs have been conducted and similar conclusions have been reached. Zhou *et al* have expressed and characterized various P-domain-related mutants and chimeras in HEK293 cells and alpha-TC1-6 cells (Zhou *et al.*, 1998). They used a series of C-terminal truncations of PC1 and PC1-Thr594 was identified as the shortest active form, thereby defining the functional C-terminal boundary of the P-domain of this convertase. Substitutions at Thr594 and nearby sites indicated that residues 592-594 are crucial for activity. They also used different chimera proteins like PC1 with interchanged P-domains of PC2 (PC1/PC2) and furin (PC1/furin), which demonstrated dramatic changes in several properties. Compared with truncated wild-type PC1 ending at Asp616, both PC1/PC2 and PC1/furin fusions had elevated activity on several synthetic substrates as well as reduced calcium ion dependence, whereas furin chimera with PC2 P-domain (furin/PC2) showed a slight decreased in activity as compared with truncated furin stop at Glu583. Of the three active PC chimeras tested, all had more alkaline pH optima. When PC1/PC2 fusion was expressed in alpha-TC1-6 cells, it accelerated the processing of proglucagon similar to wild-type PC1. They found that P-domain exchanges generated fully active chimeric proteases in several instances but not in all, for example PC2/PC1 was inactive. These observed property changes indicate a role for the P-domain in regulating the stability, calcium dependence, and pH dependence

of the convertases (Zhou *et al.*, 1998). A molecular model of these structural domain interactions has been proposed (Lipkind *et al.*, 1998). In this model, the P-domains consist of 8-stranded beta-barrels with well-organized inner hydrophobic cores. independently folded. The P-domain is integrated through strong hydrophobic interactions with the catalytic domain, conferring structural stability and maintaining the properties and activity of the proprotein convertases (Lipkind *et al.*, 1998).

1.4. Unique properties of PACE4

PACE4 is encoded on the same chromosome as furin, and some early reports suggested that PACE4 was not secreted and had a wide tissue distribution, the latter has been regarded as a soluble intracellular version of furin. However, further examination of PACE4 shows its importance in several aspects. First, its tissue distribution indicates that it may have an important role in endocrinal and neuronal functions (Dong *et al.*, 1995; Johnson *et al.*, 1994; Nagamune *et al.*, 1995; Tsuji *et al.*, 1994b). Second, PACE4 is very strongly regulated by endocrine status (Johnson *et al.*, 1994). Third, expression of PACE4 in cells lacking furin can partly restore wild-type characteristics (Sucic *et al.*, 1999). Fourth, endoproteases in the constitutive pathway clearly participate in the processing of pro-nerve growth factor (Bresnahan *et al.*, 1990), *Aplysia* egg-laying hormone (Chun *et al.*, 1994), pro-7B2 (Paquet *et al.*, 1994) and proparathyroid hormone (Hendy *et al.*, 1995).

PACE4 has yet to be characterized to the same extent as furin. However, recent studies have shed some light on the biochemical properties and physiological roles of this enzyme (Mains *et al.*, 1997; Sucic *et al.*, 1999), taking use of RPE.40 cells to examine the role of PACE4 in processing several proproteins in a furin-null background. When human PACE4 was expressed in RPE.40 cells, the phenotype of these cells was partly restored to wild type (Sucic *et al.*, 1999). These included the sensitivity to Sindbis virus and the ability to process the low-density-lipoprotein receptor-related protein but not the sensitivity to *Pseudomonas* exotoxin. Coexpression of human PACE4 with either a secreted form of the human insulin pro-receptor or the precursor form of von Willebrand factor resulted in both proprotein being processed, while RPE.40 cells were unable to

process either without coexpression of PACE4. The restoration of distinct wild-type characteristics *ex vivo* was correlated with the activity of PACE4 *in vitro*. In terms of biochemical characterization of the PACE4 activity, like furin, PACE4 is a Ca^{2+} dependent enzyme. Unlike furin, PACE4 is sensitive to temperature changes between 22 °C and 37 °C (Sucic *et al.*, 1999). PACE4 is thus an endoprotease with more stringent substrate specificity and more limited operating parameters than furin. However, some disagreements exist in the literature on the enzyme's cleavage specificity and its inhibition profile. While two reports claimed that PACE4 is not sensitive to α_1-PDX *in vitro* (Jean *et al.*, 1998; Mains *et al.*, 1997), four others revealed that it is well inhibited by this serpin (Decroly *et al.*, 1996; Tsuji et al., 1999; Vollenweider *et al.*, 1996; Zarkik *et al.*, 1997).

1.5. Alternative splicing and protein diversity

For a number of eukaryotic genes, primary heteronuclear RNA (hnRNA) can be spliced in several alternative forms, thus generating multiple transcripts, eventually resulting in different protein isoforms from a single gene. Excision of introns and splicing of mRNA precursors not only affects the message encoded by these molecules, but also their transport out of the nucleus, stability in the cytoplasm, as well as efficiency of translation (Maniatis, 1991; Newman, 1994; Sharp, 1994). All intron/exon boundaries follow the AG/GT consensus rule for splicing (Hodges & Bernstein, 1994). Different isoforms of PACE4 (Figure 1.5 and 3.2) that vary in size and coding sequence have been reported (Mori *et al.*, 1997; Tsuji *et al.*, 1994a; Zhong *et al.*, 1996). In order to determine the origin of these isoforms, the entire human PACE4 gene has been isolated as a set of overlapping genomic DNA fragments, and analyzed by restriction enzyme digestion and nucleotide sequence determination. The human PACE4 gene spans at least 250 kb and is distributed over 25 exons that range in size from 39 to 1,422 base pairs. The human PACE4 gene is the largest proprotein convertase gene reported to date. The most striking feature of its genomic structure is the size of the introns and the number of exons, although the general organization of signal peptide, prosegment, and catalytic domains, which are conserved in this family, is very similar to that reported for other kexin-like protease genes. The structural analysis of PACE4 genomic DNA indicates that multiple

PACE4 transcripts are produced as a consequence of alternative RNA splicing events, including exon skipping, and differences in the usage of the inner 5'-splicing donor and polyadenylation sites. A major transcriptional start site was detected 314 bp upstream from the ATG translational start site by primer extension analysis. Sequence analysis of the 5'-flanking region revealed that the PACE4 gene lacks TATA and CCAAT boxes in the proximal upstream region of the start site, although potential binding sites for several transcription factors including SP1, AP1, AP2, PEA3, Ets-1, GHF (growth hormone factor)-1, CREB (cyclic AMP response element binding protein), and basic helix-loop-helix proteins, were present. An unusual sequence of six tandem repeats of a nonadecamer (GGCCTGGGGGTTCACCTGC) containing an E box is found in the 5'-flanking region. These results suggest that PACE4 is not a constitutive gene product and that its expression is regulated by various transcription factors (Tsiji *et al.*, 1997). This may be the reason for the generation of so many PACE4 isoforms. Thus far, different splicing of convertase transcripts resulted in multiple active forms not only of PACE4 but also of PC5 and PC4 where functional active convertases can be derived from a single gene.

1.6. Prosegment mediated PCs inhibition

Although no three-dimensional structure is available for PCs yet, their catalytic subunits have been modeled on the basis of the x-ray structure of subtilisin (Lipkind *et al.*, 1995). Like subtilisin, PCs become active by autocatalytic cleavage of the N-terminal prosegment which is required for folding of the enzyme. In chapter 3 we demonstrate that the downstream P-domain is also required for folding and activity. The P-domain plays a regulatory role influencing both Ca^{2+} dependence and pH profile (Zhou *et al.*, 1998). A prosegment is not a general requirement of a serine proteinase activity but cleavage of the prosegment is a prerequisite for generating a fully functional enzyme. Why did nature chose to provide such a prosegment? One reasonable explanation may come from the fact that all eukaryotic proteinases that possessing such a prosegment are generally found within the secretory pathway, where many substrates can colocalize with them. In chapter 2, we have clearly shown that the prosegments of furin and PC7 are very potent inhibitors

to their parent enzyme. The presence of the prosegment usually at the N-terminal of the nascent polypeptide not only helps the correct folding of such an enzyme in the ER, but also keeps its activity in check as it traverses the secretory pathway till it reaches its final destination. It is likely that one of the important functions of the prosegment is to prevent unwanted proteinase activity.

The study presented in chapter 2 characterized the nature of the prosegment inhibition of proprotein convertases both *in vitro* and *ex vivo*. We presented data on the differential inhibition of PCs *in vitro* by the full-length prosegments of furin (pFurin) and PC7 (pPC7) purified from bacterial lysates. Using prosegment-derived synthetic peptides, we established that the inhibitory potency of pFurin and pPC7 lies mainly within the 10 amino acids located at the C-termini of these prosegments and confirmed that the last basic residue Arg is critical for effective inhibition. We then go on to ask whether the prosegments of furin or PC7, expressed *ex vivo* as independent domains, can function as inhibitors in a cellular environment. Using vaccinia virus constructs or transient transfectants of these prosegments, we show that they specifically prevent the maturation of the neurotrophins NGF and BDNF in Schwann or COS-1 cells, respectively. This is the first report demonstrating that PC prosegments, expressed *ex vivo* as independent domains, can act *in trans* to inhibit precursor maturation by intracellular PCs. What happens to the prosegment after its dissociation from the enzyme complex? Will it remain in the ER? Will the prosegment be broken down after the dissociation of the complex? Our data using vaccinia virus overexpressing prosegments showed at least some portions of the prosegments seem to traverse the secretory pathway and eventually get secreted. This, however, may be an artifact generated by vaccinia virus overproduction. Our specific prosegment antisera are currently being used to study the biosynthesis of the prosegment to address the fate of the prosegments under physiological conditions.

1.7. PCs and HIV viral infection

The viral envelope glycoproteins mediate the entry of the viral capsid into the cytoplasm of cells by a process implying both the fixation of the virion to a cellular receptor and the fusion of the viral membrane with that of the host cell. Viral surface

glycoproteins are usually synthesized as inactive precursors that are cleaved and activated by one or more host-cell proteinases. The envelope glycoprotein of human immunodeficiency virus (HIV) initiates infection by mediating fusion of the viral envelope with the cell membrane. In the HIV virus, the endoproteolytic cleavage of gp160 occurs within the Golgi apparatus at the conserved REKR↓AVGI sequence, thereby generating the two fragments gp120 and gp41. The N-terminal fragment (gp120) can interact with a specific CD4+ cellular receptor mediating infection of lymphocytes, and might allow entry of HIV into macrophages and monocytes, whereas the membrane-anchored C-terminal segment (gp41) contains an N-terminal hydrophobic sequence capable of promoting the fusion of the virion with cellular membranes. Both fragments are highly *N*-glycosylated with the gp120 and gp41 containing 24 and 4 *N*-glycosylation sites respectively. This intracellular proteolytic processing of HIV envelope glycoprotein gp160 into gp120/gp41 is an essential step for virus fusion (McCune *et al.*, 1988). Chapter 2 presents our studies of prosegments inhibition of furin mediated gp160 processing (Zhong *et al.*, 1999). In real life, there is currently a debate as to which proprotein convertase(s) is physiologically involved in the cleavage and activition of HIV-1 gp160. Earlier cellular coexpression and *in vitro* studies have shown that furin can cleave gp160 to yield gp120 and gp41. This, along with furin expression in $CD4^+$ cell lines, has led to the proposal that furin is the best protease activiting gp160 (Decroly *et al.*, 1994; Hallenberger *et al.*, 1992). However, for the following two reasons, other convertases may also be involved physiologically on gp160 cleavage: (1) In LoVo cells, which was recently demonstrated to be furin defective, gp160 was found to be processed almost as efficiently as in normal cell lines. On the other hand, as a control, the same cell line was almost totally incapable of processing Newcastle disease virus fusion glycoprotein with a similar cleavage recognition motif, providing a strong case for furin-mediated processing (Ohnishi *et al.*, 1994). (2) The furin-deficient Chinese hamster ovary (CHO)-K1 strain RPE.40 processed gp160 as efficiently as wild-type CHO-K1 cells *ex vivo* (Inocencio *et al.*, 1997). Although PC7 can process gp160 *in vitro*, this processing is probably not physiologically relevant, because it occurs with very low efficiency. PACE4 can process gp160 when co-expressed in RPE.40 cells. Further, PACE4 participated in

the activation of a calcium-independent protease activity in RPE.40 cells, which efficiently processed the gp160 precursor *in vitro*. Biosynthesis of the envelope glycoprotein gp160 and its intracellular processing by the subtilisin/kexin-like convertases furin, PACE4, PC5 and its isoform PC5/6-B demonstrate that furin and to a much lesser extent PACE4 and PC5/6-B are candidate enzymes capable of processing gp160 intracellularly (Vollenweider *et al.*, 1996). Others like PC5 and PC7 have been proposed as candidate gp160 processing enzymes (Decroly *et al.*, 1996; 1997). These studies raise a further need to search for and identify the proteinases involved in HIV-1 gp160 processing rather than supporting the notion that furin is the only cognate enzyme responsible. To date, on the basis of expression in $CD4^+$ cell lines and T-lymphocytes, and cleavage activity toward gp160, it was suggested that furin, PC5 and PC7 are the major gp 160 converting enzymes in T-lymphocytes (Hallenberger *et al.*, 1997; Miranda *et al.*, 1996).

1.8. Endocytic delivery of inhibitors

The modulation of proprotein convertases in order to control the processing of their substrates can potentially be used in various disease states. Examples of maturation of surface glycoproteins of numerous enveloped viruses have revealed that this process is dependent on the activity of PC-like enzymes. Processing of these glycoprotein precursors is essential to their membrane fusion function and hence in viral infectivity (McCune *et al.*, 1988). One aspect of the question is always how to effectively deliver such a candidate inhibitor into specific targets. Recently, a strategy using endocytic drug delivery to modulate kexin in yeast has been reported (Henkel *et al.*, 1999). At steady state, kexin is predominantly localized in late Golgi compartments and initiates the proteolytic maturation of proprotein precursors that transit the distal secretory pathway. However, kexin localization is not static, and its itinerary apparently involves transiting out of the late Golgi and cycling back from post-Golgi endosomal compartments during its lifetime. This observation led to the testing of whether the endocytic pathway could deliver small molecules to kexin from the extracellular medium. Intramolecularly quenched fluorogenic substrates taken up into intact yeast revealed fluorescence due to

specific cleavage by kexin in endosomal compartments. Furthermore, the endocytic delivery of protease inhibitors interfered with the ability of kexin to process precursor proteins. These observations revealed that the endocytic pathway does intersect with the cycling itinerary of active kexin protease. This strategy of endocytic drug delivery has implications for modulating the PC's activity needed precursor processing in human cells (Henkel *et al.*, 1999). The accessibility of PCs to endocytic delivery of inhibitors in combination with more effective inhibitors holds promise for treatment of PC dependent viral infections and pathological processes.

We can cite another example of the use of α1-PDX as a therapeutic agent. *Pseudomonas aeruginosa* is a clinically important pathogen constituting a major complication in burn patients and people afflicted with cystic fibrosis (Bodey *et al.*, 1983). Animal studies show that *Pseudomonas* exotoxin A contributes to the pathogen virulence (Miyazaki *et al.*, 1995). It is a 67 kDa protoxin that requires cleavage within endosomes by furin at the R-N-P-R↓ site to become cytotoxic. Inhibition of *Pseudomonas* exotoxin A cytotoxicity can be achieved by adding α1-PDX exogenously (Jean *et al.*, 1998). This observation of α1-PDX blocking furin-dependent cell killing by *Pseudomonas* exotoxin A demonstrates its therapeutic potential.

1.9. Future directions

The identification of the novel family of mammalian subtilisin/kexin like proprotein convertases demonstrated a major breakthrough in the proprotein processing field. Since their discovery, more and more evidences suggested their involvement in both normal and pathological conditions. If, as expected, PCs have direct attributive link to the HIV infection, specific inhibitors of PCs would form a new and promising class of antiviral agents. Continued technological progresses have provided meaningful tools in controlling a specific convertase activity in target cells or tissues. In Chapter 2 we demonstrate good candidates for PCs-specific inhibitors. The prosegments model can be exploited to find more potent and even better specific inhibitors of PCs to be used for the treatment of PC-regulated pathologies. Another aspect relates to the development of a reliable drug delivery system of therapeutic agents to treat diseases. Gene therapy as a

drug delivery system has both theoretical and practical advantages over current protein delivery systems, including the ability to target therapies to individual tissues or cell types, to locally produce proteins that can act intracellularly and to sustain new protein synthesis for periods up to weeks after a single administration (Moldawer *et al.*, 1999). Gene therapy initially conceived for replacing defective genes in diseases and inborn errors with gene encoding the normal or wild-type gene product has expanded into other novel applications such as cancer or cardiovascular disease (Pilaro & Serabian, 1999). Different types of vectors including modified retroviruses, adenoviruses, adenovirus-associated viruses and herpesviruses are used to transfer foreign genetic material into patients'cell or tissues. The adenovirus approach appears most suited as drug delivery systems due to their rapid onset and short duration of transgene expression (Moldawer *et al.*, 1999). We can take advantage of the adenovirus delivery system and our knowledge on the inhibitory prosegments to introduce them in specific cells and tissues to temporally inhibit the activity of the certain PCs without affecting the others.

The last ten years have been very productive in the field of proprotein convertases, but the most interesting questions are still unanswered. What are the normal natural substrates of each PC and their tissue-specific expression? The answers to these questions are critical for developing specific therapeutic agents to treat PC regulated diseases. In the meantime, many factors contribute to the safety and clinical activity of these agents, including the route, frequency, and duration of exposure and the type of vector employed. Knowledge of the actual PCs responsible for cognate substrate cleavages in specific tissues, together with structural features of the precursors determine where it will be processed and how it is recognized by the sorting machinery. The ability to interfere with the tissue-specific processing of pathological molecules holds the key for regulating the activity of PCs which will find in the future numerous biotechnology, pharmacological and clinical applications.

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CHAPTER 5

Claims to Original Research

2. Claims to original research

The significant contributions to research and development cited in the published papers concentrate on the characterization of the structural elements in proprotein convertases that are crucial for their enzymatic function and cellular trafficking. These include the definition of the structure-function of the pro- and P-domains controlling the folding, zymogen activation, cellular sorting and localization and enzymatic activity of the proprotein convertases.

Claims related to the report *"Functional analysis of human PACE4-A and PACE4-C isoforms: identification of a new PACE4-CS isoform"*

- Isolation of a novel isoform of PACE4 called PACE4-CS (the shortened form of PACE4-C that lacks 11 amino acids at the end of its chaperone-like P-domain) generated by alternative splicing of its heteromuclear RNA (hnRNA), which broadened the diversity of this PC' isoforms.
- Examintion of the biosynthesis, cellular localization and functional activity of the newly isolated PACE4-CS isoform compared with those of PACE4-A (intact P-domain).
- Cellular expression results demonstrated that PACE4-A codes for a functional secretable enzyme capable of precursor cleavage. In contrast, PACE4-CS (disrupted P-domain) is not secreted and remains in the endoplasmic reticulum as an inactive zymogen.
- The different fate of the two isoforms emphasizes the importance of the integrity of the P-domain and sharpens the boundary of this structural element and its indispensability.
- Consistent with studies on PC5 isoforms, this work showed that differential splicing regulates the sorting of different isoforms. Thus PACE4-A codes for a functional secretable enzyme, while isoform PACE4-CS codes for a nonfunctional zymogen and remains in the ER. The physiological significance of this isoform is yet to be defined.

Furin and PC7, the major convertases of the constitutive secretory pathway, process precursors either within the TGN or at the cell surface. As such, they mediate a

wide range of processing events, which in pathological situations may exacerbate a disease state. Examples include the participation of furin, and possibly PC7, in the processing of the viral surface glycoprotein gp160 of HIV and GP of Ebola virus as well as some bacterial toxins. Experiments in which the activation of these proteins has been prevented through the inhibition of furin and possibly other PCs indicate that there is considerable promise in treating such pathologies by the use of these novel approaches.

Claims related to the report *"The prosegments of furin and PC7 as potent inhibitors of proprotein convertases: in vitro and ex vivo assessment of their efficacy and selectivity"*

- Isolation and characterization of the prosegments of furin and PC7 as inhibitors of these enzymes. Although it has been known that the prosegments can function as inhibitors, this is the first study to test purified prosegment *in vitro*.
- Detailed studies on the inhibitory potency and specificity of prosegments using a series of synthetic peptides. Studies on the synthetic peptides provided clear evidence that the inhibition potency resides at the C-termini of the prosegments.
- Peptide truncation series showed that 10 amino acid peptides derived from the C-terminus of the prosegments remain as nanomolar inhibitors toward their parent enzymes while exhibiting reasonable selectivity among the others.
- Mutagenensis studies revealed that Arginine at this P1 position is crucial for such inhibition.
- Our cellular studies are the first to show that neurotrophins NGF and BDNF processing can be blocked by overexpression of an exogenous prosegment with comparable efficacy to α_1-PDX.
- Demonstration for the first time that proprotein convertase prosegments expressed *ex vivo* as independent domains can act *in trans* to inhibit precursor maturation by intracellular PCs.

Since so far no dominant negative contributions were indentified, this study not only represents a stepping stone for the use of prosegments of convertases as models to design potent and specific convertase inhibitors but also provides the first evidence that these polypeptides can inhibit the intracellular processing of precursors. This technology represents a novel enzyme silencing strategy that will enhance our understanding of the basic cellular functions of these proteinases. It paves the road for future work on improving the performance of these prosegments using site-directed mutagenesis, leading to the design of more selective and powerful convertase inhibitors that may provide novel approaches to the treatment of a variety of pathologies including proliferative, microbial and viral diseases.

Appendices

Preface

The first part of the appendices presents a study on the *in vitro* characterization of the novel proprotein convertases PC7 compared to that of furin. In this work, I helped the preparation of active PC7 and furin from culture media, studied the enzymatic activity of those enzymes using fluorogenic peptidyl substrates, compared the pH optimum, calcium dependence as well as inhibition profile of furin and PC7.

Recently, the first member of a new class of subtilisin/kexin like convertases, called SKI-1, was identified. Its structure is closer to pyrolysin than to mammalian PCs and it exhibits a specificity for cleavage at (R/K)-X-X-(L,T)↓ motif according to its ability to process sterol regulatory element binding proteins and pro-brain derived neurotrophic factor. SKI-1 cleaves proproteins that are critical for the control of cholesterol and fatty acid metabolism and for neuronal protection and growth. The second part of the appendices presents a study on the characterization of this newly discovered enzyme. My participation includes the following:

Cloned of human SKI-1 prosegment His-tag construct into PET 24b(+) vector with the N-terminal T7 Tag removed, expressed it in bacterial BL21 cells, purified by Ni^{2+} affinity column and reverse phase HPLC. This material was then used to generate specific antibodies with the help of a lab technician, Andrew Chen. I also tested the titer and specificity of this antibody. This antibody was used to study the zymogen activation of the new enzyme SKI-1 and the biosynthesis of the prosegment by Suzanne Benjannet in the lab.

To carry out the study on the prosegment of SKI-1, I obtained the "prepro" constructs of SKI-1 in pcDNA3 vector in both sense and antisense orientation. These constructs were used to generate stable transfectants in HK293 cells. The prosegment antiserum and stable transfectants in HK293 cell together with transfectants of SKI-1 substrates: sterol regulatory element binding proteins (SREBP) and pro-brain derived neurotrophic factor (BDNF) will provide us meaningful tools to study the role of SKI-1 in the control of cholesterol and fatty acid metabolism and in neuronal protection.

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In Vitro Characterization of the Novel Proprotein Convertase PC7*

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Biochemical and enzymatic characterization of the novel proprotein convertase rat PC7 (rPC7) was carried out using vaccinia virus recombinants overexpressed in mammalian BSC40 cells. Pro-PC7 is synthesized as a glycosylated zymogen (101 kDa) and processed into mature rPC7 (89 kDa) in the endoplasmic reticulum. No endogenously produced soluble forms of this membrane-anchored protein were detected. A deletion mutant (65 kDa), truncated well beyond the expected C-terminal boundary of the P-domain, produced soluble rPC7 in the culture medium. Enzymatic activity assays of rPC7 using fluorogenic peptidyl substrates indicated that the pH optimum, Ca^{2+} dependence, and cleavage specificity of this enzyme are largely similar to those of furin. However, with some substrates, cleavage specificity more closely resembled that of yeast kexin, suggesting differential processing of proprotein substrates by this novel convertase. We examined the rPC7- and human furin-mediated cleavage of synthetic peptides containing the processing sites of three proteins known to colocalize *in situ* with rPC7. Whereas both enzymes correctly processed the pro-parathyroid hormone tridecapeptide and the pro-PC4 heptadecapeptide, neither enzyme cleaved a pro-epidermal growth factor hexadecapeptide. Thus, this study establishes that rPC7 is an enzymatically functional subtilisin/kexin-like serine proteinase with a cleavage specificity resembling that of hfurin. In addition, we have demonstrated that rPC7 can correctly process peptide precursors that contain the processing sites of at least two potential physiological substrates.

Mammalian prohormone convertases comprise a family of serine proteinases whose function is the cleavage of peptide precursor molecules at distinct single or pairs of basic residues (1, 2). These enzymes are related to bacterial subtilisins and to the yeast prohormone processing protease kexin. There are presently seven known members of this family that have been grouped under the generic name proprotein convertases (PCs).[1] These include furin (also known as PACE), PC1 (also known as PC3), PC2, PACE4, PC4, PC5 (also known as PC6) (for reviews, see Refs. 1–4), and, most recently, PC7 (5) (also known as LPC (6), PC8 (7), or SPC7 (8)). Tissue distribution analyses of these enzymes indicate that PC1 and PC2 are expressed mainly in neural and endocrine tissues, PC4 exclusively in reproductive germ cells, and PC5 and PACE4 to varying degrees in many tissue types (1–3). Similar to furin (3, 4), PC7 has a nearly ubiquitous tissue distribution as assessed by mRNA expression (5, 7, 8), suggesting that it could, like furin (4), be involved in the processing of precursors within the constitutive secretory pathway. Interestingly, in lymphoid-associated tissues such as thymus, spleen, and lymph nodes, the expression of PC7 appears to be especially high (5).

The sites where PCs cleave peptide precursors of various hormones, growth factors, and viral envelope glycoproteins have generally been defined as (Arg/Lys)-(*X*)*n*-(Arg/Lys)↓ where n = 0, 2, 4, or 6 residues (9–11). Due to this wide-ranging specificity and the overlapping expression of various convertases in different tissues, it is often difficult to assign cleavage of a given precursor to a particular enzyme. Although it is clear that PC1, PC2 (12–14), and PC5-A (15) are the enzymes most likely to be active in secretory vesicles (9), processing within the compartments of the early secretory pathway may be best performed by furin, PACE4, PC5-B (16, 17), and, most likely, PC7.

This paper characterizes the *in vitro* properties of full-length and soluble rat PC7 (rPC7) overexpressed in mammalian cells infected with the corresponding vaccinia virus (VV) recombinants. A polyclonal rPC7 antiserum has been produced and used to identify the biosynthetic forms, likely cellular location, and site of zymogen cleavage of rPC7 in these cells. In addition, processing of several synthetic fluorogenic and peptidyl substrates is examined to elucidate the cleavage specificity of this newest member of the PC family.

EXPERIMENTAL PROCEDURES

Vaccinia Virus Constructs—The full-length cDNA of rPC7 (5) that had been inserted into the pBluescript vector (Stratagene) was digested with (5′) *Hin*dIII and (3′) *Bbs*I to remove a pair of extra 5′ ATG codons (5). Following the addition of a linker to close the sequence, the insert (5′) *Hin*dIII/(3′) *Xba*I was ligated into the *Hin*dIII/*Nhe*I sites of the VV transfer vector PMJ602 (18). This produced the rPC7 full-length recombinant virus (VV:rPC7). The native initiator sequence of this construct, CTG**ATG**C, was subsequently modified by polymerase chain reaction to GTG**ATG**G to generate a favorable Kozak consensus sequence for the initiation of protein translation (19).

Three soluble forms of rPC7 were constructed from this full-length VV:rPC7$_K$ clone by digestion with restriction enzymes followed by addition of the appropriate linkers. Thus, digestion with *Bsu*36I produced a form called BTMD- (before the transmembrane domain) rPC7$_K$, which

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[1] The abbreviations used are: PC, proprotein convertase(s); rPC7, rat PC7; hfurin, human furin; VV, vaccinia virus; BTMD, before the transmembrane domain; BCRD, before the cysteine-rich domain; ECL, enhanced chemiluminescence; MAP, multiple antigenic peptide; MCA, 4-methylcoumarin-7-amide; pro-EGF, pro-epidermal growth factor; pro-PTH, pro-parathyroid hormone; ykexin, yeast kexin; BFA, brefeldin A; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reverse phase-high performance liquid chromatography; MES, 4-morpholineethanesulfonic acid; pfu, plaque-forming units.

ends at Gly-Tyr-Ser^{622}. Similarly, digestion with *Hga*I generated two shorter forms of rPC7, called BTMD-S-rPC7, ending at Tyr-Gly-Ser^{578} or at Val-Asp-Ile^{565}. Vaccinia virus recombinants of the three constructs were then isolated as described previously (12, 14). Human BCRD-(before the cysteine-rich domain) furin (BCRD-hfurin), ending at Ser-Ser-Gly^{566}, has been described previously (20). The yeast recombinant VV:ykexin was kindly provided by Dr. D. Thomas (Biotechnology Research Institute, Montreal, Canada).

Cellular Infections and Enzyme Preparations—BSC40 cells, an African green monkey kidney epithelial cell line, were grown to confluence in 10% CO_2 at 37 °C on 15-cm tissue culture dishes using minimum essential medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and 28 μg/ml gentamycin. Following a brief rinse in phosphate-buffered saline, cells were infected with recombinant viruses at 2–4 plaque forming units (pfu)/cell in 5 ml of phosphate-buffered saline containing 0.01% (v/v) bovine serum albumin for 30 min at room temperature. They were then returned to minimum essential medium plus fetal bovine serum and incubated overnight at 37 °C.

Membrane preparations of $rPC7_K$ and yeast kexin (ykexin)-infected cells were made according to the procedure of Bresnahan *et al.* (21) with several modifications: 1) cells were harvested at 18 h post-infection by scraping in 20 mM Tris acetate, pH 7.0; 2) suspensions from the Dounce homogenizer were centrifuged at 16,000 × *g* for 15 min at 4 °C in a microcentrifuge; 3) the supernatants, representing soluble cellular material, were removed and stored at −20 °C for enzymatic activity and Western blot analyses; 4) the membrane pellets, resuspended in the above buffer, were diluted 1:1 (v/v) with glycerol and aliquoted for storage at −20 °C. Under these conditions, enzymatic activity was stable for at least 6 months. For the soluble VV:BTMD-$rPC7_K$ construct, cells at 18 h postinfection were washed three times with serum-free minimum essential medium and then incubated in the same medium for an additional 6 h. Following centrifugation at 1,500 × *g* for 10 min to remove cell debris, the media were concentrated approximately 20-fold on Centriprep-30s (Amicon), diluted 1:1 with glycerol, and stored at −20 °C. BCRD-hfurin, produced in the same manner, was further purified on DEAE-Bio-Gel A agarose (Bio-Rad) as reported (22). A similar further purification of BTMD-rPC7 consistently resulted in very poor recoveries of activity, most likely due to oxidative damage. Nonetheless, using the small amount of active BTMD-rPC7 recovered from this purification, we were able to ascertain that the properties (pH optimum and $K_{m,\ \text{app}}$ using a pentapeptide fluorogenic substrate (see below)) of the DEAE-purified BTMD-rPC7 were essentially identical to those of the enzyme obtained from concentrated media (results not shown).

Western Blots—Media and membrane extracts from rPC7-infected BSC40 cells were run on a 7.5% SDS-PAGE and the separated proteins electroeluted onto polyvinylidene difluoride membranes (Schleicher and Schuell). Protein bands were visualized by enhanced chemiluminescence (ECL) (Boehringer Mannheim) using a primary rabbit antiserum raised against a synthetic multiple antigenic peptide (MAP) (23) based on the amino acid sequence (Ala^{449}-Ser-Tyr-Val-Ser-Pro-Met-Leu-Lys-Glu-Asn-Lys-Ala-Val-Pro-Arg-Ser^{465}) located in the P-domain of rPC7 (5, Fig. 1). The specificity of the antiserum was verified by demonstrating that the bands visualized in the rPC7-infected cells could be blocked by preincubation with 200 μg/ml linear synthetic peptide of the MAP that had been prepared separately using normal, solid phase Fast-Moc chemistry (23). In addition, the antiserum did not cross-react when used on membrane pellets of BSC40 cells infected with vaccinia virus recombinants of other known membrane-associated convertases such as hfurin, mPC5/6-B, or ykexin. For quantitative analyses, densitometric readings of ECL films were carried out using the Gel Plotting macro of a Macintosh image analysis software (a public domain National Institutes of Health Image program).

Biosynthesis Assays and Amino Acid Sequencing—BSC40 cells were infected with the VV:rPC7 construct at 4 pfu/cell. Seventeen hours postinfection, the cells were pulse- or pulse-chase labeled with L-[^{35}S]Met as described (12, 16). When present during the preincubation as well as the pulse periods, the concentrations of tunicamycin and brefeldin A (BFA) were 5 and 2.5 μg/ml, respectively. As described previously (24), cells were lysed, and the proteins therein, along with those in the culture media, were immunoprecipitated by incubation with either the rPC7 antiserum (see above) plus protein A-agarose or with lentil lectin-Sepharose (25). Following SDS-PAGE, the bands were visualized by exposure to x-ray film.

For microsequence analyses, BSC40 cells infected with the VV:rPC7 construct were pulse-labeled for 4 h in the presence of [^{3}H]Leu or [^{3}H]Ile and processed as above. The labeled proteins were then run on an SDS-PAGE, after which the appropriate bands were eluted and analyzed on an Applied Biosystem gas-phase sequenator (model 470A) as described previously (12, 25).

Enzymatic Activity Determinations—Enzymatic activity was assessed using the fluorogenic synthetic peptide substrate pERTKR-MCA (Peptides International) (26) as follows. The enzyme preparation (usually 5 μl) was added to the assay reaction mixture consisting of (final concentration) 50 mM Tris acetate (pH 6.5), 1 mM $CaCl_2$, 1% Triton X-100 (v/v) in the case of membrane-bound enzyme preparations, and 50 μM 2-mercaptoethanol in the case of BCRD-hfurin. The reaction (in a total volume of 100 μl) was initiated at room temperature in a microtiter plate by the addition of pERTKR-MCA at a final concentration of 0.1 mM. Fluorescence was monitored at 0, 30, 60, and 90 min using a Perkin-Elmer spectrofluorometer (model LS 50 B) set to an excitation wavelength of 370 nm and an emission wavelength of 460 nm. In all cases, the rate of substrate hydrolysis was constant for at least 60 min.

pH, Ca^{2+}, and Inhibitor Profiles—For pH sensitivity experiments, enzymatic activity was evaluated as above using a ternary buffer system containing 17.3 mM sodium acetate, 17.3 mM MES, and 17.3 mM Tris acetate adjusted to the appropriate pH with acetic acid or NaOH. To determine the Ca^{2+} activation requirement of PC7, increasing concentrations of EDTA were added to the assay mixture described above for enzymatic activity measurements but lacking $CaCl_2$. Based on these results, the assay mixture was then supplemented with 1 mM EDTA and varying concentrations of $CaCl_2$. For the inhibitor profiles, selected compounds were added to the fluorogenic assay medium 30 min before the addition of pERTKR-MCA. In the case of EDTA and EGTA, the indicated concentrations of the chelator were added to the assay mixture without $CaCl_2$.

Fluorogenic Substrates and K_m Determinations—Fluorogenic assays were carried out substituting various MCA substrates for pERTKR-MCA. For K_m determinations, increasing amounts of pERTKR-MCA were added to the reaction mixture. The data, plotted as hydrolysis activity *versus* [pERTKR-MCA], were subject to nonlinear regression analysis (KaleidaGraph) to determine the K_m and V_{max} values. For the representative assays depicted in the figures, the data were expressed as a percentage of the calculated V_{max} and replotted.

Synthesis of Peptide Substrates—The pro-PC4 and pro-epidermal growth factor (pro-EGF) peptides were synthesized on an automated solid phase peptide synthesizer (Applied Biosystems, model 431A) using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate)-based FastMoc chemistry (22, 23). The following side chain protecting groups were used: *tert*-butyloxycarbonyl for Lys; *tert*-butyl for Ser, Thr, Tyr, Asp, and Glu; and 2,2,5,7,8-pentamethylchroman 6-sulfonyl for Arg. At the end of the synthesis, the peptides were cleaved from the resin and fully deprotected by treating the resin with reagent K (22, 23) for 5 h, followed by lyophilization and repeated washings with ether. The peptides were purified by RP-HPLC (Beckman, model 5500) on a semi-preparative Vydac column 218TP510G8 (1.0 × 25 cm) (Chromatographic Specialty Corp., Canada). The buffer system consisted of an aqueous phase containing 0.1% trifluoroacetic acid (v/v) and an organic phase of acetonitrile also containing 0.1% trifluoroacetic acid (v/v). The elution was carried out at a flow rate of 2 ml/min beginning with a 5-min isocratic step of 5% organic phase followed by a linear gradient of 5–60% organic phase over 60 min. Eluted materials were monitored at a wavelength of 225 nm. Both peptides were fully characterized by FAB mass spectrometry as well as by amino acid analysis (23). The synthetic pro-parathyroid hormone (pro-PTH) tridecapeptide was a gift from Dr. G. N. Hendy, Calcium Research Laboratory, Royal Victoria Hospital, McGill University, and has been described previously (27).

RP-HPLC Analysis of Synthetic Peptide Substrate Digests—The pro-PTH tridecapeptide (KSVKKRSVSEIQL), the pro-PC4 heptadecapeptide (YETLRRRVKRSLVVPTD), and the pro-EGF hexadecapeptide (HLREDDHHYSVRNSDS) were reacted at room temperature with enzymes in the reaction mixture as described above for enzymatic activity determinations. Time courses of substrate digestion by BCRD-hfurin, rPC7, and BTMD-rPC7 were first carried out to optimize the digestion conditions and to define the period during which the reaction proceeded at a constant rate (not shown). Thus, the reaction times chosen for the K_m determinations were from 10 to 20 min. The digestion products were analyzed using RP-HPLC separation (Varian, model 9010) on a Beckman 5-μm Ultrasphere C18 column (0.2 × 25 cm) as described above for peptide purification except that the buffer system also contained 0.01% triethylamine in both the aqueous and organic phases, the flow rate was 1 ml/min, the linear gradient of acetonitrile was 5–30% over 45 min, and monitoring was carried out at a wavelength of 210 nm. The collected peptides (two product peaks along with that of the undigested peptide, not shown) were identified and quantitated by amino acid

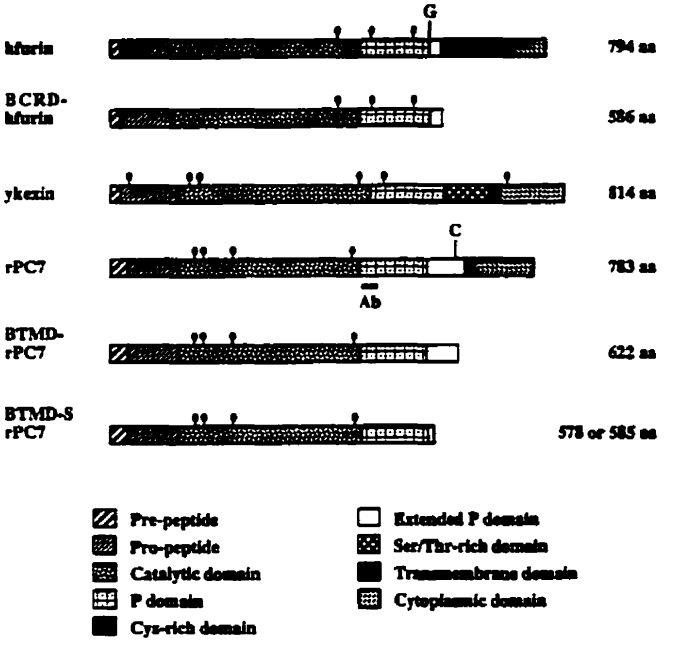

FIG. 1. **Schematic representation of selected forms of hfurin, rPC7, and ykexin.** The structural domains of the PCs studied in this work are depicted according to the legend at the *bottom* of the figure. The number of amino acids comprising each protein is indicated at the *right*. The *bar* indicates the region of the P-domain of rPC7 corresponding to the synthetic MAP used to produce an rPC7-specific antiserum (*Ab*, antibody). Also indicated are the Gly^{577} residue (*G*) denoting the C-terminal boundary of the P-domain of hfurin and the potentially unpaired Cys^{603} residue (*C*) located in the extended P-domain of rPC7. *aa*, amino acids.

composition as described (27). The K_m determinations were based on the amounts of the C-terminal cleavage product of each substrate peptide, and the resulting data were analyzed as described above for the pERTKR-MCA peptide.

RESULTS

Western Blot Analyses and in Vitro Enzymatic Activities of rPC7 and BTMD-rPC7—A schematic representation of the sequence of the full-length rPC7 and three truncated versions lacking the putative transmembrane domain and cytosolic tail (5), denoted BTMD-rPC7 and C-terminally shortened versions called BTMD-S-rPC7, is shown in Fig. 1. These molecular structures are compared with those of ykexin, the full-length hfurin, and its truncated version lacking the cysteine-rich domain, called BCRD-hfurin. The 17-amino acid peptide sequence indicated within the P-domain was used as an immunogen to obtain a polyclonal rPC7 antiserum, permitting the identification and characterization of the various forms of rPC7 obtained via overexpression in BSC40 cells. Thus, following overnight incubation of cells infected with VV recombinants expressing either $rPC7_K$ or $BTMD\text{-}rPC7_K$, the cell extracts, and media were analyzed by Western blotting. As shown in Fig. 2, an rPC7-specific doublet (101 and 89 kDa, at a ratio of approximately 1:3 as determined by densitometric analysis of the ECL film) is visible only in the membrane fraction of cell lysates obtained from $VV\text{:}rPC7_K$-infected cells. (The construct $VV\text{:}rPC7_K$ produced 2–3 times more enzyme than that containing the native initiator sequence, results not shown.) These two proteins were resistant to solubilization with 0.1 M Na_2CO_3 and even low (<5%) concentrations of Triton X-100 (not shown), indicating that rPC7 is an integral membrane protein. These findings were further supported by enzymatic activity assays of crude cellular fractions (Table I), which showed that 93% of the total rPC7-specific pERTKR-MCA hydrolytic activity was present in cell membranes. Analysis of BSC40 cells infected with

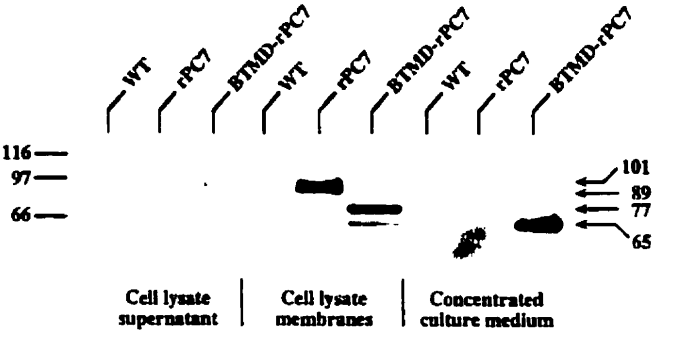

FIG. 2. **Immunodetection of rPC7 and BTMD-rPC7.** Samples from VV:wild type-, rPC7-, and BTMD-rPC7-infected BSC40 cells were processed as described under "Experimental Procedures" and run on 7.5% SDS reducing gels. Following electrotransfer to polyvinylidene difluoride membranes, protein bands were visualized via ECL detection using a primary rabbit antiserum raised against the synthetic rPC7 MAP (indicated in Fig. 1).

TABLE I
Comparative distribution of selected convertase enzymatic activities

Enzyme	Culture medium	Cell lysate supernatant[a]	Cell lysate membranes
	nmol/h[b] (% of total activity[c])		
WT[d]	1.2 (61)	0.8 (39)	0.0 (0)
ykexin[d]	6.0 (21)	3.6 (13)	18.6 (66)
rPC7[e]	0.4 (2)	0.9 (5)	18.2 (93)
BTMD-rPC7[e]	80.6 (89)	4.5 (5)	5.5 (6)
BCRD-hfurin[d]	43.7 (100)	0.0 (0)	0.0 (0)

[a] Supernatant from microcentrifuge spin at 16,000 × *g* of cells disrupted by Dounce homogenizer (see "Experimental Procedures").
[b] EDTA inhibitable pERTKR-MCA hydrolysis.
[c] Represents percentage of the sum of the total activities from the various cell fractions collected from a P-15 tissue culture dish.
[d] Infection at 2 pfu per cell.
[e] Infection at 4 pfu per cell.

VV:ykexin revealed that 21% of its activity is shed into the medium during a 7-h collection (subsequent to an overnight incubation), whereas 66% remains membrane-associated (Table I). Similarly, infections of BSC40 cells with VV:hfurin (full-length) resulted in the secretion of active, soluble furin (28, 29). These data thus confirm that rPC7, unlike hfurin and ykexin, remains as a membrane-associated protein which is not significantly shed into the medium.

In comparison, Western blot analysis of cells infected with $VV\text{:}BTMD\text{-}rPC7_K$ revealed an intracellular doublet in the membranes (77 and 65 kDa, at a ratio of about 5:1), as well as a 65-kDa form in the lysate supernatant and in the cell culture medium (Fig. 2). Densitometric quantitation and correction for the fractional amount deposited on the gel revealed that during a 7-h collection (subsequent to an overnight incubation), 90% of the total 65-kDa BTMD-rPC7 produced during this period was present in the cell culture medium. Similarly, upon measurement of the pERTKR-MCA cleavage activity of BTMD-rPC7 *in vitro*, we estimate that 89% of the total enzymatic activity is released into this 7-h medium (Table I). This value compares with the secretion of 100% of the activity of the soluble BCRD-hfurin (Table I). In contrast, neither form of BTMD-S-rPC7, comprising 578 or 585 amino acids (Fig. 1), was detectable by Western blotting or enzymatic activity measurements in the

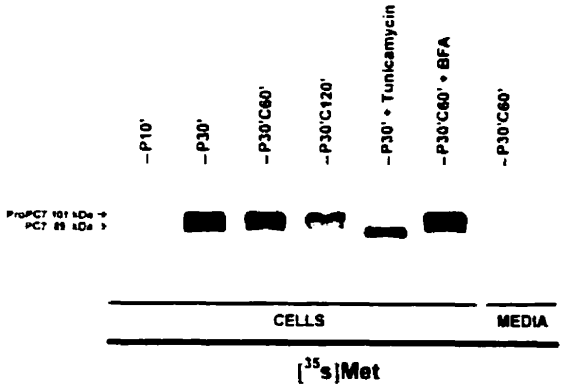

FIG. 3. **Biosynthesis of rPC7 in BSC40 cells.** VV:rPC7-infected BSC40 cells were pulse-labeled with [^{35}S]Met for 10 or 30 min and then chased as indicated. Cell lysate and media samples were immunoprecipitated with the rPC7 antiserum and then resolved by SDS-PAGE followed by autoradiography. The effects of 5 μg/ml of brefeldin A and 2.5 μg/ml of tunicamycin on rPC7 processing are depicted.

medium (not shown).

Biosynthesis and Zymogen Processing of rPC7—The biosynthesis of rPC7 was studied in BSC40 cells infected with VV:rPC7 and pulsed with [^{35}S]Met for either 10 or 30 min followed by immunoprecipitation of cell lysates. Under these conditions, two intracellular immunoreactive proteins, having molecular masses of 101 and 89 kDa, were observed on SDS-PAGE (Fig. 3). Based on densitometric scanning, the intensity of the upper band was slightly less than that of the lower one (at a ratio of about 0.7:1). Following the 30-min pulse period, chase times of up to 120 min altered this ratio to approximately 2:1, suggesting that the conversion of the early 101-kDa form into the 89-kDa protein may be followed by further post-translational modifications which raise the apparent molecular mass of the processed form to ~100 kDa. Furthermore, digestion of cell lysates by endoglycosidase H revealed that the 101- and 89-kDa forms are completely processed at early times (<45 min) and become progressively insensitive to this enzyme at times later than 120 min (not shown). Thus, at later pulse times, the ~100-kDa form would be expected to comprise a mixture of pro-PC7 (in the ER; endoglycosidase H-sensitive) and mature PC7 (in the Golgi apparatus) which underwent carbohydrate trimming and remodeling. This is very similar to the mass changes reported for hfurin (28, 30) or ykexin (31) during their transit along the secretory pathway. It should be noted that at no time did we observe any of the PC7-related forms released into the medium (Fig. 3). To clearly identify the PC7-immunoreactive proteins, we subjected the forms obtained from a 45-min pulse to microsequence analysis. Thus, the results with the [^{3}H]Leu-labeled 101-kDa form revealed a major sequence with Leu at positions 1, 7, 10, and 15, as well as a minor sequence with Leu at positions 3, 6, 11, and 17 (Fig. 4), both of which correspond to the zymogen form of rPC7 (5). We interpret these data to mean that the signal peptidase cleavage site can occur either at the predicted (5) sequence (QVMG ↓ LTEAGGLDTLGAGGLSWAV) or at an alternative site four residues later (LTEA ↓ GGLDTLGAGGLSWAVHLDS). The Leu^{15} and Ile^{2} sequence of the 89-kDa protein shows that its N terminus corresponds to the predicted zymogen cleavage site of rPC7 (5), proposed to be at the sequence RAKR ↓ SIHFNDPKYPQQWHLNNRR. Therefore, these data clearly demonstrate that the 101-kDa form detected after a pulse of 45 min is pro-rPC7 and that the 89-kDa form is rPC7.

In addition, we were able to immunoprecipitate both forms of rPC7 after either a 10-min pulse (Fig. 3) or 1-min pulse (not

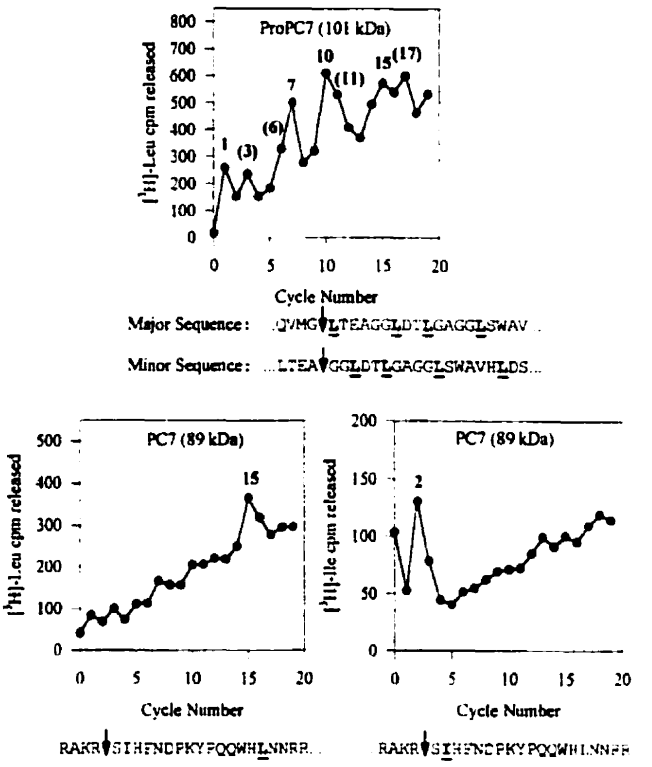

FIG. 4. **Microsequence analysis of pro-rPC7 and rPC7.** [^{3}H]Leu or [^{3}H]Ile-labeled proteins from VV:rPC7-infected BSC40 cells were run on SDS-PAGE, eluted, and subjected to microsequence analysis as described under "Experimental Procedures." The major and minor sequences obtained for the 101-kDa (*upper panel*) and the 89-kDa (*lower panel*) proteins are indicated *below* the figures. The cleavage sites are represented by the *arrows*.

shown). It is thus plausible that zymogen cleavage is an event that occurs very early along the secretory pathway. This was indeed confirmed when a pulse of 30 min, followed by a chase of 60 min in the presence of BFA, showed that the ratio of the two bands was similar to that obtained in the absence of BFA (Fig. 3). Since the fungal metabolite BFA is known to disassemble the Golgi complex and produce fusion of the *cis*-, *medial*-, and *trans*-Golgi (but not the *trans*-Golgi network) with the endoplasmic reticulum (ER) (32), these results suggest that, like hfurin (32), PC1 (14), and PC5 (15), the processing of pro-rPC7 into rPC7 most likely occurs within the ER. Finally, in the presence of tunicamycin, a pulse of 30 min revealed the synthesis of two proteins with estimated masses of 83 and 75 kDa at a ratio of about 12:1. This suggests not only that rPC7 is *N*-glycosylated, but that, similar to PC1 (24), non-*N*-glycosylated rPC7 is more rapidly metabolized than non-*N*-glycosylated pro-rPC7.

In Vitro Properties of rPC7—The enzymatic activity of rPC7 was investigated using the fluorogenic substrate pERTKR-MCA (the best hydrolyzed fluorogenic substrate of those tested, see Table I). Employing the ternary buffer system described under "Experimental Procedures," we observed that the pH optima of both rPC7 and BTMD-rPC7 reside in the range of pH 6–7 (Fig. 5), a value similar to that of hfurin (11, 33). Interestingly, full-length rPC7 maintained a significantly greater percentage of its maximal activity than BTMD-rPC7 at nearly all of the pH values examined. Assay controls using membranes or media of VV:wild-type-infected BSC40 cells displayed <5% of the maximal rPC7 activity (results not shown). Moreover, addition of 10 mM EDTA completely abolished the activity of both forms.

A precise, direct determination of the Ca^{2+} requirement of

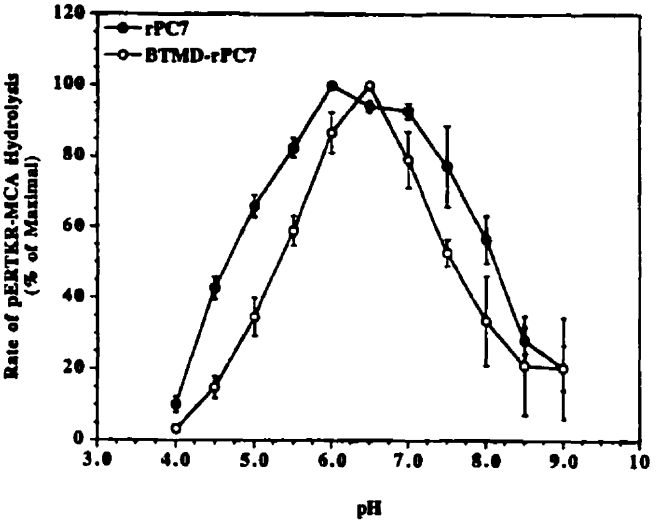

FIG. 5. **pH activation profile of rPC7 and BTMD-rPC7.** Preparations of VV:rPC7- and VV:BTMD-rPC7-infected BSC40 cells were assayed as described under "Experimental Procedures" using a ternary buffer system containing sodium acetate, MES, and Tris acetate along with the fluorogenic peptidyl substrate pERTKR-MCA. The results represent the average ± S.D. (indicated as *error bars*) of three separate determinations.

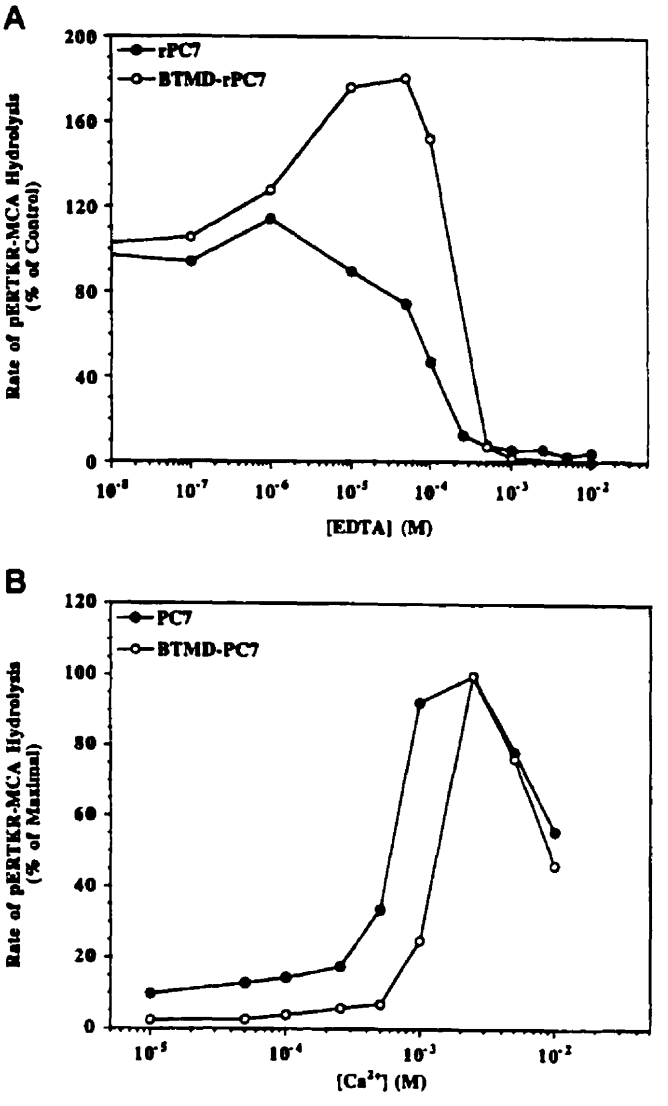

FIG. 6. *A*, effect of EDTA on rPC7 and BTMD-rPC7 enzymatic activity. Increasing amounts of EDTA were added to the reaction mixture (see "Experimental Procedures") containing enzyme preparations from VV:rPC7- and VV:BTMD-rPC7-infected BSC40 cells. However, Ca^{2+} was omitted to determine the amount of chelator required to inhibit PC7 activity. *B*, Ca^{2+} activation profile of rPC7 and BTMD-rPC7 enzymatic activity. Following a 15-min preincubation with 1 mM EDTA, enzyme preparations were assayed as above with increasing concentrations of Ca^{2+}.

rPC7 was not possible, since membrane preparations appear to contain high affinity Ca^{2+} binding sites. Thus, in experiments employing membranes to which no additional Ca^{2+} was added, the measured pERTKR-MCA hydrolytic activity was nearly 90% that of membranes to which 1 mM $CaCl_2$ had been added (not shown). Since preliminary attempts to wash out this residual Ca^{2+} were unsuccessful, an assay was carried out to determine how much EDTA would be required to abrogate the activity of rPC7. As shown in Fig. 6*A*, 1 mM EDTA added to the assay mixture was sufficient to nearly eliminate the hydrolysis of the MCA substrate by both rPC7 and BTMD-rPC7. We note that between 10 and 50 μM EDTA, the BTMD-rPC7 activity increased nearly 2-fold, whereas that of the membrane-bound rPC7 decreased by about 20%. Presumably, this is due to the presence of metal ions in the medium of BSC40 cells, which could inhibit BTMD-rPC7 activity as has been reported for PC1 and PC2 (34) as well as for furin (11, 33). Hence, the Ca^{2+} activation profile for rPC7 (Fig. 6*B*) was determined in the presence of 1 mM EDTA to begin the analysis at a minimal level of rPC7 enzymatic activity. The optimal Ca^{2+} concentration of the rPC7 and BTMD-rPC7 activities appears to be in the 1–2 mM range (Fig. 6*B*), again reminiscent of hfurin (11).

Tests using general protease inhibitors (Table II) indicate that rPC7's inhibitory profile is very similar to that of hfurin (11, 33). The only responses to serine protease inhibitors included modest inhibitory effects by 4-amidinophenylmethanesulfonyl fluoride and 1-chloro-3-tosylamido-7-amino-2-heptanone, which were probably nonspecific since they were dose-independent (*not shown*). Cysteine protease inhibitors and most mixed serine/cysteine protease inhibitors had no significant effects. An exception was antipain, which is a good inhibitor of ykexin (Table II) and is also known to slightly inhibit hfurin at 1 mM (33). As expected, the cation chelators EDTA, 1,10-phenanthroline, and, in particular, EGTA, were inhibitory, thus confirming that these enzymatic activities are indeed Ca^{2+}-dependent. Similarly, the strong inhibitory effects of oxidizing metals such as Zn^{2+} and Cu^{2+}, probably reacting with a metal-sensitive residue, have been previously described for PCs (33–35). Finally, a differential effect of reducing agents was observed. Thus, whereas 10 mM 2-mercaptoethanol, dithiothreitol, and glutathione enhanced the activity of rPC7, dithiothreitol inhibited BCRD-hfurin and ykexin, and glutathione activated ykexin but inhibited hfurin.

Cleavage Profile of Fluorogenic Substrates—The substrate specificity of rPC7 was examined and compared with those of BCRD-hfurin and ykexin using a series of synthetic fluorogenic MCA peptides of the general sequence ((R/K)XXR))-MCA. As indicated in Table III, the substrate best hydrolyzed by either rPC7 or ykexin during a 1-h reaction is the pentapeptide pERTKR-MCA. By comparison, a slightly better substrate for hfurin is RKKR-MCA, similar to findings reported using HIV gp160-related peptides (36). Interestingly, while cleavage of the model substrate RFAR-MCA is best afforded by hfurin, we note that all three enzymes can cleave to some degree at this monobasic site. The data also reveal that when the P-3 position is occupied by the acidic Glu residue (REKR-MCA), both hfurin and rPC7 do not cleave it very efficiently compared with ykexin. Also, using the RKKR-MCA substrate where the P-3 position is occupied by Lys, hfurin is more sensitive to this positively charged residue than rPC7 (compare REKR-MCA

TABLE II
Effects of proteinase inhibitors on BCRD-hfurin, rPC7, and ykexin activity

Inhibitor	Concentration	pERTKR-MCA hydrolysis		
		BCRD-hfurin	rPC7	ykexin
	mM		*% control*	
Control		100.0	100.0	100.0
APMSF	1.0	85.0	66.0	9.2
PMSF	1.0	86.6	118.2	82.3
TLCK	1.0	94.2	49.5	103.9
TPCK	1.0	83.8	127.7	87.4
Aprotinin	0.1	103.0	106.4	84.4
α1-Antitrypsin	0.1	99.3	123.8	105.6
Soybean trypsin inhibitor	0.1	105.3	113.2	91.3
3,4-Dichloroisocoumarin	1.0	72.1	114.0	77.3
Iodoacetic acid	1.0	90.5	121.9	86.1
E-64	0.1	118.7	152.5	101.4
Cystatin	0.01	114.0	117.8	127.5
Antipain	1.0	75.5	83.7	19.2
Chymostatin	1.0	100.1	118.1	85.9
Leupeptin	1.0	101.4	96.0	1.7
Pepstatin	0.1	95.0	109.5	108.4
1,10-Phenanthroline	10.0	74.9	83.9	103.4
EDTA	10.0	0.0	4.6	4.2
EGTA	10.0	9.9	24.0	27.1
2-Mercaptoethanol	10.0	140.4	110.9	142.0
Dithiothreitol	10.0	78.6	156.7	41.8
Glutathione	10.0	61.9	109.3	126.1
$ZnSO_4$	1.0	26.4	5.5	4.8
$CuSO_4$	1.0	4.1	3.2	1.5

TABLE III
Comparative cleavage of fluorogenic synthetic peptidyl substrates by BCRD-hfurin, rPC7, and ykexin

Substrate	Substrate hydrolysis		
	BCRD-hfurin	rPC7	ykexin
	% pERTKR-MCA hydrolysis		
RFAR-MCA	40.6	18.2	14.5
RVRR-MCA	46.8	8.6	12.2
REKR-MCA	13.7	10.2	58.4
RKKR-MCA	149.9	31.3	*
RSKR-MCA	45.8	41.8	70.7
KSKR-MCA	5.9	5.7	53.6
pERTKR-MCA	100.0	100.0	100.0
YEKERSKR-MCA	18.2	21.7	19.7

* Not determined.

and RSKR-MCA *versus* RKKR-MCA). Comparison of RSKR-MCA to KSKR-MCA shows that Arg at P-4 is much preferred as compared with Lys by both rPC7 and hfurin. Notably, ykexin tolerates Lys at P-4 and Glu at P-3 much better than either rPC7 or hfurin. Although the octapeptide YEKERSKR-MCA, representing the junction between the pro-segment of mouse PC1 and its catalytic subunit (37), contains the RSKR-MCA sequence, it is more poorly cleaved than the tetrapeptide. Finally, although *X*(K/R)R-AMC tripeptides are well cleaved by ykexin (38), they were very poorly processed (<5% of the pERTKR-MCA hydrolysis) by either rPC7 or hfurin (not shown).

We next examined the cleavage kinetics of pERTKR-MCA by rPC7 and BTMD-rPC7. As depicted in Fig. 7, a representative experiment, the concentration dependence of the cleavage of this substrate is different for these two enzyme forms. Averaged values (three separate determinations) of the apparent K_m values ($K_{m\,(app)}$) of rPC7 and BTMD-rPC7, as well as of BCRD-hfurin and ykexin, for the pERTKR-MCA substrate are presented in Table IV. Thus, the $K_{m\,(app)}$ of BCRD-hfurin for this pentapeptide is ~5 μM, as has been reported (26, 33). Although for ykexin the $K_{m\,(app)}$ is nearly 4-fold higher than that of hfurin, those of rPC7 and BTMD-rPC7 are approximately 14- and 28-fold higher, respectively. This suggests that rPC7 has either a relatively poor affinity for the above pentapeptide structure or a low catalytic (turnover) rate.

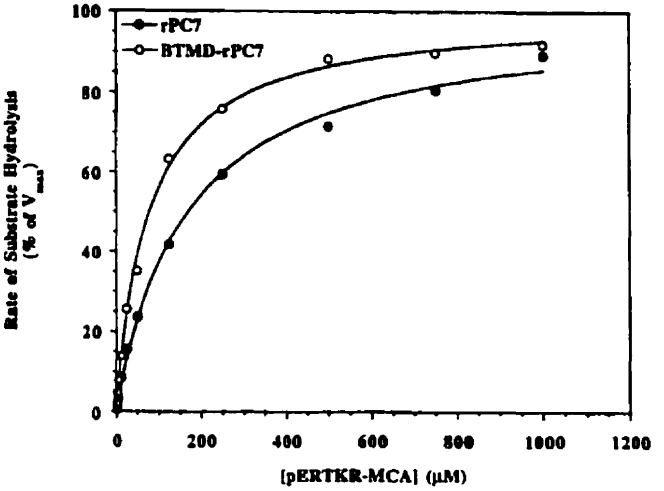

FIG. 7. **Comparative kinetic analyses of pERTKR-MCA processing by rPC7 and BTMD-rPC7.** Assays were carried out on enzyme preparations from VV:rPC7- and VV:BTMD-rPC7-infected BSC40 cells at optimal pH (6.5) and Ca^{2+} (1 mM) conditions using increasing amounts of pERTKR-MCA. The *curves* in this representative experiment were fit to the data using nonlinear regression analysis (KaleidaGraph). The calculated $K_{m\,(app)}$ values for this assay are 168.7 and 78.5 μm for rPC7 and BTMD-rPC7, respectively.

Synthetic Peptide Digestion Profiles—In an effort to determine a potential biological substrate of rPC7, three model synthetic peptides were examined. The choice of peptides was based on the colocalization of rPC7 with parathyroid hormone (PTH) in parathyroid glands (27),[2] PC4 in testicular germ cells (5, 39), and EGF in submaxillary glands (40, 42). The peptides selected were as follows: 1) a 13-amino acid peptide spanning the prohormone cleavage site of human pro-PTH having the sequence **KSVKKR** ↓ SVSEIQL; 2) a 17-amino acid peptide containing the zymogen cleavage site of mouse pro-PC4 having the sequence YETL**RRRVKR** ↓ SLVVPTD; and 3) a 16-amino acid human pro-EGF peptide having the sequence HLREDD**HH**YSV**R** ↓ NSDS.

For both the pro-PTH and pro-PC4 peptides, a single cleavage by rPC7 (Fig. 8) or BCRD-hfurin (not shown) was observed within 10–20 min of reaction at the expected physiological site, which was ascertained by amino acid analysis of the products. However, for the pro-EGF peptide, no significant processing was detected, even after a 24-h incubation. Fig. 9 depicts the relative digestion of the pro-PTH (Fig. 9*A*) and pro-PC4 (Fig. 9*B*) peptides as a function of their concentration by either BCRD-hfurin, rPC7, or BTMD-rPC7. Based on these data, kinetic constants for peptide processing were calculated and compared with those of the pERTKR-MCA substrate (Table V). The $K_{m\,(app)}$ and V_{max} values of pro-PTH peptide processing are 1.5 μM and 1.8 μM/h for BCRD-hfurin; 11.9 μM and 30.4 μM/h for rPC7; and 5.5 μM and 28.3 μM/h for BTMD-rPC7. Thus, BCRD-hfurin has an 8- or 4-fold lower $K_{m\,(app)}$ than either rPC7 or BTMD-rPC7 for this peptide. Moreover, it took at least 10 times more activity of the PC7 enzymes (as measured by hydrolysis at 100 μM pERTKR-MCA) to achieve the same extent of pro-PTH cleavage. The calculated $K_{m\,(app)}$ and V_{max} values of pro-PC4 processing are 1.4 μM and 13.0 μM/h for BCRD-hfurin, 2.2 μM and 4.3 μM/h for rPC7, and 1.5 μM and 21.1 μM/h for BTMD-rPC7 (Table V), again using approximately equal amounts of enzymatic activity measured at 100 μM pERTKR-MCA. In contrast, neither furin nor PC7 processed the pro-EGF peptide.

Using the ratio of $V_{max}/K_{m\,(app)}$, it is possible to compare the

[2] W. Dong and N. G. Seidah, unpublished results.

TABLE IV
Comparative K_m values for the cleavage of the fluorogenic peptidyl substrate pERTKR-MCA by BCRD-hfurin, rPC7, BTMD-rPC7, and ykexin

Enzyme	$K_{m(app)}$
	μM
BCRD-hfurin	5.9 ± 1.5
rPC7	164.0 ± 18.7
BTMD-rPC7	80.8 ± 12.7
ykexin	25.3 ± 1.5

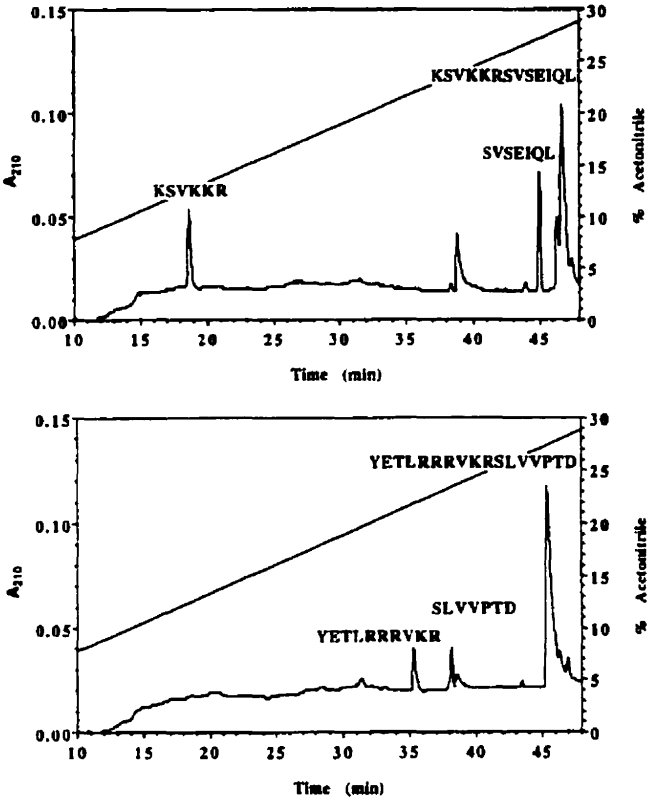

FIG. 8. **Processing of the pro-PTH and pro-PC4 peptides by rPC7.** The 13-amino acid pro-PTH and 17-amino acid pro-PC4 peptides were digested with rPC7 for 10 or 20 min, respectively, and the products were separated and purified by RP-HPLC using a 5-mm analytical Ultrasphere C18 column (Beckman), as described under "Experimental Procedures." The linear gradient of acetonitrile is depicted. The peptides contained within the indicated peaks were identified and quantitated by amino acid analysis. The unlabeled peaks represent nonpeptidic material which could be detected (absorbance at 210 nm) even in the absence of injected peptides.

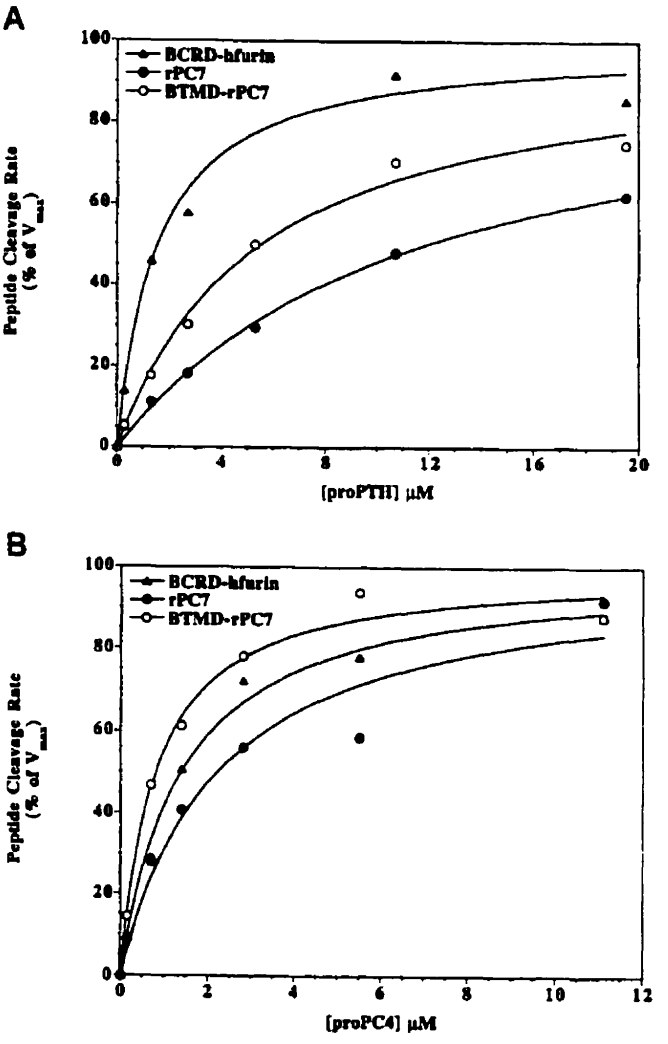

FIG. 9. *A*, comparative kinetic analyses of pro-PTH peptide processing by BCRD-hfurin, rPC7, and BTMD-rPC7. Preparations of soluble BCRD-hfurin and BTMD-rPC7 as well as rPC7 membranes were assayed as described under "Experimental Procedures" for 10 min using increasing amounts of the synthetic pro-PTH peptide. RP-HPLC analysis of the digestion reactions was carried out as described above. Peptide concentrations were quantitated by amino acid analysis. The *curves* were fit to the data using nonlinear regression analysis (KaleidaGraph). The calculated kinetic constants are indicated in Table V. *B*, comparative kinetic analyses of pro-PC4 peptide processing by BCRD-hfurin, rPC7, and BTMD-rPC7. Assays were carried out as described above except that for rPC7 and BTMD-rPC7 the reaction time was increased to 20 min. The deduced kinetic constants are indicated in Table V.

cleavage efficiency of a given enzyme preparation for various substrates. This analysis (Table V) indicates that processing of the pro-PTH peptide by furin is nearly 9 times more efficient than that of the pro-PC4 peptide. In the case of rPC7, the processing efficiency of both of these peptides appears to be similar, whereas BTMD-rPC7 exhibits a 2.7-fold preference for the pro-PC4 peptide. Interestingly, in comparison to the larger pro-PTH and pro-PC4 peptides, the cleavage efficiency of the fluorogenic pentapeptide substrate pERTKR-MCA is at least 30-fold lower for furin (27) and at least 50-fold lower for PC7.

DISCUSSION

Processing sites compatible with the cleavage selectivity of the known PCs are found in a variety of precursors that include prohormones and neural propeptides, serum proteins, cell-surface receptors, viral envelope glycoproteins, and growth factors (9). This wide spectrum of substrates is presumably the basis for the existence of a family of PCs expressed in a tissue-specific manner (3, 42) of which PC7 is the most recently discovered member (5–8). Sequence alignments suggest that PC7 is an ancestral member of the family of mammalian PCs, as it is the one most closely related to yeast kexin (5, 42). A widely expressed enzyme, PC7's tissue distribution is reminiscent of that of the ubiquitously expressed furin (5–8, 42). Moreover, like furin, PC7 is detected at high levels in lymphoid associated tissues (5, 42), suggesting that this convertase may play an important role in the immune system. Interestingly, certain human lymphomas express a modified version of PC7 mRNA (6). Until now, there have been no reports on the enzymatic properties of PC7 or identification of its potential physiological substrates. In the present work, we investigated the cleavage specificity and kinetics of rPC7 toward selected substrates *in vitro*, along with the zymogen processing and characterization of full-length and truncated forms of this enzyme. Our findings clearly demonstrate that this enzyme can cleave

TABLE V
Comparative kinetic constants for the cleavage of the synthetic peptidyl substrates pERTKR-MCA, proPTH, and proPC4 by BCRD-hfurin, rPC7, and BTMD-rPC7

Peptide	Enzyme	$K_{m(app)}$	V_{max}	$V_{max}/K_{m(app)}$
		μM	$\mu M \cdot h^{-1}$	h^{-1}
	BCRD-hfurin			
pERTKR-MCA		5.9	1.8	0.31
pro-PTH[a]		1.5	126	84.0
pro-PC4[b]		1.4	13	9.29
	rPC7			
pERTKR-MCA		164.0	6.4	0.04
pro-PTH		11.9	30.4	2.55
pro-PC4		2.2	4.3	1.95
	BTMD-rPC7			
pERTKR-MCA		74.0	7.5	0.10
pro-PTH		5.5	28.3	5.15
pro-PC4		1.5	21.1	14.1

[a] KSVKKR ↓ SVSEINL.
[b] YETLRRRVKR ↓ SLVVPTD.

substrates at paired basic residues, thus providing the first functional evidence for its membership in the family of mammalian subtilisin/kexin-like serine proteinases.

Overexpression of rPC7 in the constitutively secreting epithelial BSC40 cell line was carried out with the vaccinia virus expression system, which has previously been used to study the molecular forms of other PCs (11–14, 16, 25, 46). Using an antiserum developed against a peptide sequence located within the P-domain of rPC7 (Fig. 1), we were able to demonstrate that rPC7 is a membrane-associated protein that resists solubilization by either 0.1 M Na_2CO_3 or low concentrations (<5%) of Triton X-100. This supports the prediction from the cDNA sequence (5) that rPC7 contains a transmembrane domain. Furthermore, both Western blot (Fig. 2) and enzymatic activity analyses (Table I) of the media of cells overexpressing rPC7 failed to demonstrate the presence of a soluble, shed form of rPC7. This is in marked contrast to either hfurin (28, 29) or ykexin (31, Table I) which, when overexpressed in BSC40 cells, evince significant levels of shed protein in the culture medium. Interestingly, attempts to produce soluble forms of rPC7 ending either one (having the stop codon at Tyr-Gly-Ser^{578}) or eight (having the stop codon at Val-Asp-Ile^{585}) (5) residues after the putative C-terminal border of the P-domain (*i.e.* Gly-Ser^{578}) (9, 43, 44) resulted in an inactive enzyme that was retained as an intracellular zymogen (results not shown). We note the presence of a single Cys^{609} (see Fig. 1) within the segment separating the putative end of the P-domain and the beginning of the transmembrane sequence. In comparison, both hfurin and ykexin contain an even number of Cys residues in their corresponding segments. It is therefore plausible that Cys^{609} could be disulfide-bonded to another unique upstream Cys residue and that this interaction is critical for the proper folding and zymogen processing of rPC7 in the ER. Thus, the functionally defined P-domain of rPC7 is unique among those PCs examined, extending well beyond the conserved Gly^{577} residue (5, 43).

Biosynthetic analysis of the forms of rPC7 revealed that conversion of *N*-glycosylated pro-rPC7 to rPC7 is already detectable within 10 min after pulse labeling (Fig. 3). Furthermore, detection of both pro-rPC7 and rPC7 in the presence of BFA suggests that, like furin (30) and PC1 (14, 24), zymogen cleavage of pro-PC7 at the Arg-Ala-Lys-Arg ↓ Ser-Ile site likely occurs in the ER (Figs. 3 and 4). The predicted signal peptidase cleavage site, based on the criteria of von Heijne (45), is QVMG ↓ LTEAGGL (5). However, our protein sequencing data suggest that propeptide cleavage may also occur at an alternative site (LTEA ↓ GGL) four residues downstream from the predicted site (Fig. 4).

The data presented in Fig. 6 and Table II demonstrate that rPC7 is a Ca^{2+}-dependent serine proteinase having an optimal Ca^{2+} requirement of ~1–2 mM (Fig. 6). The membrane-bound form of this enzyme has a particularly broad pH optimum, with maximal activity occurring at pH 6–7 (Fig. 5). In contrast, the secreted mutant form, which lacks the transmembrane domain and the cytosolic tail, has a narrower pH range (albeit the same optimum of pH 6–7). While this deleted region may exert a modulatory effect on catalytic activity, as is the case for hfurin (46), it is also possible that the membrane association of rPC7 affects the interaction of this enzyme with its substrates. Taken together, the pH and Ca^{2+} optima of rPC7 resemble those of hfurin (11, 33) and ykexin (38), both of which have been shown to be active within the constitutive secretory pathway (47). In contrast, enzymes such as PC1 and PC2, which are known to be localized in secretory granules (48), have a minimal Ca^{2+} requirement of 1–2 mM and a pH optimum of 5–5.5 (23, 49). However, we also note that rPC7 exhibits 40–60% of its maximal activity at pH 5–5.5. Therefore, we cannot exclude the possibility that rPC7 may be functional within acidic compartments such as endosomes. Accurate determinations of the intracellular localization of rPC7 are required to define the organelle(s) in which the active enzyme is residing. Immunocytochemical staining of rPC7 in VV-infected BSC40 cells revealed primarily a peri- and para-nuclear localization pattern,[3] suggesting that this newest member of the PC family may reside in the ER and Golgi apparatus (15).

The cleavage specificity of rPC7 was first examined using a series of fluorogenic peptidyl substrates (Table III). A comparative analysis of hydrolysis of these MCA-containing peptides by BCRD-hfurin, rPC7, and ykexin indicates that rPC7 displays a cleavage specificity mostly resembling that of furin. For example, rPC7, like hfurin (26), does not hydrolyze tripeptide MCA substrates (even those containing a dibasic motif; data not shown), whereas ykexin does (38). Similarly, the REKR-MCA peptide, which contains a negatively charged Glu residue in the P-3 position, is poorly cleaved by both BCRD-hfurin (36) and rPC7 but is reasonably well cleaved by ykexin (Table III). With regard to hfurin (see Ref. 26) and ykexin, these findings are consistent with the proposed structures of the S3 enzymatic subsites of these enzymes based on homology modeling with subtilisin (50). Thus, in hfurin, the predicted S3 subsite contains a pair of negatively charged Glu residues (Glu^{123} and Glu^{150} in Ref. 50), whereas in ykexin, Glu^{150} is replaced by an Ala residue, presumably resulting in a lower negative charge density. However, in rPC7, the replacement of Glu^{123} by a Pro residue does not enhance this enzyme's tolerance for substrates containing Glu at the P-3 position. It is therefore possible that the identity of the amino acid corresponding to Glu^{150} in the predicted S3 subsite of PCs can influence their substrate selectivity. This hypothesis may also account for our observation that the peptide RKKR-MCA, which contains a positively charged Lys residue at P-3, is better cleaved than the REKR-MCA peptide by both hfurin and rPC7 (Ref. 26, Table III). Also, the presence of an uncharged residue at the P-3 position (*e.g.* Ser in RSKR-MCA) is well tolerated by all three enzymes (Table III). With respect to the P-4 residue, the cleavage specificity of rPC7 also resembles that of hfurin rather than ykexin. Substitution of Arg by Lys in this position nearly abolishes MCA-peptide hydrolysis by BCRD-hfurin and rPC7, yet only decreases it by ~50% for ykexin (Ref. 26, Table III). In other respects, the cleavage specificity of rPC7 more resembles that

[3] J. S. Munzer, M. Marcinkiewicz, and N. G. Seidah, manuscript in preparation.

of ykexin than of hfurin. For example, compared with BCRD-hfurin, rPC7 and ykexin rather poorly process the monobasic sequence RFAR-MCA or the double-Arg sequence RVRR-MCA (Table III).

The influence of other enzymatic subsites (P-5–P-8) may be inferred from the comparative hydrolysis of the two largest MCA-peptidyl substrates by BCRD-hfurin, ykexin, and rPC7 (Table III). The pentapeptide pERTKR-MCA, which is the best cleaved of the fluorogenic substrates tested, contains a pyro-Glu residue at the P-5 position. In comparison, the modestly hydrolyzed octapeptide YEKERSKR-MCA, representing the zymogen activation site of mPC1 (14), contains a Glu residue at both the P-5 and P-7 positions. The different cleavage rates of these two peptides by rPC7 may be due to the replacement of the Glu residue by pyro-Glu in the pentapeptide.

Upon further investigation, a more detailed kinetic analysis suggested that pERTKR-MCA is a poor substrate for rPC7 as compared with BCRD-hfurin and ykexin (Table IV). The $K_{m\,(app)}$ of rPC7 for this peptide was nearly 28 times higher than that of BCRD-hfurin and 6.5-fold higher than that of ykexin. Smaller variations were observed for the soluble BTMD-rPC7 enzyme (Fig. 7 and Table IV). This difference between the membrane-anchored and soluble forms of rPC7 may be due to an effect of the C-terminal region of this enzyme on its interaction with substrates (*cf.* Ref. 46). An alternative explanation is that nonspecific substrate adsorption to the membranes of the rPC7 enzyme preparation could decrease its availability, resulting in artificially high $K_{m\,(app)}$ values. However, addition of VV:wild type-infected BSC40 cell membranes to an assay using BCRD-hfurin had no effect on the $K_{m\,(app)}$ for the pERTKR-MCA peptide cleavage (not shown). Hence, the basis of the poor hydrolysis of this substrate by rPC7 relative to hfurin and ykexin is unclear. Perhaps, more importantly, small fluorogenic substrates such as these MCA-peptides have several characteristics that limit their usefulness as models of PC substrates. These include the observation that the MCA moiety constitutes a poor leaving group for the peptide hydrolysis reaction (26) and that these substrates fail to interact with the P′ enzymatic subsites, which could also be involved in substrate recognition (27, 51).

In view of these considerations, we synthesized three peptides, comprising at least six residues N-terminal to the cleavage site and at least four residues C-terminal to the cleavage site, to complement the studies with MCA-peptides. The peptides chosen contained the proprotein processing sites of three proteins (pro-PTH, pro-PC4, and pro-EGF) which, based on their comparative tissue distributions, could be substrates for PC7. Analysis of the cleavage efficiencies ($V_{max}/K_{m\,(app)}$) indicated that of these peptides, PC7 processed the pro-PC4 peptide (YETL**RRRVKR** ↓ SLVVPTD) either equally (for rPC7) or more (for BTMD-rPC7) efficiently than the pro-PTH peptide (**K**SVK**KR** ↓ SVSEIQL), whereas the one best processed by BCRD-hfurin was the pro-PTH peptide (Table V). It is possible that the presence of an Arg/Lys residue at the P-6 position in conjunction with a pair of basic amino acids at the cleavage site, a motif common to both peptides, is optimal for effective cleavage by rPC7. Moreover, the influence of residues extending on either side of the conventional furin consensus sequence R-*X*-(K/R)R ↓ (9–11) must be considered.

The third synthetic peptide examined, pro-EGF, consists of a monobasic P-1 Arg cleavage site along with a Tyr residue in the P-4 position and a pair of His residues in the P-5 and P-6 positions (HLREDD**HH**YSV**R** ↓ NSDS). As indicated in Table V, this synthetic peptide is clearly not a substrate for either hfurin or rPC7 in our assays. We mentioned above that the monobasic Arg-containing peptide RFAR-MCA is poorly cleaved by both rPC7 and furin (Table III). Also, cleavage studies of other monobasic substrates reported for furin indicate that it requires additional P-4 or P-6 basic (Arg or Lys) residues (52). Thus, it appears that monobasic sequences lacking these additional basic residues (52) are not good substrates for these enzymes and that His does not appear to substitute for Arg or Lys at these positions.

Investigations of the substrate cleavage specificity of rPC7 using various fluorogenic peptides and synthetic model peptides mimicking precursors colocalizing with this enzyme did not allow us to identify a PC7-specific candidate precursor that was not also well cleaved by hfurin. Previously, hfurin was shown to be more efficient than hPC1 in processing the same pro-PTH peptide used in this *in vitro* study, as well as in processing pro-PTH to PTH following coexpression of the appropriate VV construct in BSC40 cells (27). Similar coexpression experiments are currently underway to compare the *ex vivo* processing of pro-PTH to PTH by hfurin and rPC7. Given their colocalization with pro-PTH in the parathyroid gland (27)[2] and their ability to correctly process a peptide containing the pro-PTH processing site, it will be of considerable interest to determine whether both furin and PC7 are responsible for pro-PTH maturation *in vivo*.

With regard to the data on the pro-PC4 peptide, the biological relevance of these findings is not clear. Although PC7 is expressed in testicular germ cells, partially colocalizing with PC4 (5), there are as yet no reports of one PC being responsible for the zymogen activation of another. It is thus likely that the zymogen activation of PC4 is autocatalytic, as has been shown for furin (28, 30) and PC1 (53). However, as a model substrate, the sequence of the pro-PC4 peptide (. . . L**RRR**V**KR** ↓ SLV . . .) closely mimics the zymogen processing sites of both hfurin (. . . A**KRR**T**KR** ↓ DVY . . .) (11, 51) and rPC7 (. . . LL**KR**A**KR** ↓ SIH . . .) (5). If, as mentioned, zymogen activation is autocatalytic, these sequences would necessarily function to some extent as biological substrates. As such, they warrant further investigation.

In general, the issue of which PC processes a given proprotein precursor remains largely unresolved. Considering the important biological functions of many PC substrates, it is possible that, for the organism's survival, evolution has chosen to ensure sufficient processing through the redundancy (3) of PCs. This could explain why the majority of tissues express both furin and PC7 (5, 42). On the other hand, although the intracellular localization of furin and PC7 appears to overlap to some degree, the exact intracellular trafficking of PC7 has not yet been described. It is possible that these two enzymes are active in mutually exclusive environments or that their activity is modulated by as yet unidentified cofactors. Moreover, the co-regulation of cognate PCs and their substrates (36, 54) favors the notion of a physiological coupling between each PC and particular substrates. Hence, variations in the level of cellular expression of a PC or its substrate could significantly influence the nature and extent of precursor processing (8). For example, PC7 is particularly abundant in certain activatable immune cells such as $CD4^+$ T-lymphocytes (5, 42), suggesting that this enzyme may play an important role in processing precursors (*e.g.* the tumor necrosis factor receptor (55), integrins (56), and retroviral surface glycoproteins such as HIV gp160 (46)) expressed in either resting or activated immune cells. Taken together, these points illustrate the complexity of substrate recognition and cleavage by PCs and emphasize the need for multifaceted approaches to identify the cognate enzyme(s) responsible for the processing of precursor substrates under physiological conditions.

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Biochemistry

Mammalian subtilisin/kexin isozyme SKI-1: A widely expressed proprotein convertase with a unique cleavage specificity and cellular localization

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ABSTRACT **Using reverse transcriptase–PCR and degenerate oligonucleotides derived from the active-site residues of subtilisin/kexin-like serine proteinases, we have identified a highly conserved and phylogenetically ancestral human, rat, and mouse type I membrane-bound proteinase called subtilisin/kexin-isozyme-1 (SKI-1). Computer databank searches reveal that human SKI-1 was cloned previously but with no identified function. *In situ* hybridization demonstrates that SKI-1 mRNA is present in most tissues and cells. Cleavage specificity studies show that SKI-1 generates a 28-kDa product from the 32-kDa brain-derived neurotrophic factor precursor, cleaving at an RGLT ↓ SL bond. In the endoplasmic reticulum of either LoVo or HK293 cells, proSKI-1 is processed into two membrane-bound forms of SKI-1 (120 and 106 kDa) differing by the nature of their N-glycosylation. Late along the secretory pathway some of the membrane-bound enzyme is shed into the medium as a 98-kDa form. Immunocytochemical analysis of stably transfected HK293 cells shows that SKI-1 is present in the Golgi apparatus and within small punctate structures reminiscent of endosomes. *In vitro* studies suggest that SKI-1 is a Ca^{2+}-dependent serine proteinase exhibiting a wide pH optimum for cleavage of pro-brain-derived neurotrophic factor.**

Limited proteolysis of inactive precursors to produce active peptides and proteins generates biologically diverse products from a finite set of genes. Most often, such processing occurs at either monobasic or dibasic residues as a result of cleavage by mammalian serine proteinases related to bacterial subtilisin and yeast kexin (1, 2). These enzymes, known as proprotein convertases (PCs), cleave a variety of precursors at the consensus **(R/K)**-$(Xaa)_n$-**R** ↓ sequence, where Xaa is any amino acid except Cys and n = 0, 2, 4, or 6 (1–3).

Less commonly than cleavage at basic residues, bioactive products also can be produced by limited proteolysis at amino acids such as L, V, M, A, T, and S (3). This type of cellular processing has been implicated in the generation of bioactive peptides such as α- and γ-endorphin (4), the C-terminal glycopeptide fragment 1–19 of provasopressin (5), platelet factor 4 (6), the metalloprotease ADAM-10 (7), site 1 cleavage of the sterol regulatory element-binding proteins (SREBPs) (8), as well as in the production of the Alzheimer's amyloidogenic peptides Aβ40, -42, and -43 (9). Processing of this type occurs either in the endoplasmic reticulum (ER) (8), late along the secretory pathway, within secretory granules (4, 5), at the cell surface, or in endosomes (6, 7, 9). The proteinases responsible for these cleavages are not yet identified.

We hypothesized that an enzyme (or enzymes) distinct from, but related to, PCs may generate polypeptides by cleavage at nonbasic residues. To test that idea, we employed a reverse transcriptase–PCR (RT-PCR) strategy similar to the one used to identify the PCs (10), except that we used degenerate oligonucleotides closer to bacterial subtilisin than to yeast kexin. This resulted in the isolation of a cDNA fragment encoding a putative subtilisin-like enzyme from human cell lines. This partial sequence was identical to a segment of a human myeloid cell-derived cDNA reported by Nagase *et al.* (11). Preliminary results demonstrated that this putative proteinase cleaves pro-brain-derived neurotrophic factor (proBDNF) (ref. 12; S.J.M. N.G.S., and R.A.M., unpublished results).

In this paper, we show that the sequences of rat, mouse, and human orthologues of this putative type I membrane-bound subtilisin-kexin-isoenzyme, which we called SKI-1, exhibit a high degree of sequence conservation. Tissue distribution analysis by both Northern blots and *in situ* hybridization revealed that SKI-1 mRNA is widely expressed. A vaccinia virus recombinant and a stable transfectant of human SKI-1 in HK293 cells allowed the analysis of its biosynthesis and intracellular localization. Finally, we present data demonstrating that SKI-1 cleaves at a specific T ↓ residue within the N-terminal segment of proBDNF. SKI-1 is thus identified as a mammalian secretory subtilisin/kexin-like enzyme capable of cleaving a proprotein at nonbasic residues.

MATERIALS AND METHODS

PCR and Sequencing. Most RT-PCRs were performed using a Titan One Tube RT-PCR system (Boehringer Mannheim) on 1 μg of total RNA isolated from a human neuronal cell line (IMR-32), mouse corticotrophic cells (AtT20), or rat adrenal

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Abbreviations: SKI-1, subtilisin/kexin-isozyme-1; PC, proprotein convertase; ER, endoplasmic reticulum; BDNF, brain-derived neurotrophic factor; SREBP, sterol regulatory element-binding protein; RT-PCR, reverse transcriptase–PCR; vv, vaccinia virus; PDX, α1-antitrypsin Portland; WGA, wheat germ agglutinin; PMSF, phenylmethylsulfonyl fluoride.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF094820 and AF094821).

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glands. The active-site degenerate primers were as follows: His (sense) 5′-GGICA(C,T)GGIACI(C,T)(A,T)(C,T)(G,T)(T,G)IGCIGG-3′ and Ser (antisense) 5′-CCIG(C,T)IACI-(T,A)(G,C)IGGI(G,C)(T,A)IGCIACI(G,C)(A,T)IGTICC-3′, based on the sequences GHGT(H,F)(V,C)AG and GTS-(V,M)A(T,S)P(H,V)V(A,T)G, respectively. The amplified 525-bp products were sequenced on an automated laser fluorescence DNA sequencer (Pharmacia). To obtain the full-length sequence of rat and mouse SKI-1, we used PCR primers based on the human (11) and mouse sequences, in addition to 5′ (13) and 3′ (14) RACE amplifications. At least three clones of the amplified cDNAs were sequenced. The GenBank accession numbers of the 3,788-bp mouse mSKI-1 cDNA and 3,895-bp rat rSKI-1 are AF094820 and AF094821, respectively.

Infection, Transfection, and Metabolic Labeling. Human SKI-1 (nucleotides 1–4338) (11) in Bluescript (a generous gift from N. Nomura, Kazusa DNA Research Institute, Chiba, Japan; accession no. D42053) was digested with *Sac*II (nucleotides 122-4338) and inserted into the vector PMJ602, and a vaccinia virus recombinant was isolated. The PMJ602 construct was also digested with 5′ *Kpn*I/3′ *Nhe*I and cloned into the *Kpn*I/*Xba*I sites of pcDNA3 (Invitrogen), and the cDNA was transfected into HK293 cells by using Lipofectin. A number of stable transfectants resistant to G418 and positive on Western blots using an SKI-1 antiserum (see below) were isolated, and one of them (clone 9), was investigated further. Either vaccinia virus-infected or -transfected cells were pulsed for 20 min with [^{35}S]cysteine and then chased for various times in the presence or absence of either tunicamycin (5 μg/ml) or brefeldin A (2.5 μg/ml). Media and cell lysates were immunoprecipitated with SKI-1 antisera directed against either amino acids 634–651 or 217–233, or a pro-SKI-1 antiserum directed against the prosegment comprising amino acids 18–188 (Fig. 1). Immune complexes were resolved by SDS/PAGE on an 8% polyacrylamide/*N*-[tris(hydroxymethyl)methyl]glycine gel (15).

Northern Blots, *in Situ* Hybridizations, and Immunocytochemistry. Northern blot analyses (16) were done on total RNA from adult male rat tissues by using either a TRIzol reagent kit (Life Technologies, Gaithersburg, MD) or a Quick Prep RNA kit (Pharmacia) and on poly(A)$^+$ RNA of (male + female) rat adult tissues (BIO/CAN, Montreal). The blots were hybridized overnight at 68°C in the presence of [^{32}P]UTP SKI-1 cRNA probes, which consisted of the antisense of nucleotides 655-1249 of rat SKI-1. For *in situ* hybridization on newborn rats, the same rat sense and antisense cRNA probes were labeled with uridine and cytosine 5′-[γ-[^{35}S]thio]triphosphate (1, 16). For immunofluorescence staining we used a rabbit anti-SKI-1 antiserum at a 1:100 dilution and rhodamine-labeled goat anti-rabbit IgGs diluted 1:20 (16). Red SKI-1 immunostaining was compared with green-staining patterns of both fluorescein-labeled concanavalin A (Con A; Molecular Probes), an ER marker, or fluorescein-conjugated wheat germ agglutinin (WGA; Molecular Probes), a Golgi marker (17).

***Ex Vivo* and *in Vitro* proBDNF Processing.** A vaccinia virus recombinant of human SKI-1 (vv:SKI-1) was isolated as described for human proBDNF (vv:BDNF) (15). The vaccinia virus recombinants of α1-antitrypsin Pittsburgh (α1-PIT; vv:PIT) and α1-antitrypsin Portland (α1-PDX; vv:PDX) (18) were generous gifts from G. Thomas (Vollum Institute, Portland, OR). COS-7 cells (4×10^6) were coinfected with 1 plaque-forming unit (pfu) per cell of vv:BDNF and either the wild-type virus (vv:WT) alone at 2 pfu per cell or with 1 pfu per cell of each virus in the combinations [vv:SKI-1+vv:WT], [vv:SKI-1+vv:PIT], and [vv:SKI-1+vv:PDX]. At 10 h postinfection, cells were pulsed for 4 h with 0.2 mCi of [^{35}S]cysteine/[^{35}S]methionine (DuPont). Media and cell extracts were immunoprecipitated with a BDNF antiserum (ref. 19; provided by Amgen) at 0.5 μg/ml, and the proteins were resolved on SDS/PAGE 13–22% gradient gels (15). [^{35}S]Met-labeled 32-kDa proBDNF and [^{3}H]Leu-labeled 28-kDa BDNF were sequenced as described (20). For *in vitro* analysis, 32-kDa proBDNF obtained from the media of LoVo cells infected with vv:BDNF was incubated overnight with the shed form of SKI-1 obtained from cells coinfected with vv:SKI-1 and vv:PDX, either at different pH values or at pH 6.5 in the presence of selected inhibitors: pepstatin (1 μM), antipain (50 μM), cystatin (5 μM), E64 (5 μM), soybean trypsin inhibitor (SBTI, 5 μM), 0.5 M phenylmethylsulfonyl fluoride (PMSF) + 50 μM *para*-amino-PMSF (pAPMSF), *o*-phenanthroline (5 mM), and EDTA (10 mM). The products were resolved by SDS/PAGE on a 15% polyacrylamide gel, blotted, and then probed with a BDNF antiserum (Santa Cruz Biotechnology) at a dilution of 1:1,000.

RESULTS

Protein Sequence Analysis of SKI-1. We first aligned the protein sequences within the catalytic domain of PC7 (21), yeast subtilases, and bacterial subtilisins, together with that of a novel *Plasmodium falciparum* subtilisin-like enzyme called pf-SUB2 (J.-C.B., unpublished results). This led to the choice of conserved amino acids GHGT(H/F)(V/C)AG and GTS(M/V)A(T/S)P(H/V)V(A/T)G around the active sites His and Ser, respectively. Thus, using degenerate oligonucleotides coding for the sense His and antisense Ser consensus sequences, we initiated a series of RT-PCRs on total RNA and isolated a 525-bp cDNA fragment from the human neuronal cell line IMR-32. This sequence was found to be 100% identical to that reported for a human cDNA called KIAA0091 obtained from a myeloid KG-1 cell line (11) and 88% identical to that of a 324-bp expressed sequence tag (accession no. H31838) from rat PC12 cells. The full-length rat and mouse cDNA sequences were obtained after RT-PCR amplifications of total RNA isolated from rat adrenal glands and PC12 cells and from mouse AtT20 cells. As shown in Fig. 1, alignment of the protein sequence deduced from the cDNAs revealed that rat and mouse SKI-1 share 98% sequence identity and a 96% identity to human SKI-1. Interestingly, within the catalytic domain ($Asp^{218} \rightarrow Ser^{414}$) the sequence similarity between the three species is 100%. Analysis of the predicted amino acid sequence suggests a 17-aa signal peptide, followed by a putative prosegment beginning at Lys^{18} and extending for some 160–180 aa. The proposed catalytic domain encompasses the typical active sites Asp^{218}, His^{249}, and Ser^{414} and the oxyanion hole Asn^{338}. This domain is followed by an extended C-terminal sequence characterized by the presence of a conserved growth factor/cytokine receptor family motif C849LDDSHRQKDCFW^{861}. This sequence is followed by a potential 24-aa hydrophobic transmembrane segment and a less-conserved 31-aa cytosolic tail that, remarkably, consists of 35% basic residues. Some of the clones isolated from rat adrenal glands suggested the existence of alternatively spliced rSKI-1 mRNAs in which the segments coding for amino acids 430–483 or 858–901 are absent. Finally, the phylogenetic tree derived from the alignment of the catalytic domain of SKI-1 with subtilases (22) suggests that it is an ancestral protein that is closer to plant and bacterial subtilases than to either yeast or mammalian homologues (not shown).

Tissue Distribution of SKI-1 mRNA. Northern blot analyses of SKI-1 mRNA in adult male rat reveal that rSKI-1 mRNA is widely expressed and is particularly rich in anterior pituitary, thyroid, and adrenal glands (Fig. 2*A*). A Northern blot of poly(A)$^+$ RNA obtained from mixed adult male and female rat tissues also showed a wide distribution and a particular enrichment in liver (Fig. 2*B*). Similarly, analysis of 24 different cell lines (23) revealed a ubiquitous expression of SKI-1 mRNA (not shown).

In situ hybridization data obtained in a day 2 postnatal rat also provided evidence of a widespread, if not ubiquitous, distribution of rSKI-1 mRNA. Fig. 3 shows at the anatomical level the presence of SKI-1 mRNA in developing skin, striated

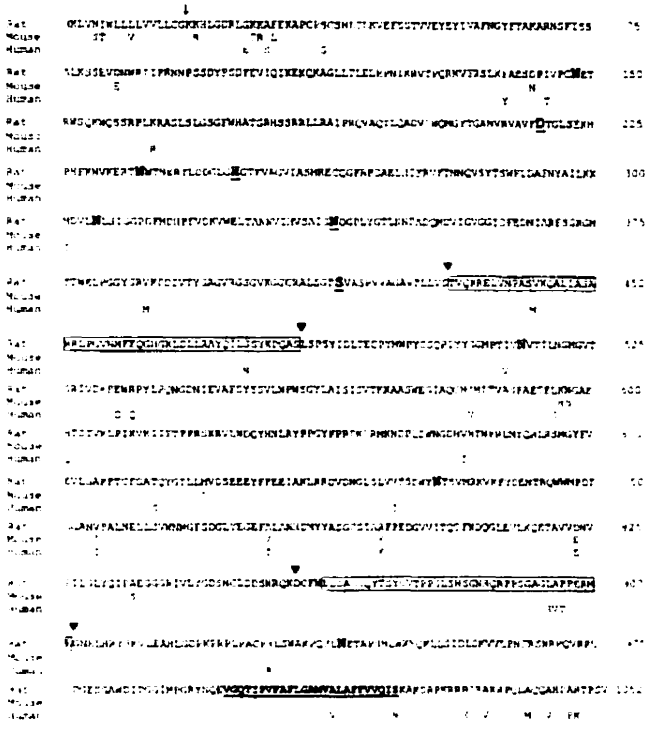

FIG. 1. Comparative protein sequences of SKI-1 deduced from rat, mouse, and human cDNAs. The position of the predicted end of the 17-aa signal peptide is shown by an arrow. The active sites Asp^{218}, His^{249}, and Ser^{414} as well as the oxyanion hole Asn^{338} are shown as bold, shaded, and underlined characters. The positions of the six potential N-glycosylation sites are emphasized in bold. The conserved shaded sequence fits the consensus signature for growth factors and cytokine receptor family. Each of the two boxed sequences was absent (▼) in a number of rat clones. The predicted transmembrane segment is in bold and underlined.

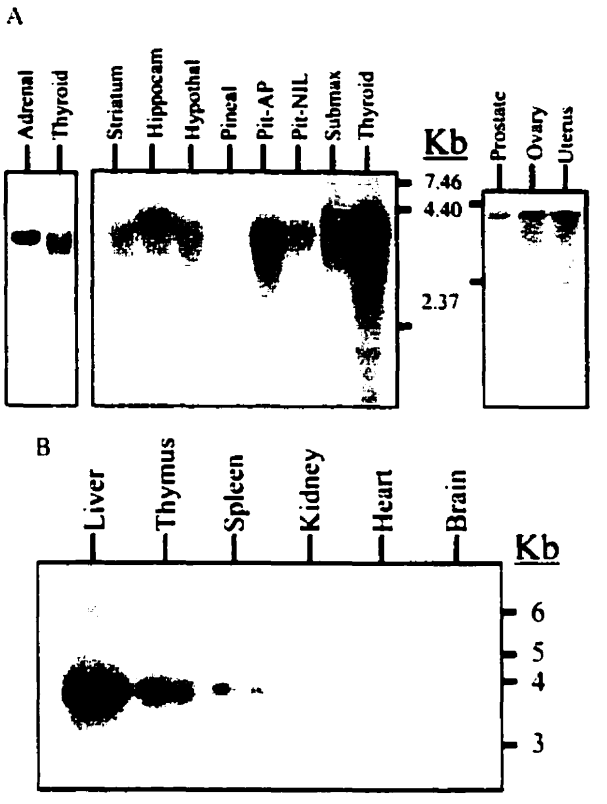

FIG. 2. Northern blot analysis of the expression of SKI-1 in adult rat tissues. (*A*) Five micrograms of male rat total RNA was loaded in each lane: pituitary anterior (AP) and neurointermediate (NIL) lobes and submaxillary gland (submax). (*B*) Two micrograms of poly(A)$^+$ RNA of (male + female) Sprague–Dawley rat adult tissues. The estimated size of rat SKI-1 mRNA is about 3.9 kb.

muscles, cardiac muscles, bones, and teeth as well as brain and many internal organs. Strong hybridization signals were detectable in the retina, cerebellum, pituitary, submaxillary, thyroid, and adrenal glands, molars, thymus, kidney, and intestine. Evidence for the cellular expression of rSKI-1 mRNA was obtained from analysis of the relative labeling densities per cell in selected tissues, based on a semiquantitative analysis of emulsion autoradiographs (not shown). In the central nervous system rSKI-1 mRNA labeling was mostly confined to neurons, whereas ependymal cells and supportive glial cells, such as presumed astrocytes, oligodendrocytes, and microglia, exhibited 5- to 30-fold-less labeling per cell. In addition, within the peripheral nervous system, trigeminal ganglia revealed a 5- to 10-fold greater expression in neurons as compared with presumptive Schwann cells. Labeling was observed in most of the glandular cells in the anterior and intermediate lobes of the pituitary as well as in the pituicytes of the pars nervosa. A semiquantitative comparison in the adult and newborn rat pituitary gland, submaxillary gland, thymus, and kidney demonstrated an overall 2-fold-decreased labeling of rSKI-1 mRNA with age (not shown).

Biosynthesis of hSKI-1. To define the molecular forms of human SKI-1 we generated both a vaccinia virus recombinant (vv:SKI-1) and a stable transfectant in HK293 cells. Three antisera were produced against amino acids 18–188 (prosegment), 217–233, and 634–651 of SKI-1. Expression of vv:SKI-1 in four different cell lines revealed that the enzyme is synthesized as a 148-kDa proSKI-1 zymogen, which is processed progressively into 120-, 106-, and 98-kDa proteins (Fig. 4). Only the 148-kDa form is recognized by the prodomain antiserum, whereas all four forms react with the other two antisera. Processing of the 148-kDa proSKI-1 into the 120- and 106-kDa forms occurs in the ER based on the presence of these proteins in cells preincubated with the fungal metabolite brefeldin A (Fig. 4; ref. 24). Furthermore, preincubation with tunicamycin revealed only two bands (Fig. 4), suggesting that the presumably membrane-bound 106- and 120-kDa forms differ by their N-glycosylation. At the 3-h chase time, results reveal the secretion of a 98-kDa shed form (sSKI-1) recognized by both SKI-1 antisera (Fig. 4) but not by the proSKI-1 antiserum (not shown). Similar SKI-1-related forms were seen in stably transfected HK293 cells after a 4-h pulse labeling with [^{35}S]methionine (not shown).

Intracellular Localization of SKI-1. Double-staining immunofluorescence was used to compare the intracellular localization of the stably transfected human SKI-1 in HK293 cells with that of either the ER or Golgi markers Con A and WGA (17), respectively. The data show that SKI-1 exhibits (*i*) perinuclear staining, colocalizing with Con A fluorescence, presumably corresponding to the ER (not shown); (*ii*) paranuclear staining colocalizing with WGA fluorescence, suggesting the presence of SKI-1 in the Golgi (Fig. 5 *A* and *B*), and (*iii*) punctate staining observed in the cytoplasm and within extensions of a few cells (Fig. 5*A*). Some, but not all, of the punctate immunostaining matched that observed with WGA. This suggests that SKI-1 localizes in the Golgi but may sort to other organelles, including lysosomal and/or endosomal compartments. An indication of lysosomal/endosomal localization was provided by the analysis of SKI-1 immunofluorescence within cells preincubated for 4 h with 10 mM leucine methyl ester, a specific lysosomal/endosomal protease inhibitor (25). The results showed a net increase in the proportion of cells exhibiting punctate staining as compared with control cells (Fig. 5 *A* and *B*). The relative proportions of SKI-1 in cellular organelles and their dependence on culture conditions are now amenable to evaluation by subcellular fractionation and electron microscopy.

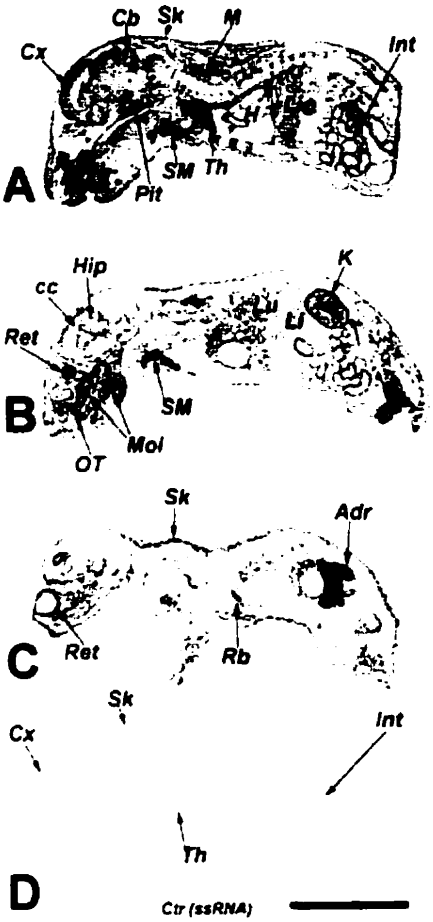

FIG. 3. *In situ* hybridization of rSKI-1 mRNA in a 2-day-old rat. *In situ* hybridization is shown at anatomical resolution on x-ray film using an ^{35}S-labeled antisense riboprobe (*A–C*) and sense control riboprobe (*D*). *Adr*, adrenal gland; *Cb*, cerebellum; *cc*, corpus callosum; *Cx*, cerebral cortex; *H*, heart; *Int*, intestine; *K*, kidney; *Li*, liver; *Lu*, lungs; *M*, muscles; *Mol*, molars; *OT*, olfactory turbinates; *Pit*, pituitary gland; *Rb*, ribs; *Ret*, retina; *Sk*, skin, *SM*, submaxillary gland; *Th*, thymus. [×4; bar (*D*) = 1 cm.]

Enzymatic Activity and Cleavage Specificity of SKI-1. To prove that SKI-1 is a proteolytic enzyme we examined its ability to cleave five different potential precursor substrates, including pro-opiomelanocortin, pro-atrial natriuretic factor, HIV gp160, pro-nerve growth factor, and proBDNF. Cellular coexpression of vv:SKI-1 with the vaccinia virus recombinants of each of the above precursors revealed that only proBDNF was cleaved intracellularly by SKI-1. Thus, upon expression of vv:BDNF alone in COS-7 cells we observed a partial processing of proBDNF (32 kDa) into the known, major 14-kDa BDNF product (15) and the minor production of a previously observed (ref. 16; S.J.M., N.G.S., and R.A.M., unpublished results) but still undefined 28-kDa product (Fig. 6*A*). Upon coexpression of proBDNF and SKI-1, a net increase in the level of the secreted 28-kDa BDNF is evident, without significant alteration in the amount of 14-kDa BDNF (Fig. 6*A*). To examine whether the 28-kDa product results from cleavage at a basic residue or at an alternative site, we first coexpressed proBDNF, SKI-1, and either α1-PIT or α1-PDX, which are inhibitors of thrombin and PC cleavages, respectively (18, 26). The results show that different from α1-PIT, the serpin α1-PDX selectively blocks the production of the 14-kDa BDNF and that neither α1-PIT nor α1-PDX affects the level of the 28-kDa product. This finding shows that α1-PDX effectively inhibits the endogenous furin-like enzyme(s) responsible for the production of the 14-kDa BDNF (15), but does not inhibit the ability of SKI-1 to generate the 28-kDa product. Thus, it is likely that the generation of the 28-kDa BDNF takes place via an alternate cleavage. Incubation of the cells with brefeldin A or the Ca^{2+} ionophore A23187 revealed that the 28-kDa proBDNF is

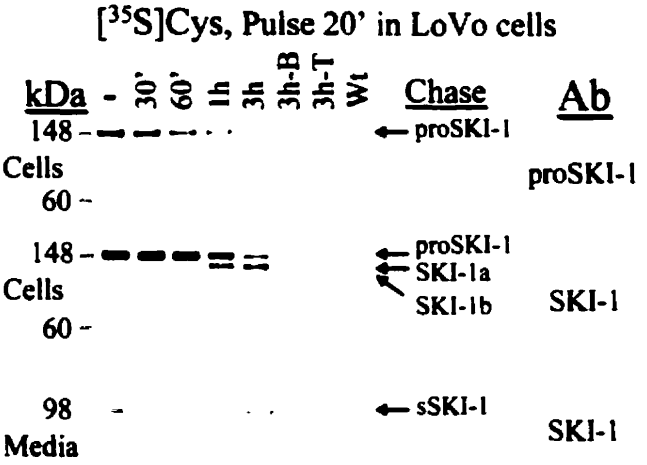

FIG. 4. Biosynthetic analysis of SKI-1 in LoVo-C5 cells overexpressing vv:SKI-1. Cells were pulsed for 20 min with [^{35}S]cysteine and chased for 30 min, 1 h, and 3 h in the absence or presence of either brefeldin A (3h-B) or tunicamycin (3h-T). The control represents the 3-h chase period for cells infected with the wild-type virus (Wt). Media and cell lysates were immunoprecipitated with either a SKI-1 antiserum (Ab: SKI-1; against amino acids 634–651) or a proSKI-1 antiserum. The arrows point to the 148-, 120-, 106-, and 98-kDa forms immunoprecipitated.

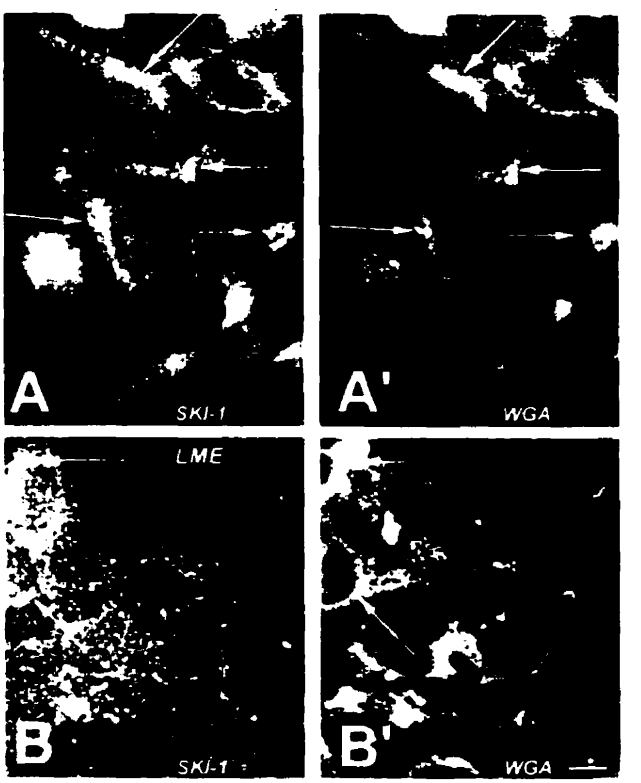

FIG. 5. hSKI-1 immunoreactivity in stably transfected HK293 cells. Black and white representation of the comparative double (red and green) fluorescence staining using an SKI-1 antiserum (directed against amino acids 634–651) (*A* and *B*) and fluorescein isothiocyanate-labeled WGA (*A'* and *B'*) in control (*A* and *A'*) and leucine methyl ester (LME)-treated (*B* and *B'*) cells. Thin arrows emphasize the observed punctate staining, which is enhanced in the presence of LME. Large arrows point to the coincident staining of SKI-1 and WGA. [×900; bar (*B'*) = 10 μm.]

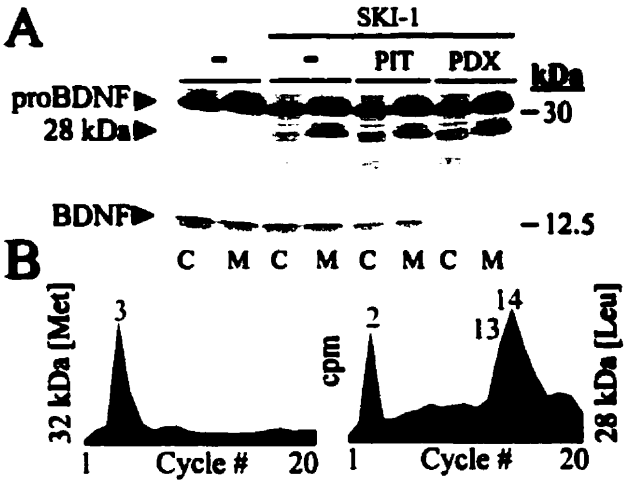

FIG. 6. Processing of proBDNF by SKI-1. (*A*) COS-7 cells were infected with vv:BDNF and either vv:WT (−) or vv:SKI-1 in the presence of either vv:PIT or vv:PDX. The cells were labeled metabolically with [^{35}S]cysteine/[^{35}S]methionine for 4 h, and the media (M) and cell lysates (C) were immunoprecipitated with a BDNF antiserum before SDS/PAGE analysis. The autoradiogram shows the migration positions of proBDNF (32 kDa), the 28-kDa BDNF produced by SKI-1, and the 14-kDa BDNF. (*B*) Microsequence analysis of the [^{35}S]Met-labeled 32-kDa proBDNF (maximal scale, 1,000 cpm) and [^{3}H]Leu-labeled 28-kDa BDNF (maximal scale, 250 cpm).

formed in the ER and that this cleavage is Ca^{2+}-dependent (not shown).

In Fig. 6*B*, we present the N-terminal microsequence analysis of [^{35}S]Met-labeled 32-kDa proBDNF and [^{3}H]Leu-labeled 28-kDa BDNF. The sequence of the 32-kDa form revealed the presence of an [^{35}S]Met at position 3 (Fig. 6*B*), which is in agreement with the proposed sequence of human proBDNF (27) resulting from the removal of an 18-aa signal peptide cleaved at GCMLA^{18} ↓ APMK site. The N-terminal sequence of the 28-kDa product revealed a [^{3}H]Leu at positions 2, 13, and 14 (Fig. 6*B*). This result demonstrates that the 28-kDa BDNF is generated by a unique cleavage at Thr^{57} in the sequence RGLT^{57} ↓ SLADTFEHVIEELL (27).

To prove that SKI-1 is directly responsible for the production of the 28-kDa BDNF at the novel Thr-directed cleavage, proBDNF was incubated at various pH values with concentrated medium of vv:SKI-1-infected Schwann cells. A similar preparation obtained from wild-type vaccinia virus-infected cells served as control. The data show that SKI-1 exhibits a wide pH-dependence profile revealing activity at both acidic and neutral pH values from pH 5.5 to 7.3 (Fig. 7*A*). Analysis

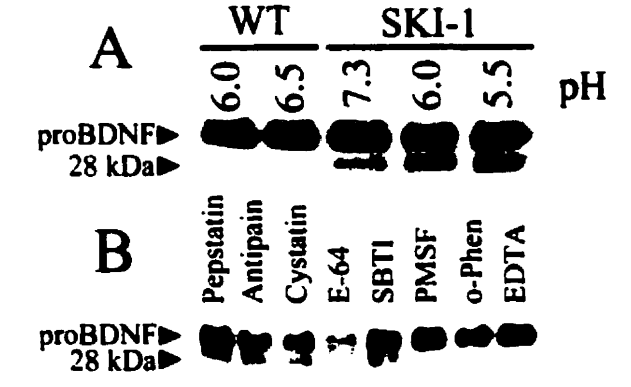

FIG. 7. *In vitro* processing profile of proBDNF by SKI-1. (*A*) pH dependence of the processing of proBDNF by SKI-1. (*B*) Inhibitor profile of the processing of proBDNF to the 28-kDa BDNF by the same SKI-1 preparation as in *A*. The reaction was performed overnight at 37°C, pH 6.0.

of the inhibitory profile of this reaction revealed that metal chelators such as EDTA and *o*-phenanthroline or a mixture of the serine proteinase inhibitors PMSF and pAPMSF effectively inhibits the processing of proBDNF by SKI-1. The inhibition by EDTA is expected because, like all PCs, SKI-1 is a Ca^{2+}-dependent enzyme. The unexpected inhibition by 5 mM *o*-phenanthroline may be a result of excess reagent because at 1 mM only 25% inhibition is observed (not shown). All other class-specific proteinase inhibitors (aspartyl-, cysteinyl-, and serine proteases- of the trypsin type) proved to be inactive.

DISCUSSION

This work provides evidence for the existence of a mammalian secretory Ca^{2+}-dependent serine proteinase of the subtilisin/kexin type that selectively cleaves at nonbasic residues. Thus, SKI-1 processes the 32-kDa human proBDNF at an KAGSRGLT ↓ SL sequence, generating a 28-kDa form, which may have its own biological activity (S.J.M., N.G.S., and R.A.M., unpublished results). Such a cleavage site is close to the consensus site deduced from a large body of work done with the PCs, whereby an (R/K)-(X)$_n$-R ↓ X-(L/I/V), (where n = 0, 2, 4, or 6) motif is favored by most PCs (1–3, 28). Note that in the SKI-1 site, P1 Arg is replaced by Thr and an aliphatic Leu is present at P2′, an amino acid also favored by PCs (1–3, 28). Several proteins are known to be cleaved after Thr. These include human antiangiogenic platelet factor 4 (ref. 6; QCLCVKTT ↓ SQ), the neuroendocrine α-endorphin (ref. 4; KSQTPLVT ↓ LF), and ADAM-10 metalloprotease (ref. 7; LLRKKRTT ↓ SA).

Interestingly, comparison of the phylogenetically highly conserved sequence of proBDNF revealed an insertion of hydroxylated amino acids (threonine and serine) just after the identified SKI-1 cleavage site of human proBDNF. Thus, in rat and mouse proBDNF, two threonines are inserted (RGLTTT—SL), and in porcine proBDNF, five serines are added (RGLTSSSSS—SL) (27). These observations raise a number of questions: (*i*) Do these insertions affect the kinetics of proBDNF cleavage by SKI-1? In that context, it was published recently that rat proBDNF is also cleaved into a 28-kDa protein (29). (*ii*) Does SKI-1 recognize both single and pairs of Thr and Ser and combinations thereof? (*iii*) Is the presence of a basic residue at P4, P6, or P8 critical for cleavage?

Another question that arises is whether SKI-1 can cleave at residues other than Thr. In that context, after submission of this manuscript, Sakai *et al.* demonstrated that the sequence of hamster S1p responsible for the site 1 cleavage of SREBPs is almost identical to the presently reported human, mouse, and rat SKI-1 (30). In this model, within the lumen of the ER, S1p cleaves SREBP-2 at an RSVL ↓ SF sequence, where Arg at P4 is very critical, whereas the P1 Leu could be replaced by a number of other amino acids (8). Our *in vitro* data show that sSKI-1 does not cleave small fluorogenic substrates of sequence RGLT-MCA, RGLTTT-MCA, or RSVL-MCA (MCA is 4-methylcoumaryl-1-amide), suggesting that it has an extended substrate-specificity pocket. In agreement, preliminary data show that SKI-1 specifically cleaves at neutral pH a 27-mer synthetic peptide of sequence GGAHDSDQHPHSGSGRSVL ↓ SFESGSGG, representing the luminal amino acids 504–530 of human SREBP-2 (8) (B.B.T. and N.G.S., unpublished data). We have shown that this synthetic peptide is efficiently processed by SKI-1 *in vitro*, paving the way for refined kinetic analyses.

Biosynthetic analysis of the zymogen processing of proSKI-1 demonstrated an ER-associated removal of the prosegment (Fig. 4). Furthermore, analysis of the $^{35}SO_4$-labeled SKI-1 demonstrated the presence of only sulfated 106- and 98-kDa forms but not that of either the 148 proSKI-1 or the 120-kDa SKI-1a forms (not shown). Because sulfation occurs in the trans-Golgi network, this confirms that the removal of the prosegment occurs in the ER. As with furin and PC5-B (1–3,

24) the membrane-bound 106-kDa SKI-1 is transformed into a soluble 98-kDa form. The secreted 98-kDa sSKI-1 is enzymatically active because it processes proBDNF *in vitro* (Fig. 7). Attempts to sequence the SDS/PAGE-purified [^{3}H]Leu- and Val-labeled 148- and 98-kDa forms resulted in ambiguous results, suggesting that SKI-1 is refractory to N-terminal Edman degradation. Presently, we are unable to define the zymogen cleavage site leading to the formation of the 120-kDa SKI-1a and 106-kDa SKI-1b deduced by pulse–chase studies (Fig. 4). Examination of the prosegment sequence (Fig. 1), the species-specific proBDNF motif potentially recognized by SKI-1, the sequence of the luminal portion of SREBP-2 (see above), and the alignment of SKI-1 with other subtilases (22) suggests three possible conserved sites: **R**ASL^{167} ↓ SLGS, **R**HSS^{182} ↓ RRLL, and **R**RL**L**^{186} ↓ RAIP. These predict cleavages at motifs containing a P4 Arg and a P1 either Leu or Ser.

Phylogenetic structural analysis of the predicted amino acid sequence of SKI-1 reveals that this serine proteinase is closer to plant and bacterial subtilases than it is to yeast and mammalian PCs. The 100% conservation of the catalytic domain sequence, although striking and suggestive of an important function, is not far from the 98% similarity between human and rat PC7 (3, 21). The sequence C-terminal to the catalytic domain of SKI-1 is very different from that of any of the known PCs. In fact, although PCs have a typical P-domain critical for the folding of these enzymes (for reviews see refs. 1–3), we did not find the hallmark sequences (3, 31) of the P-domain within the SKI-1 structure. Instead, different from the PCs, we find a conserved growth factor/cytokine receptor motif of which functional importance will need to be addressed, especially because this motif is partly missing in alternatively spliced forms (Fig. 1). Finally, the highly basic nature of the cytosolic tail of SKI-1 (Fig. 1) may be critical for its probable cellular localization within endosomal/lysosomal compartments (Fig. 5), similar to the importance of basic residues for the accumulation of the α-amidation enzyme PAM in endosomal compartments (S. L. Milgram, personal communication).

The wide tissue distribution of SKI-1 mRNA transcripts suggests that this enzyme processes numerous precursors in various tissues. Furthermore, the observed developmental down-regulation of the level of its transcripts also suggests a functional importance during embryonic development. That SKI-1 can cleave C-terminal to Thr, Leu, and, possibly, Ser residues suggests that, like the combination of PCs and carboxypeptidases E and D (32), a specific carboxypeptidase also may be required to trim out the newly exposed C-terminal hydroxylated or Leu residues.

SKI-1 is closest to the pyrolysin branch of the six-membered family of subtilisin-like proteinases (22) and we believe is the first known mammalian subtilase cleaving at sites other than basic amino acids. That other eukaryotic subtilases exist is supported by a recent report on the structure of a soluble subtilisin-like enzyme called PfSUB-1 found in *Plasmodium falciparum* (33) and exhibiting a 29% sequence identity to SKI-1. This enzyme, which is closest to the subtilisin branch of subtilases (22), localizes to granular-like compartments and presumably cleaves at a Leu ↓ Asn bond (33). Therefore, because only mammalian members of the kexin (PCs) and pyrolysin (SKI-1) subfamilies have been identified, could it be that the other four subtilase subfamilies (22) have their mammalian counterparts?

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