Structure-function analysis of the prosegment and the cysteine-rich domain of the proprotein convertase PC5A: its interaction with TIMP-2

By

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Abstract

The mammalian proprotein convertases (PCs) of the secretory pathway are calcium-dependent serine proteinases related to bacterial subtilisin and yeast Kexin. As Kexin and subtilisin, all basic aa-specific convertases contain a comparable N-terminal structure starting with a signal peptide followed by a prosegment and a conserved catalytic domain. The convertases possess additional domains at their C-terminus following the catalytic domain. All PCs exhibit a P-domain prior to the C-terminal architecture, which is specific to each convertase. Three of the basic-aa-specific convertases, furin, PACE4 and PC5 display the presence of a Cysteine-rich domain (CRD). The function of the CRD has not been studied extensively and remains unclear whereas the inhibitory function of the prosegments has been investigated previously.

In this manuscript, both the function of the prosegment and the CRD of PC5A were investigated as very little was known about the zymogen activation of PC5A, the inhibitory role of its prosegment and the characterisation of the function of its CRD. Previous subtilisin- and furin-based studies demonstrated that the prosegment acts as an intramolecular chaperone and as a potent inhibitor of its cognate enzyme. From these results, the inhibitory function of the prosegment of PC5A was investigated. The data revealed that the prosegment of PC5 is a potent inhibitor *in vitro* and *ex vivo* of its parent enzyme, but is not

selective. Introduction of single point mutations within the prosegment did not increase the potency or the selectivity of the inhibitor. We next characterised the relevance of the CRD of PC5A as it implication in the cell surface localisation of the enzyme. The plasma membrane retention was demonstrated to be via the interaction of the CRD of PC5A with TIMP-2, an endogenous MMP inhibitor. This study also showed that PC5A may have an anti-proliferative role as its overexpression diminishes cell proliferation while its inactivation by siRNA causes an increase in the proliferation rate.

This study on the structure-function analysis of both the prosegment and the cysteine-rich domain of PC5A will aid us to define the biological function of this enzyme.

Sommaire

Les convertases des proprotéines qui résident dans la voie de sécrétion sont des serine protéases de mammifères, apparentées par leur structure aux subtilisines bactériennes et à l' enzyme de la levure kexine. Comme ceux-ci, les convertases possèdent un peptide signal, un segment pro et une région catalytique conservée. De plus, ces protéases possèdent deux domaines supplémentaires, appelés domaines P et C-terminal qui sont absents chez les subtilisines mais présents chez la kexine. Le domaine C-terminal est unique à chaque convertase. Trois membres de la famille des convertases, furine, PACE4 et PC5 démontrent la présence d' un domaine riche en cysteine (CRD). La fonction de ce domaine ne fut jamais sérieusement étudiée et demeure inconnue, toutefois, l' activité inhibitrice du segment pro de la subtilisine ainsi que de certaines convertases furent étudiées extensivement auparavant.

Dans ce mémoire, la fonction du segment pro ainsi que du domaine riche en cysteine de PC5A furent étudiées. Des études précédentes sur la subtilisine et la furine ont demontrées que le segment pro agit à la fois de chaperone et d' inhibiteur de l' enzyme parental. Inspirée de ces études, la fonction inhibitrice du segment pro de PC5 fut investiguée et révéla que ce domaine est un inhibiteur puissant de PC5 *in vitro* et *ex vivo*, cependant l' inhibition n' est pas spécifique à une seule convertase. De plus, des mutations ponctuelles

introduites dans la région pro n' ont augmenté ni la puissance, ni la spécificité de l' inhibiteur. Aucune fonction du CRD n' avait été définie chez les PCs. Ce domaine joue un rôle important dans la localisation de l' enzyme puisqu' il permet à PC5A d' être retenu à la surface cellulaire. Sans ce domain riche en cysteine, PC5A n' est plus retrouvée à la surface car c' est au niveau de ce domaine que PC5A interragit avec TIMP-2 qui se trouve à la surface cellulaire. De plus, la surexpression de PC5A dans des cellules tumorales cause une diminution de la proliferation, par contre, les cellules traitées avec un siRNA contre PC5 prolifèrent d' avantage. Cette recherche sur la structure-fonction du segment pro et du domaine riche en cysteine de PC5A va aider à définir la fonction biologique de cet enzyme en plus de définir le role de ces deux domaines chez les autres convertases.

Preface

This thesis is submitted to the department of Graduate and Postdoctoral studies, McGill University. There are two types of thesis formats accepted for submission. The first option is the traditional thesis format and as an alternative, the second format consists of a dissertation based on a collection of submitted publications. This present dissertation follows the second format consisting of a manuscript-based thesis conforming to the specifications of the faculty of Graduate and Postdoctoral studies.

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.

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 preceeding and following each manuscript are mandatory.

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts: a table of contents; a brief abstract in

both 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; a thorough bibliography; Appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.

As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

When previously published copyright material is presented in a thesis, the candidate must include signed waivers from the publishers and submit these to the Graduate and Postdoctoral Studies Office with the final deposition, if not submitted previously. The candidate must also include signed waivers from any co-authors of unpublished manuscripts.

This thesis is manuscript-based on two publications corresponding to chapter C and D respectively of this dissertation. The first manuscript in chapter C is entitled " Structure-Function Analysis of the Prosegment of the Proprotein Convertase PC5A" , produced by Nadia Nour, Ajoy Basak, Michel Chretien and Nabil G. Seidah, published in the Journal of Biological Chemistry, 2003; 278: 2886-2895. Chapter D corresponds to the second publication entitled " TIMP-2 Mediated Cell Surface Localisation of the Convertase PC5A: Implication in Cancer" , by Nadia Nour, John S. Mort, Christopher M. Overall, Majambu Mbikay and Nabil G. Seidah (submitted for publication).

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Dr. Nabil G. Seidah, Director of the Laboratory of Biochemical Neuroendocrinology, thesis supervisor.

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List of abbreviations

aa amino acids

ACTH adrenocorticotropin hormone

α 1-PDX α 1-antitrypsin Portland

α 2m α 2-macroglobulin

BDNF brain-derived neurotrophic factor

BFA brefeldin A

BMP bone morphogenic protein

BSA bovine serum albumin

BPN Bacillus protease N'

BTMD before transmembrane domain

Cb cerebellum

CCK Cholecystokinin

ConA concavalin A

CRD cysteine-rich domain

Ct cytoplasmic tail

CTL control

DNA deoxyribonucleic acid

ECM extracellular matrix

EDTA ethylenediaminetetracetic acid

EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
EGTA	ethyleneglycotetraacetic acid
EPA	erythroid-potentiating activity
ER	endoplasmic reticulum
ERBB4	erythroblastic leukemia viral oncogene homolog 4
FASL	Fas ligand
FBS	fetal bovine serum
FD11	furin deficient CHO cells
GHRH	growth hormone releasing hormone
GLP	glucagons-like peptide
GPI	glycosylphosphatidylinositol
GRPP	glicentin-related pancreatic peptide
h	human
Hip	hippocampus
HRP	horseradish peroxidase
IgG	immunoglobulin
In	intestine
IC50	inhibitory concentration 50%
ISG	immature secretory granule

KCl	potassium chloride
kDa	kilo Dalton
Ki	inhibitory constant
LP	lysosomal protein LAMP1
LPC	Lymphoma proprotein convertase
LPH	lipoprotein hormone
Lu	lung
m	mouse
M	molar, pM, nM, µ M, mM
MCH	melanin-concentrating hormone
MIS	mullerian inhibiting substance
MMP	matrix-metalloproteinase
MPGF	major proglucagon fragment
MS	mass spectrometry
MSH	melanotropin
MT-MMP	membrane-type matrix metalloproteinase
NARC-1	neural apoptosis-regulated convertase 1
NGF	nerve growth factor
P	position N-terminus to cleavage site
P’	position C-terminus to cleavage site

PACE	paired basic aa cleaving enzyme
PC	proprotein convertase
PC5-Δ C	C-terminally truncated PC5
PC5-CT	C-terminus of PC5A
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PNGaseF	peptide N Glycosidase F
POMC	proopiomelanocortin
pPC7	prosegment of PC7
ppPC7	preprosegment of PC7
r	rat
RNA	ribonucleic acid
s	soluble
SDS	sodium dodecyl sulfate
SG	secretory granule
SKI-1	subtilisin-kexin isozyme
TGF-β	transforming-growth factor β
TGN	Trans Golgi Network
Tha	thalamus
TIMP	Tissue inhibitor of matrix-metalloproteinase

TMD	transmembrane domain
TNFR1	tumour necrosis factor receptor 1
To	tooth
TRH	thyrotropin releasing hormone
VEGF	vascular endothelial growth factor
Vn	vitronectin-like insert
vv	vaccinia virus
vWf	von Willerbrand factor
WT	wild type
Y	yeast
YXXØ	tyrosine-aa-aa-hydrophobic aa
Zn	zinc

Introduction

Since the discovery of the mammalian proprotein convertases, many studies have been performed to elucidate the biochemical and molecular functions of these enzymes. It is well accepted that the PCs are enzymes of the secretory pathway that process inactive precursors into biologically active polypeptides. Presently, the best understood PCs are mainly furin, PC1 and PC2. Some investigations were performed on PC5, but little was known about its

biological function and its structural properties. Based on previous studies on the prosegment of furin, PC7 and bacterial subtilisins, it was demonstrated that the prosegment can act as a chaperone as well as an inhibitor of its parental enzyme. Thus, it was of interest to verify if the prosegment of PC5 also behaves as a potent inhibitor of its parental enzyme *in vitro* and *ex vivo* in addition to being its chaperone. Moreover, within the prosegment of furin, a second internal cleavage site has been identified and shown to be required for maximal activity. In this study, we examine the importance of the secondary cleavage site within the prosegment of PC5A for its optimum proteolytic activity on the proVEGF-C substrate.

The structure of the C-terminal domain is very specific to each convertase. Only furin, PACE4 and PC5 exhibit a cysteine-rich domain of unknown function at their C-terminus. In order to investigate the functional importance of the CRD within PC5A, mutants were generated to examine its potential role in subcellular localisation of the enzyme and in protein-protein interactions. These were achieved through cell immunofluorescence and co-immunoprecipitations analysis. Furthermore, the CRD of any convertase has never been expressed as an independent domain in mammalian cells thus, in this work, the expression of the CRD of PC5A, PACE4 and furin was possible. Finally, we had insights that the expression of PC5A may be downregulated in tumours and that it had an

antiproliferative role. Thus, we examined if the overexpression of PC5A and its downregulation using a siRNA in cells would affect the cellular proliferation rate. These investigations demonstrate that elucidating the functional importance of specific domains within PC5A will enhance our understanding of the biological function of this convertase.

CHAPTER A

Proprotein Convertases

A.1 Identification of the proprotein convertases

The understanding over the past years of post-translational processing of numerous secretory proteins to generate biologically active moieties has greatly evolved. Processing of precursors following the removal of the signal peptide often takes place intracellularly, at the plasma membrane or in the extracellular space. Once insulin and γ-LPH were sequenced, it was obvious that these hormones resulted from a larger precursor proinsulin and β-LPH (POMC). When proinsulin and β-LPH were sequenced in turn, it seemed that the final products (insulin, γ-LPH and β-MSH) were derived from excision of their precursors via cleavage by an endoprotease specific for hydrolyzing the peptide bond on the carboxyl side of motifs consisting of a pair of basic residues (K-R↓ or R-R↓). By the late 1960s, biochemical analysis of insulin (Steiner et al., 1967) and γ-LPH (Chretien and Li, 1967) suggested that a trypsin-like protease(s) could be implicated in this process, but the limited molecular tools then available in mammalian systems prevented its identification.

Two decades later, the mammalian proteases involved in these processes were characterised and shown to belong to the family of the proprotein convertases (PCs). These enzymes are calcium-dependent serine proteinases that are related to bacterial subtilisin and yeast Kexin. The discovery of the mammalian PCs came from the initial characterization of the proteinase Kexin, which cleaves the yeast pro-α factor and pro-killer toxin (Fuller et al., 1988). A

database search revealed that Kexin shared a high degree of homology with the protein product of the c-fes/fps oncogene named furin (Roebroek et al., 1986c), thus it was the first mammalian family member to be identified. The initial discovery of furin led to the characterisation of other PCs over the last decade using PCR strategies. Currently, there are 7 known basic amino acid (aa)-specific PC family members that cleave various secretory precursors after basic residues: furin/PACE, PC1/3 (Seidah et al., 1990;Seidah et al., 1991b), PC2 (Smeekens and Steiner, 1990;Seidah et al., 1990), PACE4 (Kiefer et al., 1991b), PC4 (Nakayama et al., 1992a;Seidah et al., 1992b), PC5/6 (Lusson et al., 1993;Nakagawa et al., 1993a) and PC7/LPC (Seidah et al., 1996;Meerabux et al., 1996a;Bruzzaniti et al., 1996b) (see Figure A-1).

Recently, two other non-basic specific convertases implicated in cholesterol metabolism have been identified, namely SKI-1/S1P (Espenshade et al., 1999;Elagoz et al., 2002) and NARC-1 (Abifadel et al., 2003;Seidah et al., 2003) (see Figure A-1). The PCs cleave precursors at both single and pairs of basic residues usually post the consensus sequence [R/K]-Xn-[R/K] and n=0, 2, or 4 while SKI-1 mainly processes proteins exhibiting the consensus motif [R/K]-X-[hydrophobic]-Z↓, where Z is variable (Pullikotil et al., 2004). Since the breakthrough in PC identification, a significant amount is now known about the biochemistry and cell biology of this class of protease. Moreover, with the

elucidation of the crystal structures of both Kexin (Holyoak et al., 2003a) and furin (Henrich et al., 2003b), the structural basis behind the specificity of these proteases will be further understood.

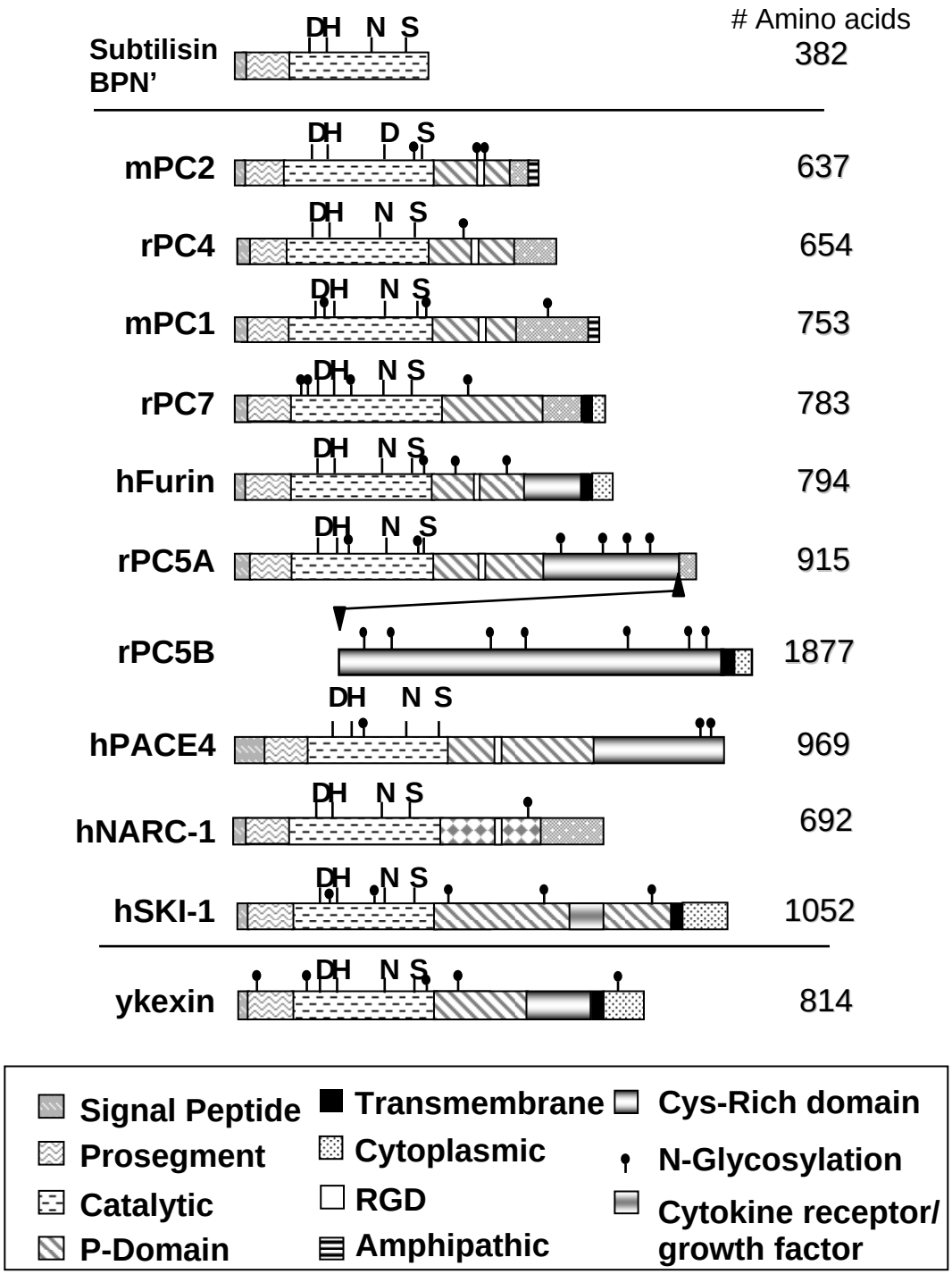


Figure A-1: Schematic representation of the mammalian proprotein convertase family members and their homologues yeast kexin and bacterial subtilisin.

Over the past years, the chromosomal assignment of the PC genes in humans was achieved (see Table A-1). The study demonstrated that both the furin and PACE4 genes are located on chromosome 15 within 5 centiMorgans (Roebroek et al., 1986a;Kiefer et al., 1991a). The other PC genes are randomly dispersed in the genome; the PC5 gene is located on chromosome 9 while the PC4 gene is found on chromosome 19 (Mbikay et al., 1995;Van de Loo et al., 1996) and the PC7 gene loci is found on chromosome 11 (Meerabux et al., 1996b). The mapping of the genes of the two convertases PC1 and PC2 that are sorted through the regulated secretory pathway revealed that the PC1 gene is localized on chromosome 5 whereas the PC2 gene is localized on chromosome 20 (Seidah et al., 1991a). The synteny between mice and humans for all chromosomal regions carrying PC loci suggests that their multiplication and divergence occurred before the branching apart of the human and murine species (Seidah et al., 1994). The newly characterized human SKI-1 and NARC-1 genes, which are involved in cholesterol homeostasis, have been mapped to human chromosome 16 (Nakajima et al., 2000) and 1 (Abifadel et al., 2003) respectively. The gene size of each PC is quite different and this variation in length results from differential gene splicing and different exon sizes and numbers. The organization of introns and exons is very similar at the N-terminal region of the various PCs while the C-terminal end segment has a variable

organization giving rise to the unique properties of each PC. The conservation of the specific domains throughout the family implies that all the PC genes evolved from a common ancestral gene (assumed to be PC7) through various mechanisms such as: duplications, translocations, insertions or deletions (Seidah et al., 1994).

Once the chromosomal assignments of the PC genes were obtained, mouse knockout strategies were undertaken for all PCs except PC5A, in order to define the specific function of each PC (see Table A-1). The PC1 knockout mice that survive suffered from chronic diarrhea and are half the size of WT animals (Zhu et al., 2002b), whereas the PC2 null phenotype resulted in retarded growth post partum, hypoglycaemia and endocrine peptide processing defects (Furuta et al., 1997). The PC4 null mice are viable and only display reduced fertility as well as a drastic reduction in litter size (Mbikay et al., 1997b). Functional inactivation of the furin gene in mice resulted in a lethal phenotype for the embryos at an early stage of development. This lethality was due to defects in ventral closure and failure to undergo axial rotation (Roebroek et al., 1998). Even though the PACE4 knockout phenotype resembles the phenotype of furin null mice, a quarter of the embryos are embryonic lethal and die at a later stage in development. The embryos, which are viable, show some heart and craniofacial defects (Constam and Robertson, 2000). PC7 seems to have a non critical

redundant function, as its inactivation in mice does not result in any detectable phenotype while the knockout of PC5B is reported to be lethal although details are not available yet. Preliminary data in our laboratory on the PC5A knockout seems to be embryonic lethal, but the reason has not been identified yet. Homozygous germ-line disruptions of SKI-1 were embryonically lethal (Yang et al., 2001) whereas the disruption of NARC-1 in mice is not yet determined but it is anticipated that the knockout of NARC-1 will also be lethal as it is involved in cholesterol metabolism.

Table A-1: Summary table of the chromosomal assignment of the human PC genes and of the phenotypes of the PC null mice models existing.

PC	Locus symbol	Human Chromosome	Mouse Chromosome	Mice null phenotype
Furin	PCSK3	15q26.1	7	Embryonic lethal Ventral closure defects Fail to undergo axial formation
PC1	PCSK1	5q15-q21	13	Dwarfism Chronic diarrhea Pre and postnatal lethality
PC2	PCSK2	20p11.2	2	Retarded growth Hypoglycaemia
PC4	PCSK4	19p13.3	10	Impaired fertility Smaller litter
PACE4	PCSK6	15q26	7	Some embryonic lethality

				Craniofacial and heart defects
PC5A PC5B	PCSK5 PCSK5	9q21.3 9q21.3	19 19	n.d Embryonic lethal
PC7	PCSK7	11q23.3	9	No reported phenotype
SKI-1	PCSK8	16q24	8	Embryonic lethal
NARC-1	PCSK9	1p32.3	4	n.d.

A.2 Tissue distribution and subcellular localisation

The basic amino acid specific PCs can be subdivided into 4 groups based on tissue distribution and intracellular localisation. The first class comprises furin and PC7, which have a widespread distribution and cleave proproteins that reach the cell surface via the constitutive secretory pathway. The second class includes the PCs that are sorted through the regulated secretory granules such as PC1, PC2 and PC5A. The third class encompasses PACE4 and PC5, which are expressed in both endocrine and non-endocrine cells and are plausibly cleaving precursors in both the constitutive and regulated secretory pathways. The final class comprises PC4, which has a distribution restricted to testicular and ovarian germ cells.

Table A-2: Tissue and cellular expression of the proprotein convertases.

Convertase	Tissue expression	Cellular localisation
Class I		
Furin	Ubiquitous	TGN/Endosomes/Cell surface
PC7	Widespread	TGN/Endosomes/Cell surface
Class II		
PC1	Neuroendocrine cells	TGN, secretory granules
PC2	Neuroendocrine cells	Secretory granules

Class III		
PC5A	Widespread	TGN, secretory granules
PC5B	Restricted, digestive system	TGN/Endosomes/Cell surface
PACE4	Widespread	TGN, secretory granules?
Class IV		
PC4	Testicular and ovarian germ cells	TGN?, Cell surface?

Furin is the first mammalian PC encountered and it is also the best understood enzyme of the family. The tissue distribution of furin shows its ubiquitous expression throughout the organism, however, it is found in greater amounts in the liver and kidney (Seidah et al., 1998). PC7 is widely expressed, but mostly detected in lymphoid-associated tissue such as the thymus, T-cells and the spleen (Bruzzaniti et al., 1996a;Seidah et al., 1998), suggesting a potential role of PC7 in host defence mechanisms. Furin (Molloy et al., 1999a) and PC7 (Munzer et al., 1997a) which are part of the first class of convertases are mainly localised within the TGN, which is a late Golgi structure responsible for the sorting of secretory proteins to their final destinations/compartments. Furin and PC7 can also cycle to the cell surface and integrate the endosomal compartments from the TGN (Molloy et al., 1999b) (see Figure A2). The roles of the proprotein convertases in diseases such as cancer have become an interesting topic of study over the last years and as novel targets in a cure for

some cancers. The mRNA levels of furin are usually very low in normal tissues whereas its expression is upregulated in several human cancers such as: breast cancer (Cheng et al., 1997), head and tumours (Bassi et al., 2001), lung cancers (Mbikay et al., 1997a) and glioblastoma (Leitlein et al., 2001). The relationship between the expression of PC7 and cancer has not been examined extensively. The only study that discuss the level of PC7 in cancer showed an increase in the mRNA level of PC7 in breast tumours as compared to the adjacent normal tissue (Cheng et al., 1997).

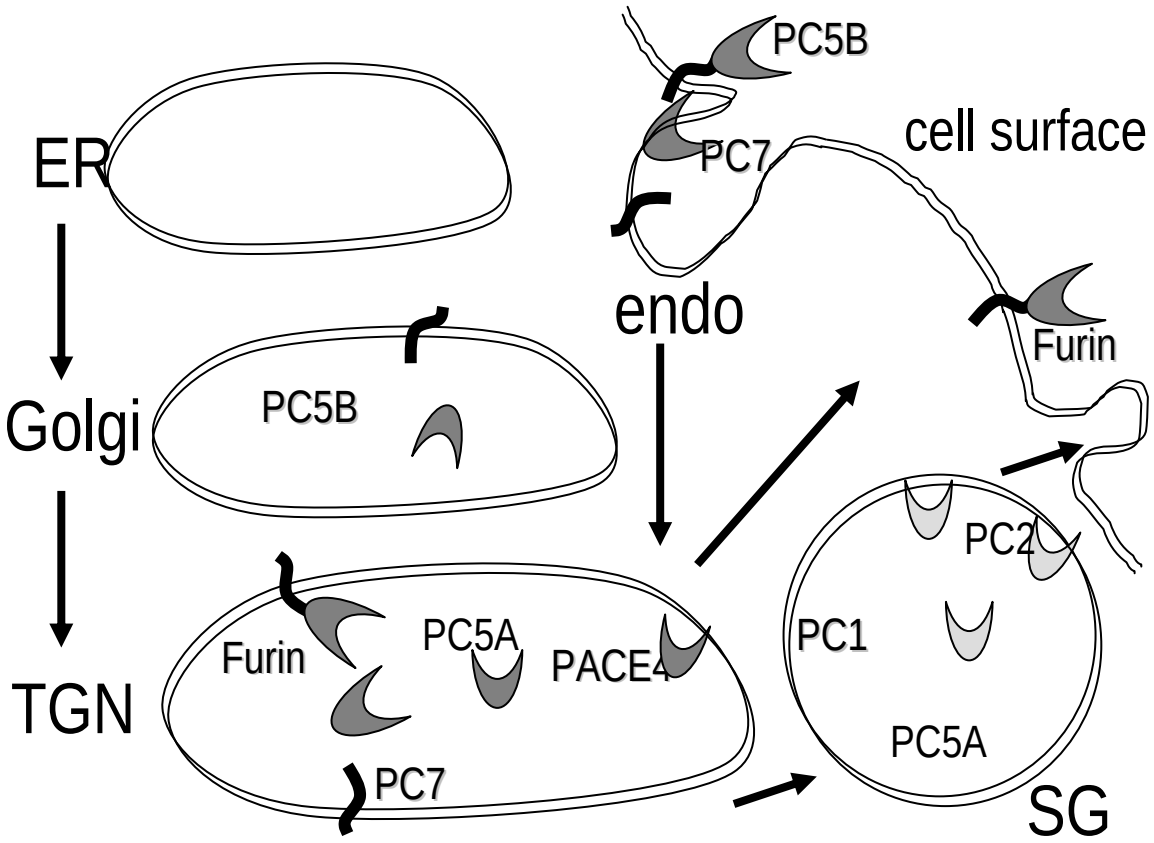


Figure A-2: Cellular localisation of the proprotein convertases

The neuroendocrine PCs that constitute class II, PC1 and PC2, show a restricted expression pattern indicating specialised functions within the tissues. They are mainly detected in neural and endocrine tissues (Seidah and Chretien, 1999). Moreover, while both PC1 and PC2 are expressed in the pituitary gland, they are detected in distinct areas: PC1 is abundant in the anterior lobe of the pituitary while PC2 is rich in the intermediate lobe (Marcinkiewicz et al., 1993b). The convertases entering the regulated secretory pathway and concentrating in secretory granules (SG) are PC1, PC2 and to a lesser extent PC5A (Malide et al., 1995) (see Figure A-2). Some researches suggested a link between high expression of PC1 and PC2 with neuroendocrine tumour development. Both mRNA levels of PC1 and PC2 were increased in pancreatic tumours (Marcinkiewicz et al., 1994) as well as in pituitary adenomas (Jin et al., 1999) and in gastrointestinal carcinoids (Tomita, 2001). On the other hand, a few types of cancer do not display an increase in expression of both PC1 and PC2. In breast cancer for example, the expression of PC1 is upregulated while PC2 mRNA is not detectable; in contrast, the PC2 mRNA level is augmented in lung cancers and not PC1 (Cheng et al., 1997;Mbikay et al., 1997a). The fact that both enzymes are not co-regulated in tumours confirms that each neuroendocrine PC displays a specific function.

The third class of PCs corresponds to enzymes that are expressed in both endocrine and non-endocrine cells and includes PACE4 and PC5. These enzymes exhibit a broad tissue distribution in the organism. PACE4 is widely expressed, but its richest sources are the anterior pituitary, heart, liver kidney, digestive track, the placenta and also in specific areas of the brain such as in the cerebellum (Seidah et al., 1994;Koide et al., 2003). PC5 is found in 2 different isoforms generated by alternative splicing, soluble PC5A and membrane-bound PC5B. PC5A is mainly expressed in the brain, adrenal gland, lung, intestine, kidney and in the ossification centers (Lusson et al., 1993) (see Figure A-3). In contrast, the distribution of PC5B seems to be more restricted as it is detected in the intestine and not in the brain, but it tissue expression has not been study extensively (Nakagawa et al., 1993b).

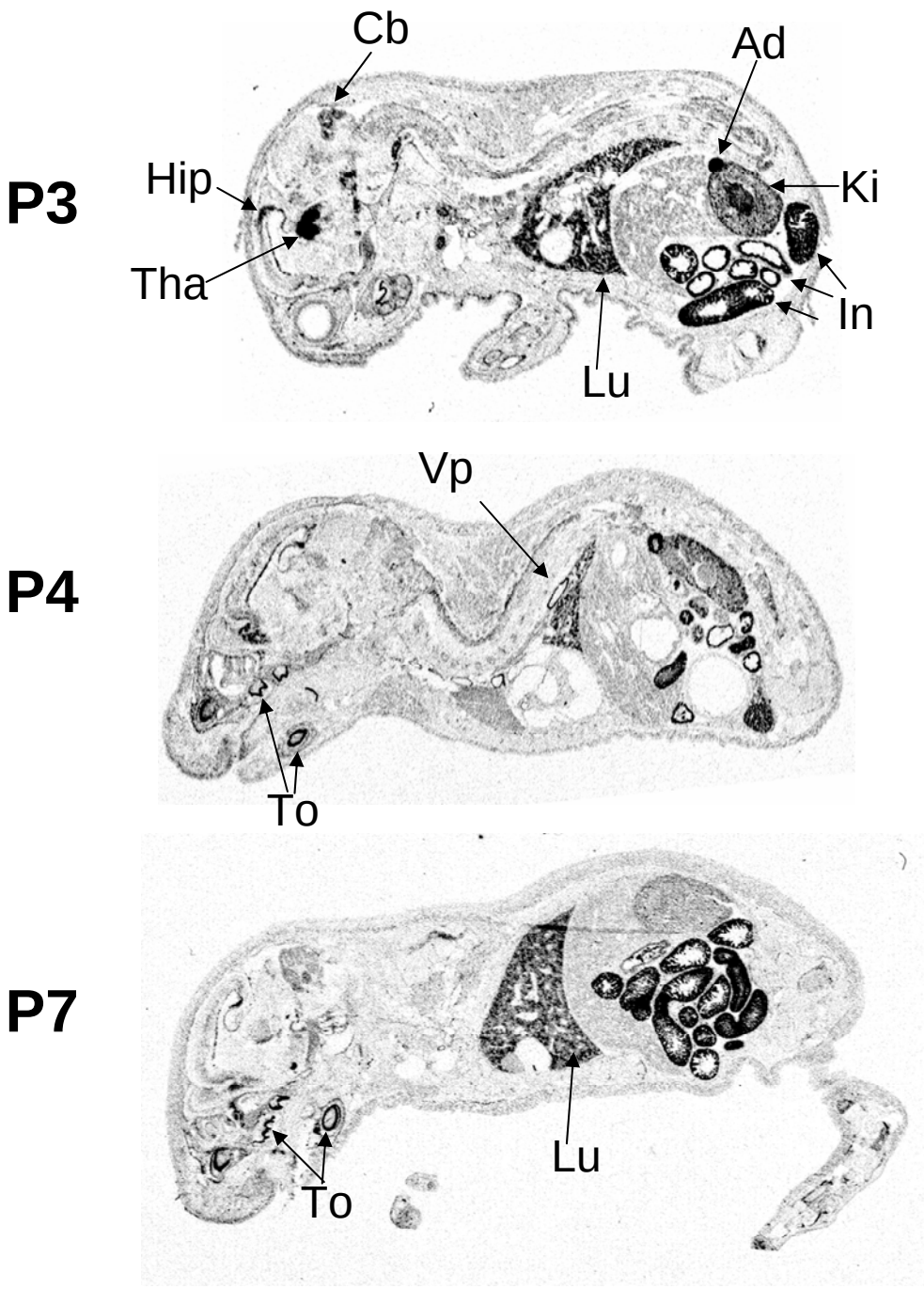


Figure A-3: In situ hybridisation of PC5. Ad, adrenal gland, Lu, lungs, To, tooth, In, intestine, Ki, kidneys, Tha, thalamus, Hip, hippocampus, Cb, cerebellum

PACE4 and PC5B are constitutive pathway convertases and concentrate in the TGN and in a late Golgi compartment respectively as depicted in Figure A-2. PC5A is quite unique compared to the other PCs since it is the only convertase that can be localised in the regulated SG as well as in the constitutive pathway more specifically in the TGN (see Figure A-2). PACE4 may also behave like PC5A, since it was shown to enter secretory granules of transfected AtT-20 cells (Mains et al., 1997). As for the other PCs, PACE4 expression was also shown to be increased in head and neck cancers, breast cancers, lung cancers, but decreased in ovarian cancer. Moreover, a simple overexpression of PACE4 in non-tumorigenic immortalized keratinocytes is sufficient to confer an invasive phenotype (Bassi et al., 2000;Mahloogi et al., 2002). This suggests that an uncontrolled increase in PACE4 expression may be sufficient to promote cancer. In addition, another study demonstrated that the expression of PACE4 is upregulated in association with the conversion of squamous cell carcinoma to a more advanced malignant spindle cell carcinoma (Hubbard et al., 1997). Conversely, PC5 has not been shown to be upregulated in any cancers. In breast cancer tissue, PC5 was not detected and it may even be downregulated in tumours as compared to the normal adjacent tissue. Further research using quantitative PCR will resolve this enigma.

The enzyme that displays the most restricted tissue expression is PC4 as this convertase is only expressed in germinal cells of testis. Moreover, PC4 is primarily expressed in round spermatids and was recently detected in macrophage-like cells of the ovary (Tadros et al., 2001). The subcellular localisation of PC4 and it expression in tumours versus normal tissue has not been studied yet. However, one study that demonstrate that PACAP can be cleaved by PC4 proposes that PC4 is localised in the Golgi compartment or in a later compartment (Li et al., 1998).

A.3 Structure of PCs

The structure of the 7 PCs can be compared against their yeast kexin and bacterial subtilisin BPN` homologues. It is apparent that various domains are conserved while others are specific to each PC family member (see Figure A-1). The N-terminal structure is similar for each PC. Their N-terminal organisation encompasses a signal peptide that is required for the entry of the enzyme into the endoplasmic reticulum (ER) and its subsequent trafficking within the secretory pathway. The signal peptide is followed by a prosegment that was shown for several PCs to behave as an intramolecular chaperone assisting in the proper folding of the zymogen as well as an inhibitor of the enzyme. (See Table A-3 for prosegment sequence identities) Next, PCs possess similar catalytic domains

where the catalytic reaction of the substrates occurs after basic residues. This domain is the most conserved region containing the catalytic triad of Asp, His and Ser and the oxyanion hole Asn. The only exception is PC2, which possesses an Asp instead of an Asn residue. Following the active site, every PC possesses a P-domain that stabilises the enzyme through hydrophobic interactions with the catalytic domain and appears to be necessary in the folding of the enzyme in the endoplasmic reticulum. Within this P-domain there is a conserved RGD motif of unknown function found in all basic amino acid specific PCs except PC7. The domains within the C-terminus region are specific to each proprotein convertase. PC1 and PC2 have a putative amphipathic alpha-helix present at their carboxyl terminus preceded by a stretch of charged amino acids that acts as a sorting signal that directs the proteases to the dense cored secretory granules (Jutras et al., 2000b). Some PCs including furin, PC7, and PC5-B are type-I membrane-bound enzymes due to the presence of a transmembrane domain at their C-terminus and their sorting to their target compartments is regulated by their cytosolic tails. (Jones et al., 1995;Xiang et al., 2000b) Finally, furin, PACE4, PC5-A and PC5-B exhibit a cysteine-rich domain (CRD) of unknown function at their C-terminus, but is not required for maximal proteolytic activity on small fluorogenic substrates (De, I et al., 1996). Even though all seven members share

conserved domains, each proprotein convertase exhibits a unique sequence suggesting divergent and shared functions.

Moreover, all convertases are N-glycosylated but at different levels and further research will determine if this is important for the proteolytic activity or more likely stability of the enzyme. While PC1 (Benjannet et al., 1993) and furin (Molloy et al., 1994) attain complex N-glycosylation, PC2 displays hybrid sugars even as a mature active enzyme (Benjannet et al., 1993). In addition to the N-glycosylation, the convertases undergo other post-translational modifications as do most of the proteins that traffic through the secretory pathway. The other types of post-translational modifications that convertases encounter will be discussed in more detail in section A.4 of this chapter.

The convertases are initially synthesised as zymogens, which signifies that the enzymes are proteolytically inactive, and are characterised by the presence of a prosegment at their N-terminus. The sequence of the prosegment is quite conserved among the PC family members except for PC7 (see Table A-3). The prosegment has two functions, it serves an auto-inhibitory role to prevent activation at the wrong compartment or time and acts as an intramolecular chaperone during protein synthesis and folding.

Table A-3: Sequence identities of the prosegment s of PCs.

Convertases	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%

Previous subtilisin-based studies revealed that the prodomain functions as an intramolecular chaperone that assists in the correct folding of the catalytic domain while acting like a competitive inhibitor of proteolytic activity (Fu et al., 2000). Based on furin studies, a model for the activation of the convertases was proposed, wherein the autoactivation is a multi-step process (Anderson et al., 1997). The first event takes place in the ER where the convertase undergoes autocatalytic processing of its prodomain at the primary cleavage site (junction of the prosegment and the catalytic site). The prodomain is presumed to remain associated with the enzyme forming a complex and thus acting as an inhibitor. When the complex reaches its target compartment with the adequate pH and calcium concentration, cleavage occurs within the prodomain at a secondary cleavage site, which causes the release of the prosegment and full activation of the enzyme as seen in Figure A-4 (Thomas, 2002). All PCs seems to follow the

same mechanism as furin except for PC2 and PC7. Of all the convertases, only PC7 and possibly PC4 do not possess a secondary cleavage site within its prosegment suggesting that its mechanism of activation is similar to the subtilisin, which requires only one cleavage (see Figure A-5). In contrast, PC2 was shown to undergo cleavage of its prosegment at the primary site not in the ER, but rather within the acidic environment of the TGN and immature secretory granules (ISG) (Benjannet et al., 1993).

PC2 is unique because its autoactivation is tightly linked to the expression of a specific binding partner that remains attached to the enzyme until it reaches the target compartment of zymogen activation. This binding protein is known as 7B2 and it is expressed mainly in endocrine and neural cells and is cellularly localized within secretory granules (Mbikay et al., 1989). This neuroendocrine polypeptide was first isolated from pituitary gland extracts (Hsi et al., 1982;Seidah et al., 1983). The presence of 7B2 is essential for the proper transport, folding and activation of PC2. Upon their entry into the ER, both proteins transiently interact together and are later cleaved by a furin-like protease during their transport to regulated secretory compartments (Braks and Martens, 1994;Benjannet et al., 1995b). 7B2 is a bifunctional molecule, it acts as a chaperone protein through its N-terminal domain while the C-terminal region is a specific inhibitor of PC2 (Braks and Martens, 1994;Zhu and Lindberg,

1995;Benjannet et al., 1995b). A model was proposed for the interaction of PC2 and 7B2 leading to the activation of PC2. In the ER, pro7B2 binds to proPC2 and then the complex exits the ER; when it reaches the TGN, a furin-like enzyme cleaves the C-terminal peptide from 7B2. This facilitates the conversion of proPC2 into mature active PC2 and the C-terminal peptide of 7B2 remains associated with PC2 acting as an inhibitor of the enzyme until it reaches a later compartment. Once in the optimal compartment/environment, the inhibition of PC2 is released by cleavage within the C-terminal domain of 7B2 by PC2 (Zhu et al., 1996;Bergeron et al., 2000).

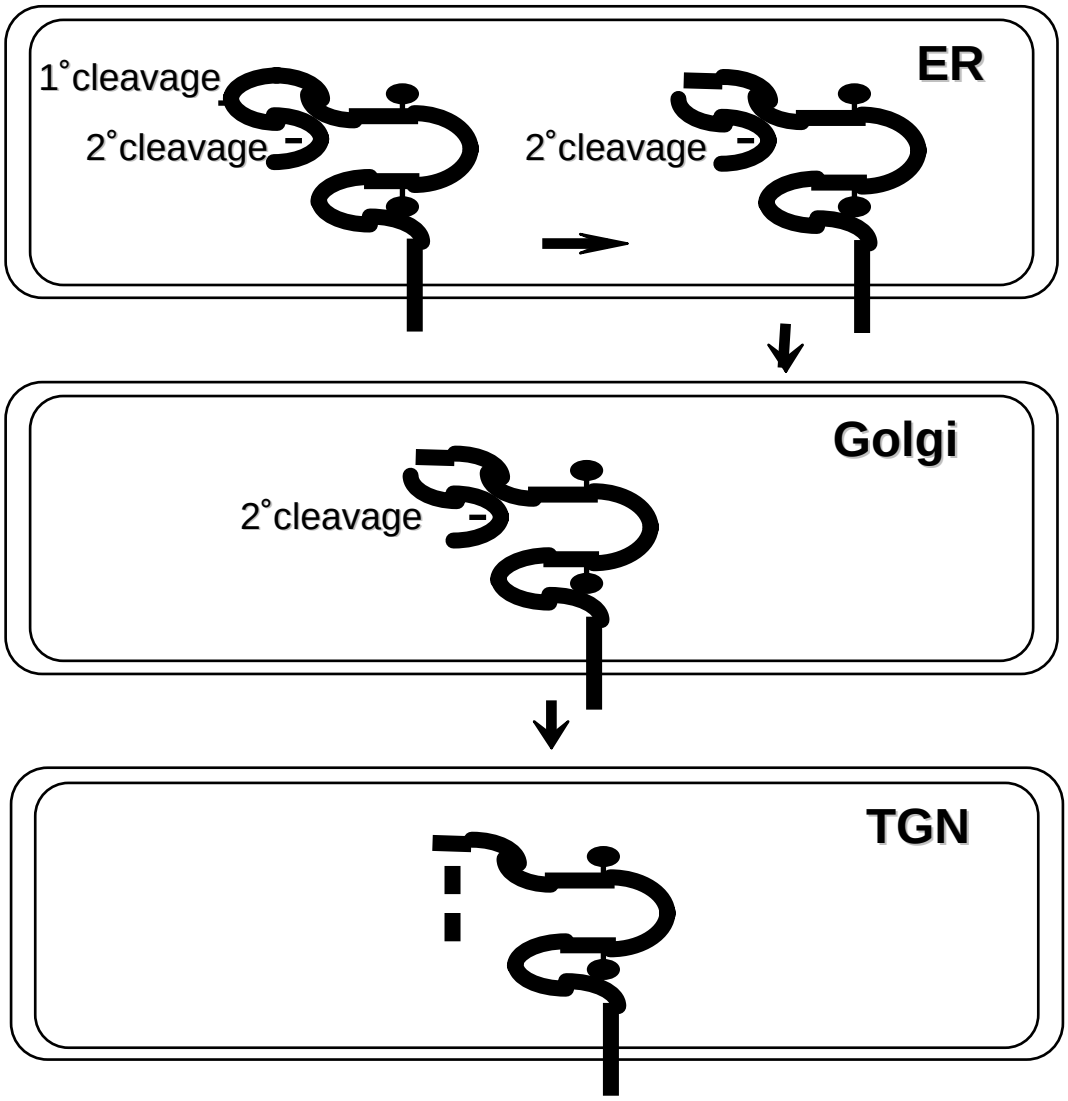


Figure A-4: The convertase autoactivation pathway (except PC2 and PC7).

In addition to its inhibitory function of the cognate enzyme, the prodomain of furin was also shown to act as an intramolecular chaperone. This was observed when a mutant of furin lacking the prosegment was retained in the ER while co-expression of the prosegment in trans restores both its trafficking and enzymatic activity indicating that the furin propeptide is an intramolecular chaperone (Anderson et al., 2002). The same results were obtained when other PCs lacking

their prodomains were expressed. The prosegment of furin, PC7 and PC1 were shown to possess an inhibitory activity in trans towards their parental enzyme and on other convertases *in vitro* and *ex vivo*. The prodomain of furin (pfurin) is ~10 fold more potent at inhibiting PC5A (IC50~0.4nM) than furin (IC50~4nM) while the prosegment of PC7 (pPC7) is a relatively selective inhibitor of PC7 (Zhong et al., 1999;Fugere et al., 2002). In contrast, overexpression of the preprosegments (ppfurin and ppPC7) in mammalian cells resulted in potent but moderately selective inhibition of their parent enzyme. The prodomain of PC1 (pPC1) is a slightly more potent inhibitor of PC1 as compared to furin *in vitro* (low nM)(Boudreault et al., 1998a). The C-terminal arginine residue of the prosegment (P1 position) is important for the inhibitory activity since a mutation into an alanine totally abolishes the inhibition (Zhong et al., 1999) (see Figure A-5).

```
            1                                                                 60
mpace4       aLpppRPVY TN...HWAVq Vl..GGp... ....gaADRV AaahGYlNLG QIGnLDD..Y
mpc5          .....rVY TN...HWAVk Ia..GGf... ....aEADRI AsKyGFINvG QIGaLkD..Y
mfurin             kIF TN...tWAVh IP..GGp... ....avADRV AqKhGFhNLG QI..FgD..Y
mpc4          sAQap.IY vs...sWAVr Vt..kGy... ....qEAERl ArKfGFVNLG QI..FpDdqY
mpc2          E...RPVF TN...HFlVe lhk.dGe... ....eEArqV AaehGFg.vr klpfaEg..l
mpc1          k...Rq.F vN...eWAae IP..GGq... ....eaAsaI AeelGYdlLG QIGsLEn..h
mpc7        lsEAgGldIL gtgglsWAVh ldsleGerke esltqqADaV AqaaGLVNaG rIGeLqg..h
Consensus   ---------- ---------- -----G---- ------A--- A---G----- ----------

            61                                       100                  120
mpace4      YHFYH...Sk tf.KRStLSs .RGpHtfLrm DPqVKWLqQQ evKRRvKR↓q. ..arsdsLYF
mpc5        YHFYH...SR tI.KRSvLSs .RGtHSfiSm EPkVeWiqQQ vvKkRtKR↓dy dlshaqstYF
mfurin      YHFWH....R aVtKRS.LSp HRpRHSrLqR EPqVKWLEQQ vaKRRaKR↓dV yqePt.....
mpc4-a      FHLRH....R gVAqqS.Ltp HWGhrlrLkk DPkVrWFEQQ tlrRRvKR↓Sl v.vPt.....
mpc2        YHFYHnglak akArRS.L.. HhkRq..LeR DPrIKmalQQ egfdRkKR↓gy rdineidinm
mpc1        YlFkHkshpR .rsrRSAL.. Hitkr..LSd DdrVtWaEQQ yeKeRsKR↓SV qkdsaldL.F
mpc7        YlFvqptghR qameveAM.. rqqaeavLaR heaVrWhseQ tllkRaKR↓SI h........F
Consensus   ---------- ---------- ---------- ---------Q ----R-KR↓-- ----------
```

Figure A-5: Alignment of the sequences of the prodomains of the PCs.

The catalytic domain is the region with the highest sequence homology among the mammalian members of this family. The similarity in sequence between the active site of PC5 and PACE4 is strong since they share 75% identity while PC2 shows the least homology to the other members. They all have in common the serine protease catalytic triad composed of Asp/His/Ser and Asn as the oxyanion hole (Asp for PC2). Mutation of the Aspartic acid residue into an Asparagine residue at the oxyanion hole of PC2 did not affect its autoactivation nor its substrate cleavage specificity, although the oxyanion mutant was no longer secreted (Zhou et al., 1995a). Moreover, the oxyanion mutant of PC2 was not able to interact with the chaperone partner 7B2 indicating that the Asp residue is critical for binding of proPC2 to pro-7B2 (Benjannet et al., 1995a). In most PCs, mutation of the catalytic triad residue causes their retention in the ER as it was shown for furin. This ER retention blocked substrate processing, but had no effect on furin prosegment removal (Creemers et al., 1993;Creemers et al., 1995). However, mutation of the Asn oxyanion hole does not impair autoprocessing but affects their in trans proteolytic activity. This was observed with the oxyanion hole mutant of kexin (Brenner et al., 1994). This mutant of kexin was shed to the same extent as the wild-type demonstrating that the mutant is able to exit the ER since shedding is a late event in the secretory pathway.

Recently, the crystal structure of mouse furin, comprising the catalytic and the P-domains, inhibited by decanoyl-RVKR-chloromethylketone peptide was obtained. From the structure, it is apparent that the catalytic domain is cross-connected through disulfide bridges and it contains 2 internally bound calcium ions, one that seems to stabilize the S1 pocket (Henrich et al., 2003a). Prior to the crystal structure of furin, the crystal structure of kexin was achieved and revealed that the catalytic site is also stabilized by 2 disulfide bridges but contains 3 calcium binding sites as opposed to 2 in the furin convertase (Holyoak et al., 2003b). All the negatively charged subsites determined for furin can only be predicted for PC5 as the other PCs should exhibit fewer negative charges. Only the crystal structure of the other PCs will help identify the subtle differences that will explain the distinct activity versus the redundant activity of each enzyme.

A few investigations were performed to elucidate the function of the P-domain also known as homo B domain. This domain is a distinctive characteristic of the proprotein convertases, as this additional downstream sequence is not found in subtilisin-like proteases. This domain was suggested to be important for both folding and stabilisation of the active site possibly through hydrophobic interaction. The first study demonstrated that truncation of this region in both kexin and furin causes a loss in enzymatic activity (Fuller et al., 1988). In the human colon carcinoma LoVo cells, the endogenous furin is proteolitically

inactive due to frameshift mutations identified within its P-domain (Takahashi et al., 1993;Takahashi et al., 1995b). Similar results to those of furin were obtained with truncated forms of PC1 (Zhou et al., 1998) and PC2 (Creemers et al., 1996). Deletion of the complete P-domain or only the C-terminus results in the abolition of the prosegment auto-processing ultimately culminating in a loss of proteolytic activity. It is assumed that the enzymes are unstable and misfolded because they are unable to exit the ER and traverse the secretory pathway. Interestingly, expression of only the P-domain of PC1 was normally sorted trough the secretory pathway, which indicates that the P domain can properly fold without the catalytic domain. Truncation after residue 593 was disruptive in the WT PC1, but not in the protein corresponding to the P-domain, lacking the catalytic domain. These results indicate that the C-teminus of the P-domain (residues 594-616) is necessary for the folding of the PC1 enzyme precursor, but not for the folding of the isolated P domain (Ueda et al., 2003). From this finding and mutational analysis, it is believed that threonine 594 is normally oriented toward the interface with the catalytic domain in PC1, possibly playing an important stabilizing role. Since the P-domain is critical for optimal enzymatic activity, swap experiments were performed to define if this domain is specific to each convertase or if it is interchangeable. Compared with truncated wild-type PC1, both PC1/PC2P and PC1/FurP exhibited an elevated activity on several synthetic substrates as well

as reduced calcium ion dependence, whereas Fur/PC2P was only slightly decreased in activity as compared with truncated furin (Zhou et al., 1998). Moreover, all three active PC chimeras had more alkaline pH optima. This indicates that the P-domain is critical for proteolytic activity but is not specific to each convertase. The crystal structure of furin and kexin revealed that the structure of the P-domain consists of a jelly-roll of β-barrels (Henrich et al., 2003a;Holyoak et al., 2003b). This closed structure suggests that only a complete P-domain can fold stably and hence confer its stabilizing effect on the catalytic domain. Furthermore, the P-domain and the catalytic domain participate in interdomain contacts through hydrophobic and salt bridge interactions (Henrich et al., 2003a).

A conserved **RGD** motif is found within the P-domain of the convertases. This tripeptide motif can be found in several proteins of the extracellular matrix. Integrins link the intracellular cytoskeleton of cells with the extracellular matrix by recognizing this **RGD** motif (Ruoslahti, 1996). All the basic aa specific proprotein convertases possess this **RGD** motif except PC7, which exhibits a different sequence with **RGS** instead of **RGD** (Seidah et al., 1996;Bruzzaniti et al., 1996a;Meerabux et al., 1996b). The functionality of this tripeptide motif is still unknown, but it is suggested that it may be involved in protein-protein interactions as for the integrins. In order to investigate the functional importance of this motif,

mutants of PC1 were generated where the **RGD** was converted to a **RGE** sequence. The **RGE** motif was used based on previous experiments that demonstrated that binding of fibronectin to its integrin receptor was dependent on the **RGD** sequence and to be abrogated when replaced by **RGE** (Ruoslahti, 1996). The WT-PC1 is well folded and well secreted whereas the PC1-**RGE** mutant remains mostly as proPC1-**RGE** trapped in the ER and the little that is secreted is via the constitutive pathway as opposed to the regulated pathway (Rovere et al., 1999). The proteolytic activity of the **RGE** mutant was tested on cleavage of POMC into β-LPH and the PC1-**RGE** was less efficient than the WT-PC1 as it is less secreted. Moreover, the **RGE** mutant was still co-immunoprecipitating with α5β1 as did the WT-PC1 indicating that the **RGD** tripeptide motif is not required for the intracellular interaction with integrins (Rovere et al., 1999). PC5 also possesses this conserved R**RGD**L motif and it is present in all species from frog (Gangnon et al., 2003) to mammal (Lusson et al., 1993) but differs in protochordate amphioxus C**RGH**L (Oliva, Jr. et al., 2000). From the furin structure, the charged side chains of the residues of the **RGD** motif, which lies in a loop, point in different directions and do not form a contiguous surface (Henrich et al., 2003a). Thus, the structure disagrees with the proposed disintegrin or cellular routing function. However, the proposed structure is representative of the folded protein and not of the unfolded-folding intermediate.

Following the P-domain, each convertase displays a distinct C-terminal structure. The uniqueness of the C-terminal region is not only based on the variable C-terminal domains, as some PCs are produced by alternative splicing, generating C-terminally modified forms. This is seen in the case of mammalian PC4 (Seidah et al., 1992a), PC5 (Nakagawa et al., 1993b) and PACE4 (Kiefer et al., 1991b;Tsuji et al., 1994;Zhong et al., 1996), but also for the protochordate amphioxus products of PC5 gene (Oliva, Jr. et al., 2000). The multiple isoforms generated by alternative splicing usually only differ in their C-terminal sequence without modifications of the other upstream domains excluding some isoforms of PACE4, which lack the P-domain or possess deletions within the signal peptide sequence and the catalytic domain (Zhong et al., 1996).

As mentioned previously, the C-terminus of PCs is quite variable with PC1 and PC2 displaying a putative amphipathic alpha-helix preceded by a stretch of charged amino acids. This specific feature is also observable within the C-terminal segment of carboxypeptidase H, which is sorted along with PC1 and PC2 in dense cored secretory granules. It was postulated that the amphipathic helix in PC1 and PC2 could play a role in membrane anchorage of the enzymes as it was described for carboxypeptidase H (Fricker et al., 1990). In 1995, it was suggested that the α-helix could be involved in the targeting of the convertases to secretory granules, but was not clearly proved (Zhou et al., 1995a). It turned out that

the α -helical structure in the C-terminal tail of these proteinases is indeed very important for their sorting into the regulated secretory pathway. A study on PC1 provided clear evidence that the C-terminal region of PC1 contains one or more specific peptide sequences capable of acting as sorting domains to the regulated secretory pathway, as deletion of the complete C-terminal domain or the α -helix block the entry of the mutant enzyme into the secretory granules (Jutras et al., 2000a). The membrane-bound convertases, Furin, PC7 and PC5B, carry a hydrophobic transmembrane domain (TMD) at their C-terminus, which anchors the enzymes to membranes. This TMD corroborates the fact that these endoproteases are always synthesized as type-1 membrane-bound enzymes. Subsequent to the TMD, a cytosolic tail is found that undergoes post-translational modifications to confer the adequate cellular localisation (Molloy et al., 1994;Bosshart et al., 1994;Chapman and Munro, 1994).

Finally, the most common domain displayed at the C-terminus of the convertases is a cysteine-rich domain (CRD). This cysteine-rich region contains multiple repeats of the following consensus motif: **Cys-Xaa2- Cys-Xaa3- Cys-Xaa2- Cys-Xaa5-7- Cys-Xaa2- Cys-Xaa8-15- Cys-Xaa3- Cys-Xaa9-16**. The mammalian convertases possessing a CRD are furin, PACE4, PC5A and PC5B. While PACE4 and PC5A possess 5-tandem repeats within their CRDs (Lusson et al., 1993;Nakagawa et al., 1993a) furin exhibits only two shortened repeats

(Roebroek et al., 1986b) and PC5B has an extended CRD that contains 22 repeats (Nakagawa et al., 1993b). The frog and amphioxus homologue of PC5 also have a conserved CRD (Oliva, Jr. et al., 2000;Gangnon et al., 2003).

Prior to our work, the function of the CRD of PC5A, PACE4 and furin was unknown. It was suggested that the CRD allows access to plasma membrane precursors (Oliva, Jr. et al., 2000), interaction with the ECM (Tsuji et al., 2003), affect cell growth and/or localisation (Lusson et al., 1993) as well as increased stability of the convertase in the case of PC5B (Wang et al., 2004). For furin and PC5A, it was shown that the cysteine-rich domain is not required for processing efficiency of the enzyme in contrast to the P-domain, whose presence is essential (Hatsuzawa et al., 1992;De, I et al., 1996). In the case of PACE4, it was reported that deletion of the entire CRD accelerated the cleavage of the prodomain and thus led to faster activation of PACE4 (Mains et al., 1997). Recently, it was determined that it is only the last 25 residues of the C-terminus of PACE4 that inhibits the cleavage of the prosegment, thus slowing down the activation process, and not the full-length CRD (Taniguchi et al., 2002). Furthermore, the same group established that PACE4 and PC5A can bind heparin in the extracellular matrix (ECM) whereas soluble furin does not. They have shown specifically that the interaction between heparin and PACE4 occurs via a cationic stretch found within the CRD of PACE4 and that PC5A can also bind heparin possibly via a

similar motif (Tsuji et al., 2003). As mentioned above, PC5A and PACE4 have the same number of cysteine repeats while furin has less and shorter repeats (see Figure A-6) and this may be an important factor for the interaction with heparin. The convertases that share the highest homology of sequence of their CRD are PC5 and PACE4 with 37% identity. The conservation of the Cys motif within the CRD of PC5A and PACE4 was also found in non-convertase proteins such as in the epidermal growth factor receptor (EGFR) (Ward et al., 1995), and in a novel ECM protein know as Fras1 (McGregor et al., 2003). Furthermore, blasting the CRD sequence of PC5A (using NCBI blastp program) resulted in a perfect pairing of the Cys residues with those of the Neu protooncoprotein, also known as the receptor protein-tyrosine kinase erbB-2 (accession # AY 116182). The CRD of the EGF receptor was established to contain sorting information that directs the receptor to the caveolae/rafts and also was involved in protein-protein interactions with other EGF receptors (Kumagai et al., 2001;Yamabhai and Anderson, 2002). Therefore, the cysteine-rich domain of the convertases possibly has protein-protein interaction properties that might regulate the cellular localisation of the enzymes.

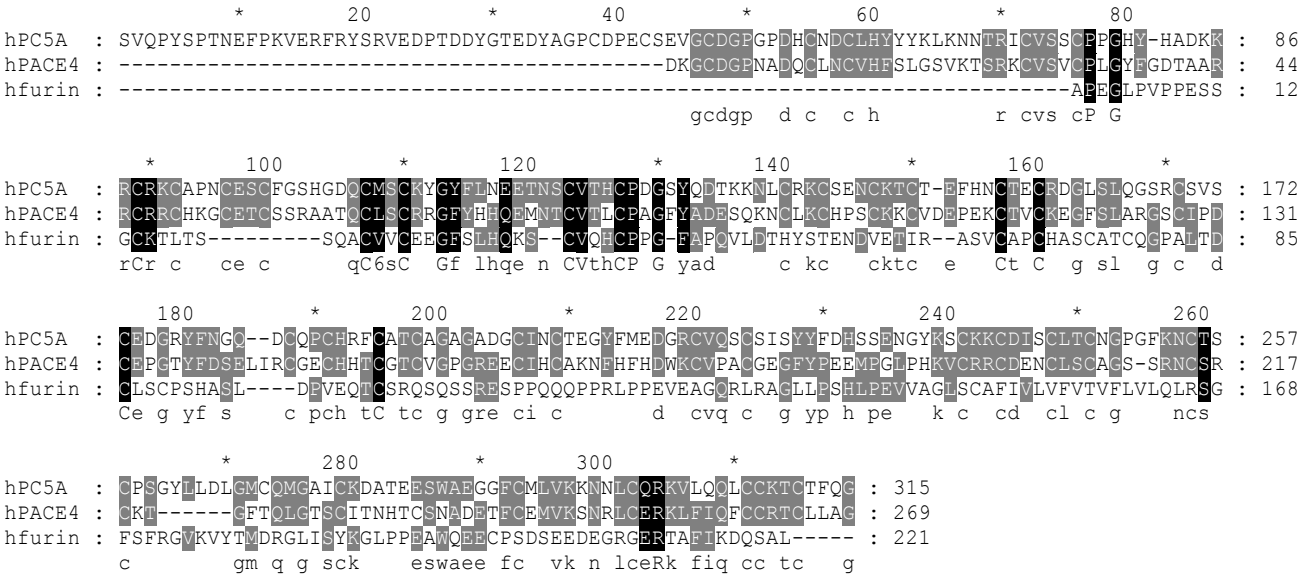


Figure A-6: Alignment of the sequences of cysteine-rich domain of PC5A, PACE4 and furin.

A.4 Sorting signals and C-terminal modifications

Similar to proteins that transit through the secretory pathway, the convertases undergo post-translational modifications such as phosphorylation, palmitoylation, sulfation, glycosylation and processing/shedding. Moreover, some PCs contain within their structure sorting signal information. In the case of furin, the sorting information is located within specific motifs of the cytoplasmic tail. The cytoplasmic tail of furin is involved in the translocation of the convertase from one organelle to another and the targeting to its optimal environment/ compartment (Bosshart et al., 1994). There are three specific determinants enclosed within the

tail of furin, which contribute to its steady state localization and trafficking. (i) The first sorting signal is comprised within two acidic clusters that correspond to casein kinase 2 phosphorylation motifs (SDSEEDE) (Jones et al., 1995;Takahashi et al., 1995a). The cycling between phosphorylated and dephosphorylated states would define the routing of furin towards the TGN, granules or the cell surface via endosomes. (ii) The other sorting signal is encompassed within a (YKGL) sequence containing a tyrosine that may function as a signal for internalization from the plasma membrane. This sequence is similar to the YXXØ, where Ø is a hydrophobic residue, consensus sequence implicated in the internalization mechanism of membrane proteins (Trowbridge, 1991). (iii) The last motif consists of adjacent leucine and/or isoleucine residues (dileucine signal) acting as an internalization signal (Johnson and Kornfeld, 1992). The distinct subcellular localization of PC5B is due in part by sorting information within its cytoplasmic domain. PC5B is localized to a paranuclear, brefeldin A-dispersible, responsive post-Golgi network (TGN) compartment distinct from furin and TGN38 (Xiang et al., 2000a). As in the cytosolic tail of furin, PC5B contains two sorting signals. Through mutational analysis, it indicates that endocytosis is predominantly directed by a canonical tyrosine-based motif (YEKL). Truncation studies reveal that two acidic clusters (AC1 and AC2) within the PC5B tail contain differential sorting information. The membrane-proximal acidic cluster (AC1)

directs TGN localization and interacts with the TGN sorting protein PACS-1. The membrane-distal acidic cluster (AC2) promotes a localization within a post-golgi compartment a characteristic of the full-length PC5B tail (Xiang et al., 2000a). The results demonstrated that acidic motifs can target proteins to distinct TGN/endosomal compartments. Conversely, the cytosolic tail of PC7 does not contain the acidic stretch nor the YXXL retrieval motif, however, it encloses a dileucine internalization motif (De, I et al., 1996). This suggests that PC7 localizes in a different compartment than PC5B and furin. PC7 may indeed pass through the TGN and localise within TGN-derived vesicles (Wouters et al., 1998).

The soluble PC5A was reported to contain sorting information within the last 38 amino acids of the C-terminus. It was shown that PC5A can enter the secretory granules while the PC5A mutant, lacking the last 38 a.a., was no longer accumulating in the secretory vesicles (De, I et al., 1996). It suggests that PC5A can be sorted to the regulated secretory pathway due to the presence of a sorting signal within the C-terminal 38 amino acids.

PC1 and PC2 also contain sorting signals within their sequences that target the enzymes through the regulated secretory pathway and to accumulate in secretory granules (Bennett et al., 1992;Kirchmair et al., 1992;Marcinkiewicz et al., 1993a;Shennan et al., 1994;Malide et al., 1995). The forms of PC1 and PC2 that are stored in dense granules correspond to the mature and active forms.

Initially, it was presumed that the C-terminus of both PC1 and PC2 contained the targeting information that directs these enzymes to secretory granules. It was speculated that the information sorting them to the granules was part of the amphipathic helices acting as membrane anchors (Smeekens et al., 1992). Moreover, it was determined that the C-terminus of PC1 could possibly act as an inhibitor of PC1 activity against certain substrates in the ER and Golgi apparatus, and its removal, occurring in secretory granules, may explain the observed granule-specific processing of certain proproteins (Jutras et al., 1997). In addition to the inhibitory role of the C-terminus, a later study revealed that there is in fact sorting information enclosed within the α -helix of the C-terminus of PC1 that directs the enzyme to the secretory granules. The authors proved this by fusing the sequence of PC1 corresponding to the α -helix to a segment of mouse immunoglobulin 2b heavy chain that does not enter granules by itself, whereas the fused protein was now targeted and concentrated in secretory granules responding to KCl that stimulates the release of secretory granule content (Jutras et al., 2000a). This study agrees with a previous investigation reporting that a C-terminally truncated form of PC1 was shown to undergo constitutive secretion from transfected AtT-20 cells instead of normally regulated secretion (Zhou et al., 1995a). A regulated pathway sorting domain was also identified in the C-terminal region of PC2. Immunofluorescence and immuno-

electron microscopy showed the presence of the furin-PC2 chimeras and PC2 in dense-cored secretory granules together with proopiomelanocortin immunoreactivity (Creemers et al., 1996). Thus, this illustrates that the C-terminus of PC2 is sufficient to direct furin chimeras into the secretory pathway. Recently, a study revealed that the C-terminus of PC2 confers raft association and is sufficient and a requirement for sorting of PC2 to the secretory granules. Moreover, the sorting signal was identified within the last six residues of the C-terminus of PC2 as deletion of these residues abolished lipid raft association and sorting to the regulated secretory granules (Assadi et al., 2004).

Table A-4: Post-translational modifications of PCs

N-linked glycosylation
Phosphorylation
Sulfation
Palmitoylation
Proteolysis/Shedding

The first post-translational modification that the convertases undergo is N-linked glycosylation. All convertases are found to be N-glycosylated but at different levels. In the ER, the convertases become N-glycosylated by the transfer of an initial oligosaccharide core to an asparagine residue within an Asn-X-Ser/Thr motif (Lennarz, 1987). The sugar moieties are then trimmed in the ER and further modified through the Golgi apparatus resulting in one of the three types of structures: high-mannose, complex or hybrid (Kornfeld and Kornfeld, 1985). The function of the glycosylation is not known apart from being important for the proper folding of the enzyme. It was reported that treatment with tunicamycin, an inhibitor of N-glycosylation, caused a dramatic intracellular degradation of PC1 and PC2 within the endoplasmic reticulum with the net effect of a reduction in the available activity of PC1 and PC2 (Benjannet et al., 1993). Thus, glycosylation is important for proper protein conformation as well as for cell trafficking. The carbohydrate structures of PC1 and PC2 are different as demonstrated by the resistance of the secreted PC1 (complex sugars) to and sensitivity of the secreted PC2 (hybrid sugars) to endoglycosidase H digestion (Benjannet et al., 1993). Furin (Molloy et al., 1994), PACE4 (Nagahama et al., 1998) and PC7 (Van de Loo et al., 1997) were also shown to be N-glycosylated and gained complex structures while PC5 exhibits hybrid sugars (Lusson et al., 1993;De, I et al., 1996).

Another modification that PCs can undergo is phosphorylation of the cytosolic tail. It was demonstrated that the cytoplasmic tail of furin (Jones et al., 1995;Takahashi et al., 1995a;Schapiro et al., 2004) and PC5B (Xiang et al., 2000a) can be phosphorylated while that of PC7 is not (Van de Loo et al., 1997). Modifications such as phosphorylation usually take place at the Golgi compartment. As previously mentioned, the phosphorylation of the cytoplasmic tail regulates the sorting of the enzyme to its appropriate compartment. Another modification that occurs within the Golgi is sulfation. Sulfation is a mechanism by which a sulphate group is added to tyrosine residues or sialic acid components of oligossacharides. This process is mediated by a sulfotransferase(s) specifically located within the trans Golgi cisternae and the TGN (Baeuerle and Huttner, 1987;Hart, 1992). Mature forms of PC5A and PC5B were shown to be sulphated, but the shorter C-terminally truncated form of PC5 (PC5-Δ C) is not (De, I et al., 1996). This suggests that the sulphate groups are only added to the C-terminus of PC5. Furthermore, a construct corresponding to the C-terminal cysteine-rich domain of PC5A is sulphated, but only on tyrosine residues as treatment with PNGaseF did not remove the sulphated groups (unpublished results). PC2 is also sulphated on a tyrosine residue (Benjannet et al., 1993) and it was determined that sulfation is not required for intracellular transport, sorting, and proteolytic processing of the enzyme (Van Kuppeveld et al., 1997). Furthemore, PC1 is also

sulphated on tyrosine residues and not on Asn-linked oligosaccharides (Benjannet et al., 1993;Boudreault et al., 1998b). Little is known about the intracellular role of protein sulfation. Sulfated tyrosine residues have been found both in constitutive and regulated secretory proteins (Huttner, 1988). The biological function of tyrosine and carbohydrate sulfation in proprotein convertases is not known, but it has been established for a few secreted biologically active peptides. It can affect the activity of hormones (Bodanszky et al., 1978) or the secretagogue activity of hormones (Jensen et al., 1980), it can confer stability to secretory proteins by protecting them against proteolytic degradation in the circulation (Mian et al., 1979) and it could be essential for their ability to undergo protein-protein interactions (Huttner et al., 1991). It is possible that the sulfation of convertases confers a similar role.

PC7 is the only member that undergoes palmitoylation of its C-terminal cytosolic tail. PC7 is palmitoylated on cysteine residues within a hydrophobic domain located within the cytosolic tail (Van de Loo et al., 1997). In other proteins, palmitoylation has been shown to be important for proper localisation and/or function (Milligan et al., 1995). Therefore, further studies will establish if palmitoylation of PC7 is indeed critical for its localisation and function.

The last modification discussed is proteolysis and shedding. PC1 undergoes autocatalytic processing of its C-terminus domain in order to gain its optimal

proteolytic activity (Zhou and Lindberg, 1994;Zhou et al., 1995b). The C-terminal segment appears to act as an inhibitor of PC1 activity against certain substrates in the ER and Golgi apparatus, and its removal, which occurs naturally in secretory granules, may explain the observed granule-specific processing of certain proproteins (Jutras et al., 1997). This also demonstrates that PC1 is present in a partially active state prior to entering the secretory granules where it is autocatalyticaly cleaved to induce a state of maximum activity. Another PC that was shown to be processed at its C-terminus is PC5. It was demonstrated in AtT20 cells that PC5A and PC5B are truncated at their C-terminus producing a 65kDa form, a late event along the secretory pathway (De, I et al., 1996). It was latter shown that this processing was not autocatalytic as in the case of PC1 since a prosegment mutant of PC5A that was inactive at processing substrates was still observed in a shorter 65 kDa form (Nour et al., 2003). Moreover, overexpression of PC5A or other PCs in FD11 cells, which are furin deficient, did not increase the PC5 C-terminal processing (unpublished results). The function of this cleavage in PC5 remains to be clarified and will be further discussed in chapter D. Membrane-bound convertases, furin and PC5B, were shown to be detected in conditioned media of the overexpressing cells indicating a C-terminal cleavage called shedding. It was first observed when cells were infected with a vaccinia virus recombinant for human furin. The authors reported the presence of

a soluble form of furin (shed furin) detected in the media that conserved its proteolytic activity as it was able to cleave a fluorogenic peptide (Vidricaire et al., 1993). Later on, the shedding site of furin was identified by mass spectrometry and N-terminal sequencing: RQSQSS↓ R683 (Plaimauer et al., 2001). In the same study, mutational analysis showed that Arg 683 is important for shedding to occur. Another investigation demonstrated that an introduction of mutations within furin' s cysteine-rich domain, which abolish TNFR1 (tumour necrosis factor receptor 1) shedding, also impaired furin shedding, indicating that the CRD of furin contains structural information necessary for the shedding of its ectodomain (Denault et al., 2002). The enzyme involved in furin' s shedding has not been identified, but to date, five metalloproteases have been implicated in membrane protein shedding: ADAM17, ADAM10, ADAM9, ADAM19 and MMP-7 thus, one of these enzymes may be the shedase of furin. PC5B is another convertase that is shed. A soluble form of PC5B was observed in the media of AtT20 cells overexpressing the enzyme (De, I et al., 1996) whereas PC7 is not shed into a secreted form (Munzer et al., 1997b). Although the importance of shedding has not been established, it was shown that it does not affect the intrinsic proteolytic activity of the convertases.

A.5 Potential substrates and cleavage specificity

Many proteins of the secretory pathway are initially synthesized as inactive precursors that undergo processing at a specific cleavage site to produce bioactive proteins. These proteins were found to be cleaved after monobasic, dibasic or multibasic residues, generally following the determined consensus sequence: (**R/K**)-Xn-(**R/K**), where n=0, 2, 4 or 6. The search for the enzymes involved in this intracellular processing lasted more than 20 years and led to the identification of the proprotein convertases. Recently, aside from cleavage after basic residues, other types of cleavage sites have been characterized such as (R/K)X(hydrophobic)Z↓, where Z is variable; a cleavage performed by the enzyme known as SKI-1/S1P (Seidah et al., 1999;Toure et al., 2000). Finally the newly characterized convertase, NARC-1, cleaves after the sequence VFAQ↓ found at the end of its prosegment and its specificity of cleavage is not determined as no other substrates than itself have been identified. (Seidah et al., 2003;Naureckiene et al., 2003). The proprotein convertases have been shown to cleave many different types of precursors since their discovery and more than one PC is usually competent at cleaving the same substrate depicting redundancy in their activity. The precursors cleaved are subdivided into four groups as depicted in Table A-5.

- Type I consists of the proproteins of the constitutive pathway processed after the P4, P2-P1 multibasic site corresponding to **R**-X-(**K/R**)-**R** ↓ . This type of cleavage usually takes place within the TGN and is preferred by furin. Precursors containing this cleavage site are mainly growth factors, receptors, bacterial toxins and viral glycoproteins (Oda et al., 1991;Hosaka et al., 1991).
- Type II includes the precursors that are cleaved after a simple dibasic site (**R/K**)-(**R/K**) ↓ . This type of processing usually occurs within immature secretory granules by PC1, PC2 and PC5A and the processed products are stored in dense core granules.
- Type III represents proproteins cleaved at a monobasic site, generally occupied by an **R** ↓ amino acid preceded by another basic residue at either P4, P6 or P8 (Watanabe et al., 1992;Nakayama et al., 1992b).
- Type IV includes all the precursors that are processed at either single or paired basic residues. This group differs from the others as it requires an **R/K** two residues downstream of the cleavage site (P2') in addition to the presence of a basic residue either at P4, P6 or P8.

Table A-5: Examples of polypeptide precursor types cleaved by members of the PC family.

Precursor Protein	Cleavage site sequence P6 P5 P4 P3 P2 P1 ↓ P1' P2'
Type I precursors [R-X-K/R-R]	
hPro-NGF	Thr-His-**Arg**-Ser-**Lys**-**Arg** ↓ Ser-Ser
Pro-BMP-4	Thr-Arg-**Arg**-Ser-**Lys**-**Arg** ↓ Ser-Pro
hInsulin proreceptor	Pro-Ser-**Arg**-Lys-**Arg**-**Arg** ↓ Ser-Leu
hPro-PDGF-A	Pro-Ile-**Arg**-Arg-**Lys**-**Arg** ↓ Ser-Ile
hIntegrin α3	Pro-Gln-**Arg**-Arg-**Arg**-**Arg** ↓ Gln-Leu
HIV-1 gp160	Val-Gln-**Arg**-Glu-**Lys**-**Arg** ↓ Ala-Val
Diphtheria toxin	Gly-Asn-**Arg**-Val-**Arg**-**Arg** ↓ Ser-Val
Pro-7B2	Glu-Arg-**Arg**-Lys-**Arg**-**Arg** ↓ Ser-Val
Notch1 receptor	Gly-Gly-**Arg**-Gln-**Arg**-**Arg** ↓ Glu-Leu
Pro-MT1-MMP	Asn-Val-**Arg**-Arg-**Lys**-**Arg** ↓ Tyr-Ala
Type II precursors [K/R-K/R]	
POMC (MSH/CLIP)	Pro-Val-Gly-Lys-**Lys**-**Arg** ↓ Arg-Pro
(LPH/END)	Pro-Pro-**Lys**-Asp-**Lys**-**Arg** ↓ Tyr-Gly
Proinsulin(B/C chain)	Thr-Pro-**Lys**-Thr-**Arg**-**Arg** ↓ Glu-Ala
(A/C chain)	Gly-Ser-Leu-Gln-**Lys**-**Arg** ↓ Gly-Ile
Prorenin	Ser-Gln-Pro-Met-**Lys**-**Arg** ↓ Leu-Thr
hIntegrin α4	His-Val-Ile-Ser-**Lys**-**Arg** ↓ Ser-Thr
hVEGF-C	His-Ser-Ile-Ile-**Arg**-**Arg** ↓ Ser-Leu
Neuronal adhesion protein L1	**Arg**-Lys-His-Ser-**Lys**-**Arg** ↓ His-Ile
Proneurotensin	Pro-Tyr-Ile-Leu-**Lys**-**Arg** ↓ Ala-Ser
Type III precursors [K/R]	
Pro-ANF	Leu-Leu-Thr-Ala-Pro-**Arg** ↓ Ser-Leu
Prosomatostatin	**Arg**-Leu-Glu-Leu-Gln-**Arg** ↓ Ser-Ala
Pro-EGF	**Arg**-Trp-Trp-Glu-Leu-**Arg** ↓ His-Ala
Pro-CCK 58	Pro-Arg-**Arg**-Gln-Leu-**Arg** ↓ Ala-Val
Type IV precursors [P2'K/R]	
Pro-MIS	**Arg**-Gly-**Arg**-Ala-Gly-**Arg** ↓ Ser-Lys

Proglucagon	Leu-Met-Asn-Thr-**Lys**-**Arg** ↓ His-Arg
Pro-PTP-μ receptor	Glu-Glu-**Arg**-Pro-**Arg**-**Arg** ↓ Thr-Lys

The substrates and cleavage specificities of furin, PC1, PC2 and PC5A have been studied in depth and will be discussed more extensively in the following section.

Furin:

The substrate specificity of furin has been investigated extensively by cellular co-expression and *in vitro* studies in comparison to the other convertases. Initial studies relying on the co-expression of furin and substrates in cultured mammalian cells have illustrated that furin is able to cleave nerve growth factor (NGF) (Bresnahan et al., 1990;Zhu et al., 2002a) and von Willerbrand factor (vWf) (van de Ven et al., 1990;Zhu et al., 2002a). In the manuscript on von Willerbrand factor cleavage by furin, the authors showed that the mutation RSK**R** into RSK**G** resulted in an unprocessed protein indicating the requirement of a basic residue at P1 (van de Ven et al., 1990;Zhu et al., 2002a). A subsequent study demonstrated that both PC1 and furin can cleave renin after a dibasic site, but when an arginine residue was introduced at P4, it became a better substrate of furin indicating that it preferentially recognizes the **R**-X-(**K/R**)-**R** sequence (Hosaka et al., 1991). Since then, furin has been shown to cleave a great variety

of precursors such as: growth factors (Siegfried et al., 2003a;Siegfried et al., 2003b), their receptors (Khatib et al., 2001;Bergeron et al., 2003), plasma proteins involved in blood clotting and complement system (van de Ven et al., 1990), protease zymogens (Pei and Weiss, 1995;Yana and Weiss, 2000), bacterial toxins (Gordon et al., 1995), viral glycoproteins (Decroly et al., 1994), prohormones (Hendy et al., 1995;Posner et al., 2004) and other types of precursor proteins (Benjannet et al., 1995b). Most of these substrates possess the **R**-X-(**K/R**)-**R** recognition motif. *In vitro* and *in vivo* mutagenesis experiments were performed to determine the specificity of cleavage of furin. From these studies, the following sequence rules that govern the cleavage by furin were established (Watanabe et al., 1992;Watanabe et al., 1993;Takahashi et al., 1994;Krysan et al., 1999). (i) The presence of an arginine residue at P1 is critical whereas a basic residue at P2 is dispensable. (ii) At least one other basic residue is required at P2, P4 or P6, in addition to the arginine at P1. (iii) A hydrophobic aliphatic side chain at P1' is not favourable for furin processing. Even though furin preferentially recognizes and cleaves the consensus **R**-X-(**K/R**)-**R** site, it was demonstrated that furin is also capable of cleaving substrates after monobasic (prosomatostatin) (Brakch et al., 1995) or dibasic (proVEGF-C) (Siegfried et al., 2003a) sites.

The evolution of technology led to the development of predictive model for PC cleavage specificity. This ProP model is publicly available and can predict a furin cleavage site in independent sequences with a sensitivity of 95% for the furin neural network and 62% for the general PC network (Duckert et al., 2004). From the furin data set, it was observed that the minimal furin cleavage site is **R**-X-X-**R**, but a less favourable residue at P4 can be compensated by a basic residue (K/R) at P2. Moreover, a hydrophobic side chain (leucine, valine or isoleucine) is never observed at P1' , which is in concordance with the previous rules established (Watanabe et al., 1993). Based on the recent crystal structure of furin, it becomes obvious that furin can discriminate against a lysine at P4 because the arginine side-chain at P4 makes contact with multiple residues. Moreover, furin prefers an arginine at P1 since it contacts other residues and hence would not be satisfied by a lysine or citrulline residue. In contrast to the specificity of P1, P2 relies on simpler interactions and there is little difference between lysine and arginine. Thus, the general consensus sequence that furin prefers is **R**-X-(**R/K**)-**R**.

PC1 and PC2:

Most of the substrates of PC1 and PC2 are part of the type II group of precursors (see Table A-5) Both enzymes cleave the majority of their substrates

in secretory granules of endocrine cells, which explains the requirement for an acidic pH and a higher calcium concentration for optimal proteolytic activity (Zhou and Lindberg, 1993;Shennan et al., 1994). PC1 behaves differently than PC2 as it was demonstrated that the conditions within the TGN can allow PC1 to initiate the processing of some precursors (Benjannet et al., 1991). The precursors shown to be processed by PC1 and PC2 include proopiomelanocortin (POMC), proinsulin, pro-THR, prodynorphin, proglucagon, pro-MCH, proneurotensin, pro-CCK and finally proenkephalin. In addition to these precursors, PC1 is capable of processing prorenin and pro-GHRH.

The precursor POMC is differentially cleaved by PC1 and PC2. POMC (Chretien et al., 1979) is a precursor synthesized in the brain and the pituitary gland that generate many active polypeptides upon cleavages: ACTH, β -lipotropin, α -MSH, γ-LPH, β-MSH and endorphin. Each of these hormones is known to lead to smaller peptides having distinct biological activities: alpha-melanotropin (α -MSH) and corticotropin-like intermediate lobe peptide (CLIP) are formed from ACTH; γ-LPH and β-endorphin are peptide components of β-LPH. β-MSH is contained within γ-LPH (Chretien and Seidah, 1981;Bennett, 1985;Benjannet et al., 1991). PC1 and PC2 are both expressed in the pituitary gland, but PC1 is more abundant in the anterior lobe whereas PC2 is richer in the intermediate lobe (Seidah et al., 1990). The processing of POMC into the various active

polypeptides occurs in two steps. Initially, in the corticotroph cells of the anterior pituitary, POMC is cleaved by PC1 to generate corticotrophin (ACTH) and β -lipotropin (β -LPH). Then, these products are further cleaved by PC2 in the melanotrope cells of the intermediate lobe generating β -endorphin and α -MSH (Zhou and Mains, 1994;Halban and Irminger, 1994). The specific and sequential roles of PC1 and PC2 on POMC corroborate with their different localization and different maturation rates. The sequential roles of PC1 and PC2 in POMC processing are shown in Figure A-7.

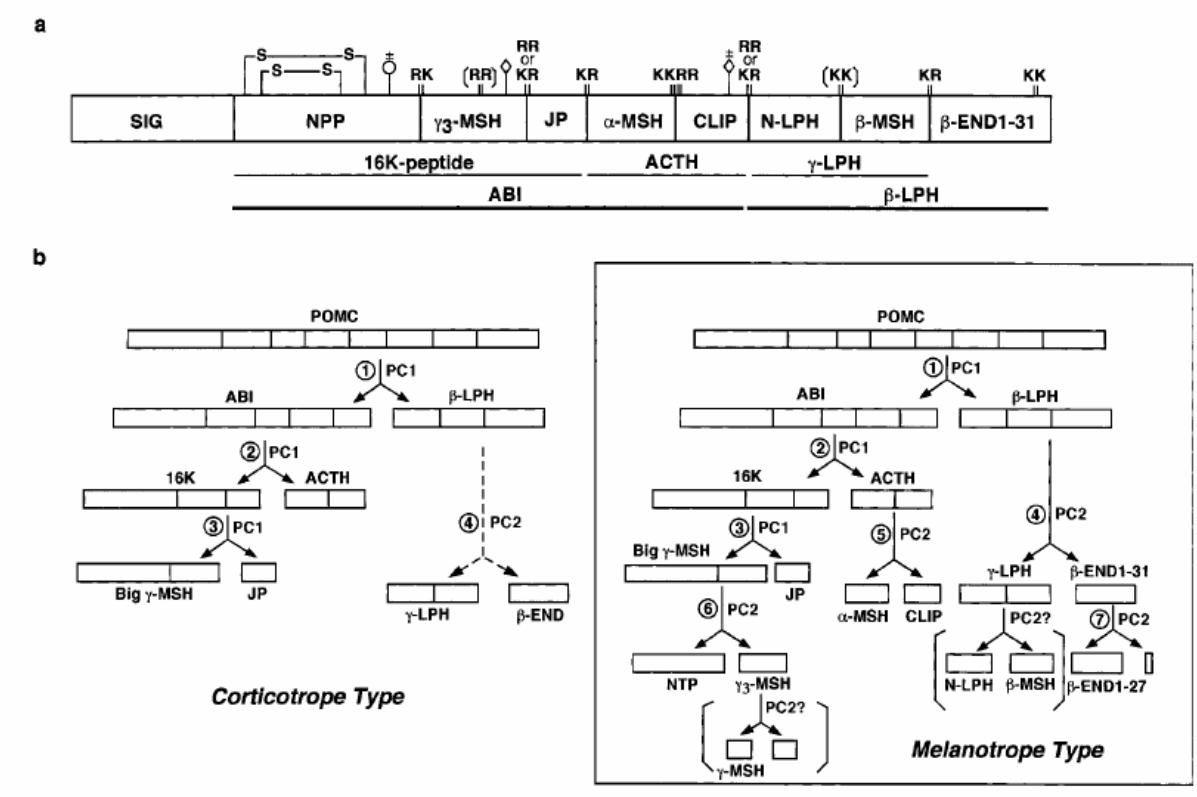


Figure A-7: Processing of POMC by PC1 and PC2. (A) Structure of POMC precursor. (B) Differential processing of POMC in corticotrope and melanotrope cells.

Proglucagon presents another example of differential processing by PC1 and PC2, which results in the secretion of two hormones with distinct activities from one precursor (Rouille et al., 1995). In mammals, proglucagon is synthesized within alpha cells of the islets of Langerhans in the pancreas as well as in the endocrine L cells of the intestinal mucosa (Bromer et al., 1972;Orskov et al., 1987) . Proglucagon is processed differentially in the pancreatic alpha cells and the intestinal L cells to yield either glucagon or glucagon-like peptide 1 respectively, structurally related hormones with opposing metabolic action (Mojsov et al., 1986). The alpha cells secrete mature glucagon to stimulate glycogenolysis and gluconeogenesis in the liver counter-balancing the action of insulin on the blood glucose level. In contrast, the intestinal L cells release insulinotropic hormone (truncated form of GLP-1) upon stimulation. Processing of proglucagon within the L cells produces glicentin, GLP-1, IP-2 and GLP-2. Glicentin is further processed to produce glicentin related polypeptide (GRPP) and oxytomodulin (Rouille et al., 1995). In the pancreatic alpha cells, proglucagon is mainly processed into glucagon, GRPP, IP-1 and MPGF, which is a peptide

encompassing GLP-1, IP-2 and GLP-2 (Mojsov et al., 1986). This differential processing is thought to result from the differential expression of the convertases in alpha cells and L cells. While PC2 is expressed in high levels in the pancreatic alpha cells, PC1 is almost unidentifiable (Marcinkiewicz et al., 1994;Tanaka et al., 1996), whereas in the L cells the opposite is observed (Scopsi et al., 1995). It has been suggested that PC2 alone would not be able to perform all the cleavages necessary for the generation of glucagon. Certain researchers assume that additional convertases might be required in the formation of glucagon due to the inability of PC2 to cleave at the KR_{32} site which is essential for the formation of the mature active glucagons (Dhanvantari et al., 1996). Various cell lines in which proglucagon is processed were demonstrated to express PC5. Moreover, in a specific cell line, PC1 and PC2 were undetectable while PC5 was expressed and proglucagon was processed in a manner reminiscent of the intestinal L-cell type, suggesting that PC5 may participate in proglucagon processing when PC2 and PC1 are absent (Blache et al., 1994). Recently, with the arrival of knockout animal models, it was shown that proglucagon processing was altered in alpha cells derived from PC2 null mouse islets and that the absence of PC2 activity resulted in a total block of the formation of mature glucagon (Webb et al., 2004). In mice lacking PC1, intestinal processing of proglucagon was impaired causing an increase in the levels of proglucagon and a marked reduction in the levels of

the products: glicentin, oxyntomodulin, GLP-1 and GLP-2 indicating that PC1 is essential for the processing of all the intestinal proglucagon cleavage sites (Ugleholdt et al., 2004). Production of the mature form of glucagon or alternative products demonstrated that each of the 2 proteases (PC1 and PC2) has distinct cleavage specificity. As such, this cleavage specificity may be tied to or a cause of their different tissue specificity. The differential processing of proglucagon is displayed in Figure A-8.

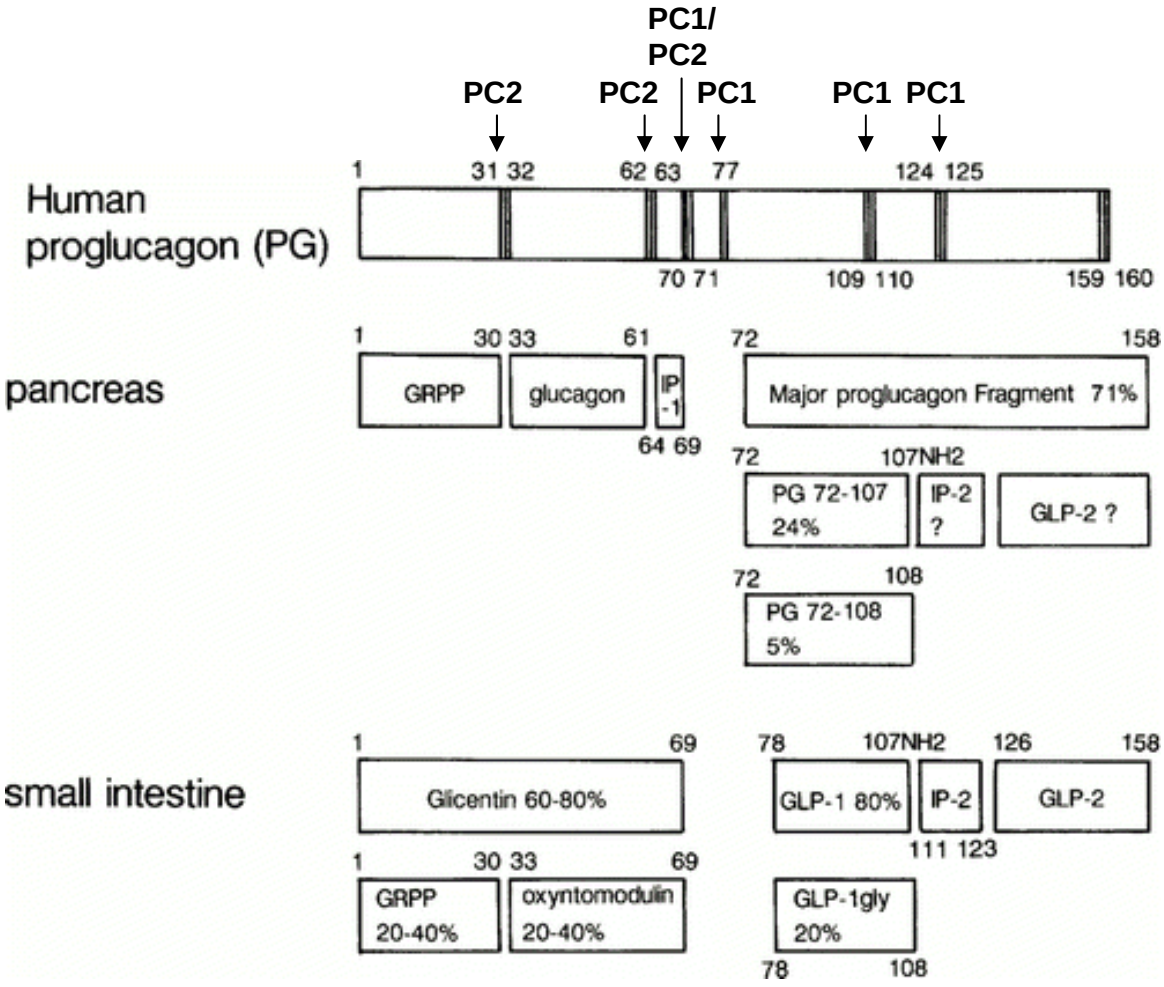
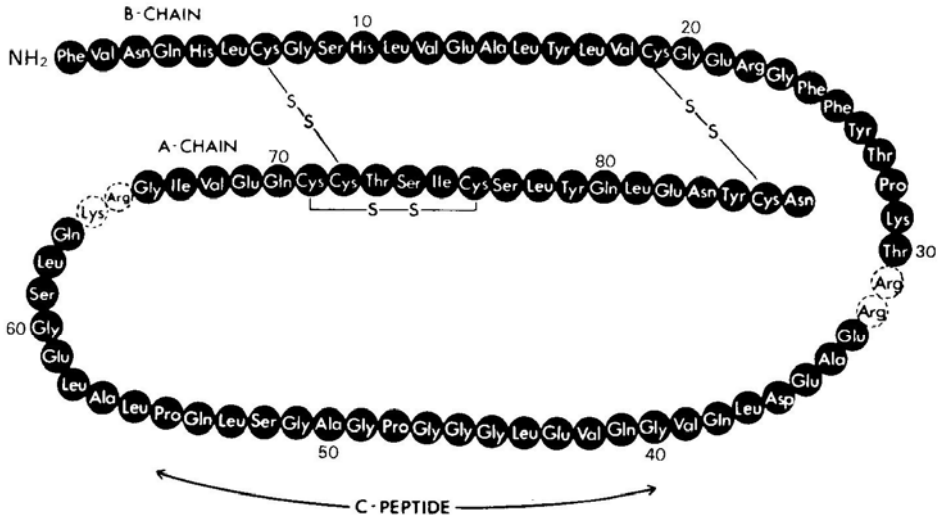


Figure A-8: Proglucagon processing in the pancreas and the small intestine.

A final example of precursors cleaved by both PC1 and PC2 is the proinsulin substrate. The conversion of proinsulin into active insulin in secretory granules of pancreatic β-cells requires the specificity of cleavage of PC1 and PC2 as observed in the case of POMC and proglucagon. There is a correlation in the expression of both PC1 and PC2 with proinsulin in the β-cells of the pancreas (Marcinkiewicz et al., 1994). The proinsulin precursor is made of the A and B chains connected together by the C-peptide that is flanked on both sides by dibasic residues. The two cleavages required for the release of mature insulin occur in a specific order, the junction of the C-peptide/B-chain (des-31.32) is cleaved initially by PC1. Then, the processed intermediate is further cleaved at a second site by PC2 at the junction between the A-chain and the C-peptide (des-64.65) releasing mature insulin and the C-peptide (Davidson et al., 1987;Bailyes et al., 1991;Bennett et al., 1992;Rhodes et al., 1992). After each cleavage, the exoprotease carboxypeptidase H rapidly removes the basic residues exposed by PC1 and PC2 (Davidson and Hutton, 1987). The mature insulin is made of the A and B chains associated through disulfide bonds. PC1 preferentially cleaves at the **RR** site, which is found at the B-chain/C-peptide junction while PC2 prefers cleaving after the **KR** found at the C-peptide/A-chain junction. The conversion of proinsulin into mature insulin is seen in Figure A-9. The importance of PC1 in the processing of proinsulin was further supported by the increased levels of des-

64.65 proinsulin generated by PC2 in mice lacking PC1. On the other hand, PC2 null mice had an increased des-31.32 proinsulin intermediate (Furuta et al., 1998;Zhu et al., 2002a). These results confirm that both PC1 and PC2 are major players in processing proinsulin and that their coordinated actions are necessary for the most efficient and complete processing of this hormone.

A. Proinsulin



B. Processing of proinsulin to insulin and C peptide

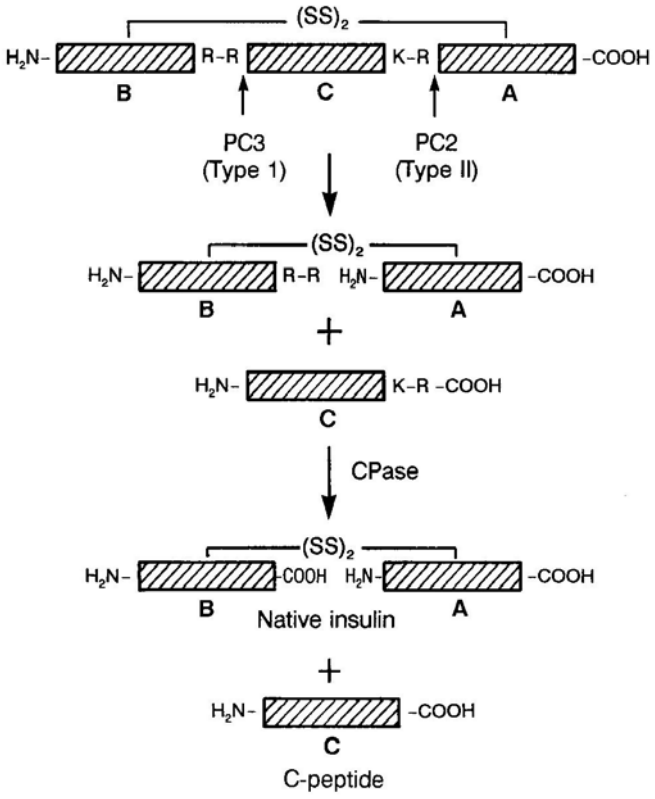


Figure A-9: (A) Structure of proinsulin. (B) Conversion of proinsulin into insulin by PC1/PC3 and PC2.

PC5:

The specific function of PC5 has not been characterized yet, but a variety of precursors have been reported as being processed by PC5A both *in vitro* and *in vivo*. Recently, PC5A was expressed and purified from baculovirus-infected insect cells and thus its cleavage specificity was determined. This showed that PC5A not only cleaves fluorogenic peptides containing the **R**-X-X-**R** consensus motif as furin does, but is also capable of cleaving peptides after dibasic and monobasic residues (Cain et al., 2002). Some substrates are cleaved by both PC5A and PC5B whereas other precursors are preferentially cleaved by one or the other. This may be explained by the fact that both enzymes are sorted to different compartments and that only PC5A can cleave precursors within both the regulated and constitutive secretory pathways. The substrates that are cleaved by PC5A include proneurotensin (Barbero et al., 1998), neuronal adhesion molecule L1 (Kalus et al., 2003), endothelial lipase (unpublished), pro-CCK (Cain et al., 2002) and prorenin (Mercure et al., 1996). Lefty and the integrin α 4, α v chains were shown to be well processed by PC5A while PC5B had a limited activity (Lissitzky et al., 2000;Ulloa et al., 2001;Bergeron et al., 2003). In contrast, BMP-4, which is part of the TGF-β family was well cleaved by PC5B (Cui et al.,

1998). Some of the first substrates shown to be processed by PC5A are the mullerian inhibiting substance and the receptor protein tyrosine phosphatase μ (RPTPμ) (Campan et al., 1996;Nachtigal and Ingraham, 1996). The specificity of PC5A cleavage is different than that of furin as PC5A easily processed proteins after single or paired basic residues without the requirement of a basic amino acid at P4. All of the substrates that have been identified as being cleaved by PC5 can also be cleaved by another proprotein convertase. Thus, the search for a specific substrate of PC5 is still in progress.

A.6 Inhibitors of the proprotein convertases

Since the proprotein convertases activate proteins implicated in various diseases (cancer, Alzheimer and viral infections), the PCs are attractive targets for the development of potent and selective inhibitors against each convertase. In

addition, the activity of the convertases is redundant on some substrates. Thus, the development of good and specific inhibitors will be useful in delineating the function and specific substrates of each convertase. Successful approaches developed include active-site-directed chloromethylketone inhibitors, reversible peptide-based inhibitors, plant derivatives, various engineered variants of protein-based inhibitors that possess a furin-like motif and endogenous inhibitors.

The first type of inhibitor, the active-site-directed chloromethylketone, consists of peptidyl sequences encompassing a furin-like motif coupled to chloromethylketone moiety. These inhibitory peptides enter the active site of the enzyme resulting in the formation of a covalent enzyme-inhibitor complex. These peptide were shown to be a highly potent irreversible inhibitors of both PC1 and furin and are also useful for the titration of the enzyme (Garten et al., 1994;Jean et al., 1995b). Reversible peptide-based inhibitors are potent competitive inhibitors. Studies of these inhibitors on furin and PC1 revealed that they were potent with Ki values in the micromolar range (Basak et al., 1995;Jean et al., 1995a). Another group has developed many peptide inhibitors with the best inhibitor against furin displaying a ki value in the nanomolar range (Angliker, 1995). The best known chlomethylketone peptide used to inhibit the proprotein convertases is the decanoyl-**Arg**-Val-**Lys**-**Arg**-chloromethylketone (dec-**R**V**KR**-cmk), but lacks selectivity.

The other type of inhibitor developed is derived from plant. The inhibitor is a chemical constituent of the medicinally active plant Andrographis paniculata (from the family Acanthaceae), also known as 'King of Bitters'. The constituent, neoandrographolide, exhibited the highest inhibitory action with an IC50 of 53μM against furin. Furthermore, although andrographolide exhibited a relatively small level of enzyme inhibition (IC50 = 1.0mM and Ki = 200μM against furin), upon succinoylation, its inhibitory action against furin, PC7 and PC1 was enhanced significantly with a Ki in the low micromolar range (Basak et al., 1999). Thus, this suggests that further modification of the andrographolide cytoskeleton may increase its inhibitory potency towards furin.

The protein-based variant inhibitors include α 2-macroglobulin (Van Rompaey et al., 1997), α 1-antitrypsin (Anderson et al., 1993;Benjannet et al., 1997;Jean et al., 1998) , proteinase inhibitor 8 (Dahlen et al., 1998), turkey ovomucoid third domain (Lu et al., 1993), englin c (Komiyama and Fuller, 2000;Komiyama et al., 2003;Liu et al., 2004) and the various convertase prodomains (Zhong et al., 1999;Fugere et al., 2002;Nour et al., 2003). α 2-macroglobulin (α 2m) is an endogenous glycoprotein that is abundant in the blood and inhibits a wide range of proteases by a unique mechanism. The inhibition starts by cleavage of a flexible and surface-accessible peptide stretch called the bait region within α 2m. The protease becomes 'trapped' by α 2m and

is thus sterically shielded from its substrate (Sottrup-Jensen, 1989). The bait region sequence of α 2m is not conserved between all the mammalian homologues, thus a group has inserted a furin-like motif within the bait region (α 2m-fur). This α 2m-fur was able to inhibit furin both *in vitro* and *in vivo* on the processing of TGF-β and the inhibitor formed a complex with the trap enzyme. The defect with this inhibitor is that even if there is a specific furin-like motif, α 2m-fur is not a specific inhibitor of the convertases as it also inhibits other proteases such as trypsin and elastase as potently as wild-type α 2m (Van Rompaey et al., 1997). The physiological inhibitor of neutrophil elastase is a serpin known as α 1-antitrypsin. A naturally occurring mutation was observed whereby the bait region of α 1-antitrypsin was mutated from AIPM to AIPR and became an inhibitor of thrombin called α 1-antitrypsin-Pittsburgh. Then, a group decided to mutate the alanine residue at P4 into an arginine in order to obtain a furin consensus site RIPR known as α 1-PDX. This engineered inhibitor was a success as it inhibits potently (Ki~ pM) several of the convertases of the constitutive secretory pathway both *in vitro* (Anderson et al., 1993;Jean et al., 1998) and *in vivo* (Anderson et al., 1993;Benjannet et al., 1997). The mechanism of inhibition of the serpin, α 1-AT, initially forms a noncovalent complex with the serine protease that involves no conformational change within the protease or the body of the serpin. Subsequent peptide bond hydrolysis results in an acyl-

enzyme intermediate (EI#) that progresses to either a kinetically trapped loop-inserted covalent complex (EI+, inhibitory pathway) or a cleaved serpin (I*) and free proteinase (noninhibitory or substrate pathway) as seen in Figure A-10. It is indeed a potent inhibitor of the proprotein convertases, but it is not selective as it can not be used to discriminate which PC is involved in a specific substrate processing. Recently, efforts have been made to increase the inhibitory selectivity of the rat α 1-antitrypsin towards furin, PC5 and PACE4 by mutagenesis of the bait reactive site loop. A mutant variant AV**RR**, instead of AV**PM**, inhibited furin and PC5 but not PACE4 *in vitro* indicating that selectivity can be achieved by mutating each position with the right amino acid (Tsuji et al., 2002). Another serpin that has inhibitory activity towards furin is the proteinase inhibitor 8. This serpin contains two sequences homologous to the minimal sequence for recognition by furin in its reactive site loop. This serpin was shown to be a potent inhibitor of furin *in vitro* with an overall Ki of 54 pM and was observed to form a SDS-stable complex with furin (Dahlen et al., 1998). This inhibitor is also not selective towards the proprotein convertases as it also inhibits trypsin-like proteases.

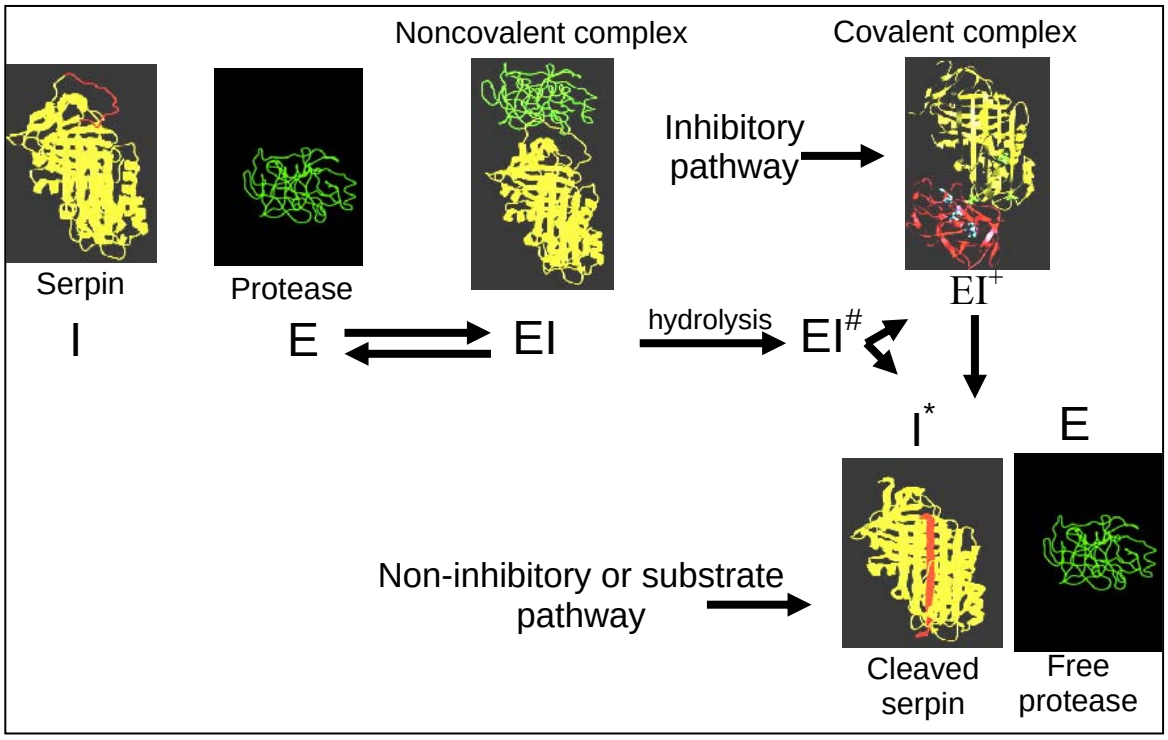


Figure A-10: Fate of the serpin (α 1-AT) and protease inhibitory complex. The serpin (I) inhibition of proteinase (E) proceeds via an initial noncovalent complex (EI) that involves no conformational change within the protease or the body of the serpin. Hydrolysis results in an acyl-enzyme intermediate (EI#) that progresses to either a covalent complex (EI+, inhibitory pathway) or a cleaved serpin (I*) and free proteinase (noninhibitory or substrate pathway).

Recently, a new family of genetically engineered inhibitors of proprotein convertases was created based on Eglin c, a polypeptide protease inhibitor from the medicinal leech Hirudo medicinalis (Komiyama and Fuller, 2000;Komiyama et al., 2003). This approach was used to develop high-affinity, selective furin inhibitors. Eglin c is thermostable and it is a potent inhibitor of degradative

subtilisins. A basic furin consensus motif and other variants were introduced within the Eglin c reactive site loop and these engineered proteins were expressed in bacteria. Their respective inhibitory actions were tested *in vitro* on furin and kexin on the processing of a fluorogenic substrate. It was shown that alteration of the loop of Eglin c by the introduction of the **R**-X-X-**R** motif was sufficient to inhibit both furin and kexin in the nanomolar range (Komiyama and Fuller, 2000). The same group has used the bacterial Eglin c variants and modified them further especially at P4' (Komiyama et al., 2003). When a tryptophan residue was introduced at P4' , the inhibitor was much more selective towards PC7 as compared to furin and kexin, in contrast, an aspartic residue at P4' makes the inhibitor more selective against furin. This aspartic mutant blocked the processing of the von Willebrand factor in COS-1 cells when added extracellularly into the culture medium of the cells (Komiyama et al., 2003). Thus, mutations at P4' of the reactive site loop of Eglin c could be optimized to increase the affinity of the inhibitor for the target protease or to discriminate between closely related target proteases.

The last type of protein variants discussed is based on the prosegment of the proprotein convertases. As mentioned earlier in the section on structure, the prosegments were shown to be potent inhibitors of the convertases, but not selective toward their cognate enzyme. The first study demonstrated that the

purified prosegment of PC1 from insect cells was a good inhibitor of PC1 and furin *in vitro* but not of PC2 (Boudreault et al., 1998a). Next, the prosegments of furin (pfurin) and PC7 (pPC7) were expressed and purified from bacteria and both were shown to be potent inhibitors (nanomolar) with pfurin being more specific toward PC5A and pPC7 and pPC5 being selective to their parent enzymes (Zhong et al., 1999;Nour et al., 2003). It was determined that the C-terminus arginine residue at P1 of both pfurin and pPC7 was essential for the inhibitory activity of the prosegment and that mutation into an alanine abolished the inhibitory effect *in vitro* and *ex vivo* see Figure A-11. Furthermore, the expression of the prosegments in mammalian cells resulted in the inhibition of the processing of NGF, BDNF and the HIV glycoprotein gp160 and the ppfurin was as potent as the general PC inhibitor α 1-PDX (Zhong et al., 1999). Moreover, another group has expressed all the convertases' prosegments and concluded that they are potent inhibitors but do not display significant specificity (Fugere et al., 2002). The mechanism of inhibition displayed by the prosegments derived from the PCs has been characterised and classified as a slow tight binding competitive inhibitor (Boudreault et al., 1998a;Zhong et al., 1999). On the other hand, the prosegment of yeast kexin behaves as a mixed inhibitor with an IC50 of 160nM (Lesage et al., 2001). Since a peptide fragment corresponding to the last 24 residues of the C-terminus of the prosegment of PC7 displayed a strong

inhibition of PC7 activity in comparison to the full-length prosegment, it was used to define the structure. It revealed a slightly kinked helical conformation for the entire peptide and it may be through this conformation that the inhibitory activity of the prosegment is conferred (Bhattacharjya et al., 2000). Therefore, the prosegments are powerful inhibitors but they lack specificity, which make them less of a good choice. The only way to increase the specificity of the prosegments would require the mutagenesis of each residue or the elucidation of the crystal structure of a convertase inhibited by its prosegment.

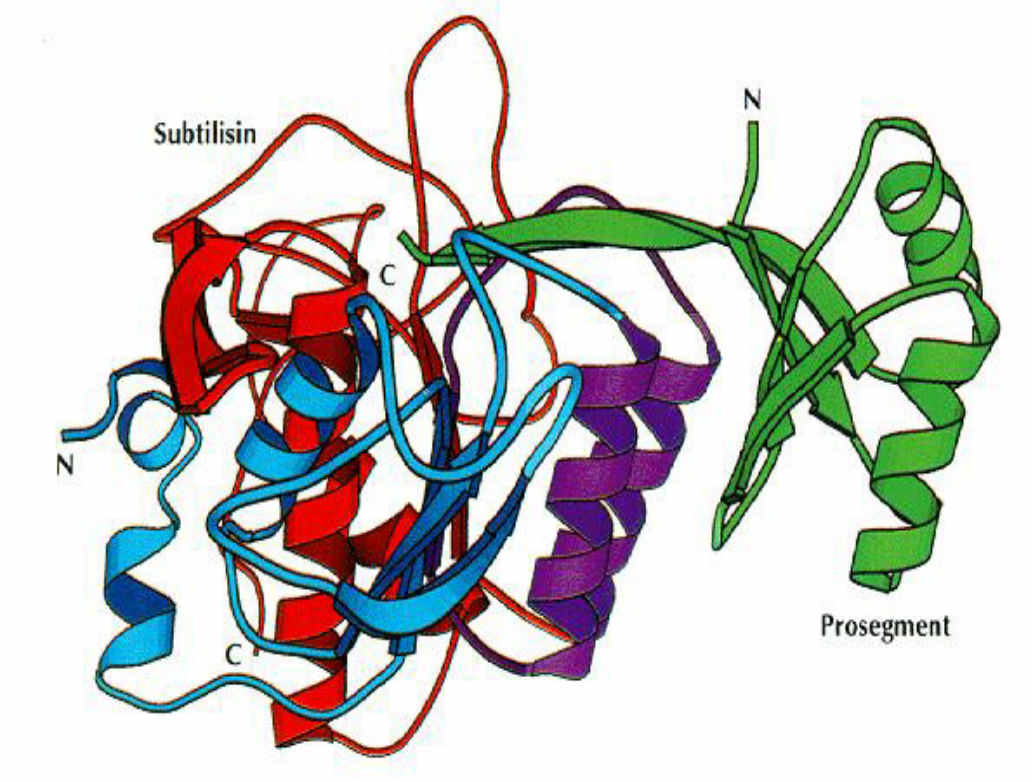


Figure A-11: The prosegment-subtilisin BPN' complex, the prodomain C-terminus binds in the enzyme active site in a product-like manner.

Some endogenous inhibitors of the convertases have been discovered in the last couple of years. The endogenous inhibitors of both PC1 and PC2 have been identified, the 7B2 protein inhibits PC2 (nanomolar) (Muller and Lindberg, 1999) while proSAAS inhibits PC1 (micromolar) (Fricker et al., 2000). ProSAAS possesses general similarities with granin-like proteins such as chromogranins A/B and 7B2. The distribution of proSAAS is similar to PC1: it is mainly found in the brain and in the gut. In the brain, the richest areas of proSAAS' expression are the hypothalamus and the pituitary, which is consistent with the brain distribution of PC1 (Sayah et al., 2001). The first study demonstrated that proSAAS was able to inhibit processing of POMC by PC1 in At-T20 cells (Fricker et al., 2000). Another group determined that the inhibitory activity of proSAAS was within a hexapeptide found at the C-terminus as 7B2 and peptides derived from that region were indeed highly potent (low nanomolar) and specific inhibitors of PC1 in comparison to PC2 and furin. The same group later showed that as 7B2, proSAAS possesses a furin-like motif that is cleaved by furin (Sayah et al., 2001). This processing does not require PC1 nor PC2 as in PC12 cells which are deficient in PC1 and PC2 (no mRNA), proSAAS was still well processed (Mzhavia et al., 2002). Moreover, mutations within the furin sequence inhibit the processing and also reduce the inhibitory potency of proSAAS (Basak et al., 2001) .Thus, the mechanism of action of proSAAS seems to be similar to 7B2.

Another endogenous protein that was shown to inhibit PC2 is the cystatin-related epididymal spermatogenic (CRES) protein (Cornwall et al., 2003). CRES is related to the family 2 cystatins part of the cystatin superfamily of cysteine protease inhibitor. CRES is different from the cystatins since it lacks sequences necessary for cysteine protease inhibition and is specifically expressed in reproductive and neuroendocrine tissues. Moreover, CRES did not inhibit the cysteine proteases cathepsin B and papain, but was a potent inhibitor (Ki of 25nM) of the serine protease PC2. CRES inhibits selectively PC2 as it did not inhibit PC1, furin, trypsin and the subtilisin (Cornwall et al., 2003). This novel inhibitor blocks PC2 activity by a mechanism that seems to differ from that of the serpin as it does not form a higher molecular mass complex of PC2 and CRES by SDS-PAGE. Recently, an endogenous secretory pathway serine protease inhibitor (serpin) from Drosophila has been identified and is called Spn4A (Osterwalder et al., 2004;Richer et al., 2004). Spn4A is involved in the regulation of peptide maturation in Drosophila and is closest vertebrate homologue is neuroserpin. Within its reactive site loop Spn4A contains a furin consensus cleavage **R**-R-**K**-**R** motif. Spn4A is a very potent inhibitor as it inhibits hfurin with a Ki of 13pM and dPC2 with a Ki of 3.5 nM and forms SDS-stable complex with each enzyme (Richer et al., 2004). This newly characterised serpin (Spn4A) is the most potent and efficient natural serpin of convertase to date. The search for

new endogenous inhibitors for the other convertase family members is still ongoing.

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CHAPTER B

Matrix-Metalloproteases

B.1 Discovery and structure of the matrix-metalloproteases (MMPs)

The metzincin superfamily, which belongs to the metalloproteinases, encodes a highly conserved zinc-binding motif containing three histidine residues that bind zinc and a conserved methionine-turn in the active-site helix. The metzincin superfamily includes serralysins, astacins, adamalysins and matrix metalloproteinases (**MMPs**) (Stocker et al., 1995). The first proteinase of the MMP family to be identified was initially named collagenase as this enzyme, produced in culture by resorbing tadpole tail, degraded gels made of fibrilar collagen (GROSS and LAPIERE, 1962). Since this discovery, a family of related enzymes belonging to the matrixin group has been characterised in species from hydra to humans and they are collectively called matrix-metalloproteinases (MMPs). At the moment, the vertebrate MMP family counts 28 members, 22 of which are found to be expressed in human tissues (Egeblad and Werb, 2002). MMPs share high protein sequence homology and are classified into eight distinct groups according to their structural properties. The secreted MMPs are divided into five groups. MMPs that possess the minimal-domain, MMPs containing a simple hemopexin domain, gelatin-binding MMPs, furin-activated secreted MMPs and vitronectin-like insert MMPs. The membrane-bound MMPs, also known as membrane-type matrix-metalloproteinases (MT-MMPs), are

divided into three classes that include type I transmembrane MMPs, glycosyl-phosphatidyl inositol (GPI)-linked MMPs and finally type II transmembrane MMPs. All these distinct subclasses with their members are described in Table B-1.

Table B-1: The MMPs family and their structural classes.

MMP designation*	Structural class	Common name(s)
MMP-1	Simple hemopexin domain	Collagenase-1, interstitial collagenase, fibroblast collagenase, tissue collagenase
MMP-2	Gelatin-binding	Gelatinase A, 72-kDa gelatinase, 72-kDa type IV collagenase, neutrophil gelatinase
MMP-3	Simple hemopexin domain	Stromelysin-1, transin-1, proteoglycanase, procollagenase-activating protein
MMP-7	Minimal domain	Matrilysin, matrin, PUMP1, small uterine metalloproteinase
MMP-8	Simple hemopexin domain	Collagenase-2, neutrophil collagenase, PMN collagenase, granulocyte collagenase
MMP-9	Gelatin-binding	Gelatinase B, 92-kDa gelatinase, 92-kDa type IV collagenase
MMP-10	Simple hemopexin domain	Stromelysin-2, transin-2
MMP-11	Furin-activated and secreted	Stromelysin-3
MMP-12	Simple hemopexin domain	Metalloelastase, macrophage elastase, macrophage metalloelastase
MMP-13	Simple hemopexin domain	Collagenase-3
MMP-14	Transmembrane	MT1-MMP, MT-MMP1
MMP-15	Transmembrane	MT2-MMP, MT-MMP2
MMP-16	Transmembrane	MT3-MMP, MT-MMP3
MMP-17	GPI-linked	MT4-MMP, MT-MMP4
MMP-18	Simple hemopexin domain	Collagenase-4 (*Xenopus;* no human homologue known)
MMP-19	Simple hemopexin domain	RASI-1, MMP-18‡
MMP-20	Simple hemopexin domain	Enamelysin
MMP-21§	Vitronectin-like insert	Homologue of *Xenopus* XMMP
MMP-22	Simple hemopexin domain	CMMP (chicken; no human homologue known)
MMP-23	Type II transmembrane‖	Cysteine array MMP (CA-MMP), femalysin, MIFR, MMP-21/MMP-22¶
MMP-24	Transmembrane	MT5-MMP, MT-MMP5
MMP-25	GPI-linked	MT6-MMP, MT-MMP6, leukolysin
MMP-26	Minimal domain	Endometase, matrilysin-2
MMP-27#	Simple hemopexin domain	
MMP-28	Furin-activated and secreted	Epilysin
No designation	Simple hemopexin domain	Mcol-A (Mouse)
No designation	Simple hemopexin domain	Mcol-B (Mouse)
No designation	Gelatin-binding	75-kDa gelatinase (chicken)

The fist category is comprised of MMPs that possess the minimal-domain, which consists of a signal peptide that directs the enzyme to the ER, a prodomain with a zinc-interacting thiol (SH) group that maintains the enzyme as an inactive zymogen and a catalytic domain that contains a zinc-binding site (Zn) (Muller et al., 1988; de Coignac et al., 2000). In addition to the aforementioned domains,

the second group, the simple hemopexin-domain containing MMPs, has a hemopexin-like domain connected to the catalytic domain through a hinge (H) region that mediates interactions with tissue inhibitors of metalloproteinases, cell surface molecules and substrates (Hunt et al., 1987). The hemopexin domain is composed of four repeats with the first and last repeat connected via a disulfide bond (S-S) (Bode et al., 1999). The gelatin-binding MMPs possess within their catalytic domain inserts similar to the collagen-binding type II repeats of fibronectin (Fi) (Collier et al., 1988; Murphy et al., 1994; Shipley et al., 1996). The furin-activated secreted MMPs have a furin recognition motif for intracellular proprotein convertases (F) between their prodomains and their active sites that permits the intracellular activation by the proprotein convertases (Sato et al., 1994). This furin motif is also found in the vitronectin-like insert (Vn) MMPs (Liang et al., 1997) and the membrane-type MMPs (MT-MMPs). The transmembrane MMPs have at their C-terminus a single-span transmembrane domain (Tm) and a short cytoplasmic tail (Ct) whereas the GPI-anchored MMPs have a glycosylphosphatidylinositol (GPI) at their C-terminus that anchors them to the membrane. The last group is composed of only MMP-23, which is very unique since it has N-terminal signal anchor (SA) that targets it to the plasma membrane and as such it is a type II transmembrane MMP. Furthermore, MMP-23 is distinctive because a cysteine array (CA) and an immunoglobulin-like domain (Ig)

are found at its C-terminus (Pei et al., 2000). The structures of the various classes are depicted in Figure B-1.

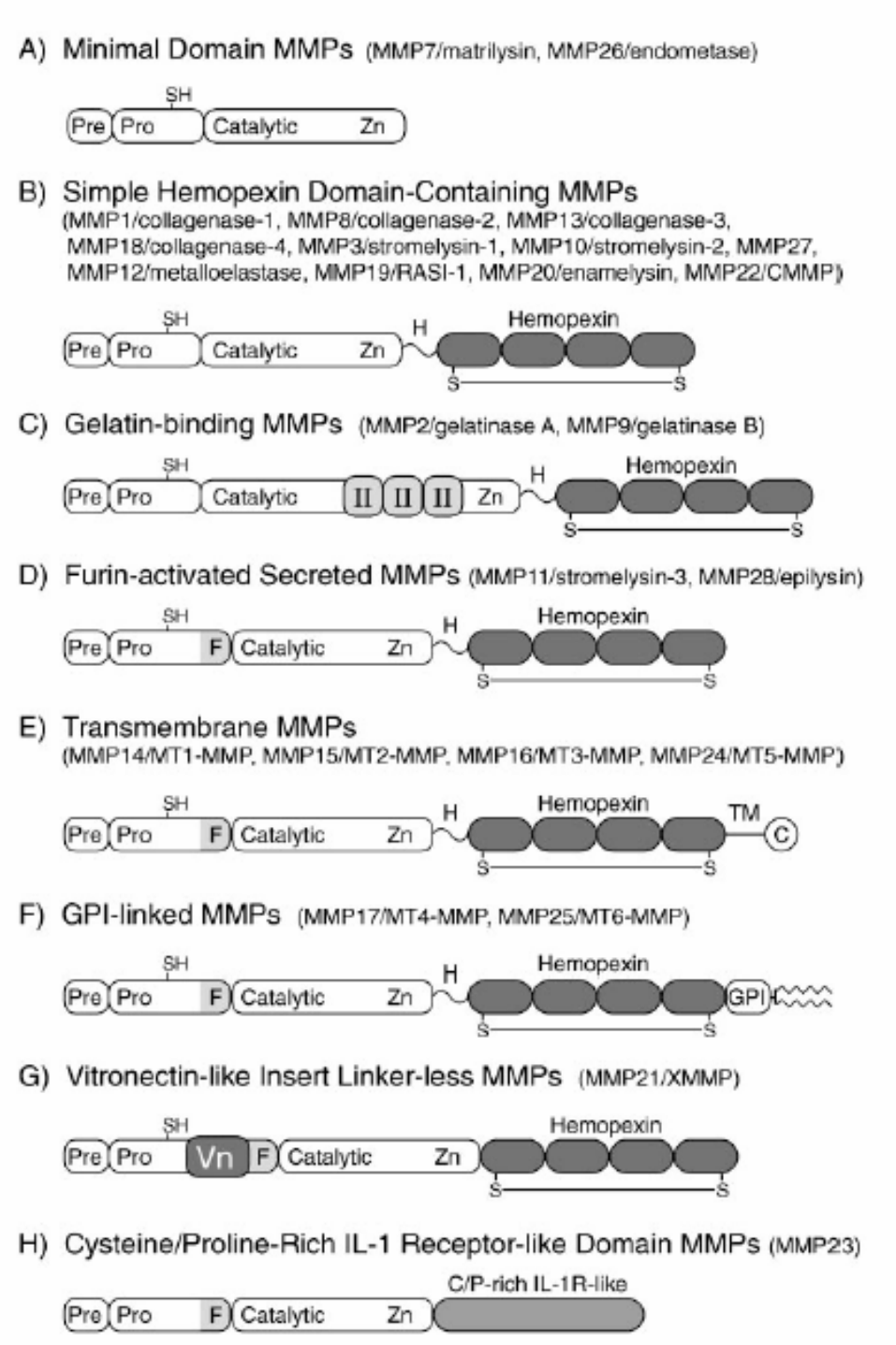

Figure B-1: The protein structure of the MMPs. Pre: signal peptide, Pro: prodomain, F: furin potential site, II: collagen-binding fibronectin type II inserts, H: hinge region, TM: transmembrane domain, C: cytoplasmic tail, GPI:

glycophosphatidyl inositol-anchoring domain, C/P: cysteine/proline, IL-1R: interleukin 1 receptor.

All MMPs are initially synthesized as a zymogen with a prodomain and most of them are secreted in their latent zymogen forms save for the furin activated MMPs. Within the prodomain, a conserved cysteine switch motif PR**C**XXPD is found that maintains the proMMP in a latent form (Springman et al., 1990; Van Wart and Birkedal-Hansen, 1990). From the crystal structures of various MMPs, it was determined that the catalytic domain is quite similar from one MMP to another, exhibiting a spherical shape with a flat active site cleft, which extends horizontally to interact with substrates and/or inhibitors (Bode et al., 1999). The proteolytic activity of the MMPs is zinc dependent and there is a conserved zinc-binding motif, **H**EF/L**GH**S/AL**G**LX**H**S, within the catalytic domains (Stocker et al., 1995). When present, the hemopexin domain is involved in TIMP (tissue inhibitor of metalloproteinase) binding, the binding of certain substrates, membrane activation and potentially in proteolytic activities. As an example, MMP-1 needs its C-terminus hemopexin domain in order to cleave native fibrillar collagens (Murphy et al., 1992; Sanchez-Lopez et al., 1993). The hinge region, rich in proline residues, varies in length and composition between the different MMPs that possess this domain. The hinge region affects the substrate specificity,

stability and proteolytic activity of the enzyme (Knauper et al., 1997). As previously mentioned, the gelatin-binding MMPs have 3 head-to-tail cysteine-rich repeats within their catalytic domain that represent fibronectin-like type II repeats. These three fibronectin inserted modules form the collagen binding domain and are thus important for the binding and cleavage of collagen and elastin (Murphy et al., 1994; Shipley et al., 1996). MMP-21 is quite unique as it is the only member having an inserted vitronectin-like domain within its prodomain. This domain is composed of four tandem repeats of a motif similar to that in vitronectin, an abundant cell adhesion protein in plasma and tissues (Otter et al., 1995). Vitronectin is an adhesive glycoprotein that is found associated to the ECM and it is involved in the regulation of blood coagulation on the basis of its ability to bind heparin, plasminogen, plasminogen activator inhibitor-1 and thrombin-inhibitor complexes (Kost et al., 1992). MMP-21 has been partially cloned from human multi-tissue gene libraries and the partial sequence is 73% identical to the sequence of XMMP from Xenopus laevis (Marchenko et al., 2001; Yang et al., 1997). The function of this domain within human MMP-21 and XMMP has not been characterised yet but it may play a role in ECM binding or simply in protein-protein interactions. Finally, MMP-9 also possesses a unique feature that consists of the insertion of a type V collagen-like domain of unknown importance at the end of its hinge region.

B.2 MMPs activation and their functions

Since the MMPs are synthesized as inactive zymogens, they need to undergo processing of their prosegments to become fully active. The furin-activated MMPs have a furin cleavage site between their prodomain and their catalytic site. These MMPs are cleaved intracellularly by proprotein convertases such as furin and PACE4 that release the prodomain and this result in the activation of the MMPs as seen in Figure B-2. The first MMP that was shown to be processed by a furin-like protease was stromelysin-3 (MMP-11) after the R-X-K-R site (Pei et al., 2000; Santavicca et al., 1996). The other MMP that was described to be activated by a furin-like convertase is MT1-MMP. It was shown that mutations within the furin cleavage site (RRKR) abolished the maturation of proMT1-MMP into MT1-MMP and that overexpression of the convertase inhibitor, α 1-PDX, efficiently blocked the conversion into the active form (Figure B-2) (Imai et al., 1996; Pei and Weiss, 1996; Yana and Weiss, 2000). Recently, the activation of MMP-28 and MT5-MMP were shown to be blocked by a synthetic peptidyl-CMK furin inhibitor in cells suggesting that MMP-28 (Illman et al., 2003) and MT5-MMP (Wang and Pei, 2001) are both cleaved by a furin-like enzyme in these cells. MT3-MMP is also activated by furin intracellularly and mutation of the

arginine residue at P1 into an alanine (RA) of the furin consensus motif, resulted in a complete loss of MT3-MMP activation. Moreover, they assayed the ability of the RA mutant to form cysts from dispersed singular cells in three-dimensional collagen gels as compared to WT. The cells transfected with WT MT3-MMP formed a bigger cyst than the control or the RA mutant; in fact, the RA mutant behaved as the catalytic site mutant indicating that furin cleavage is required for the activity of MT3-MMP (Kang et al., 2002).

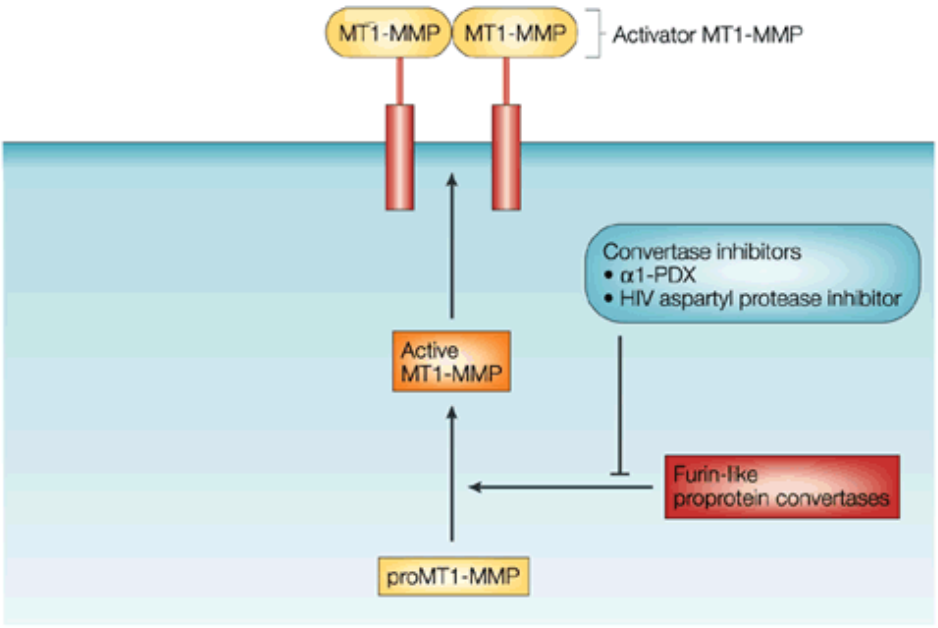

Figure B-2: Intracellular activation of MMP by the furin-like convertases.

The other MMPs that lack a furin-susceptible cleavage site are thus activated extracellularly by another mechanism upon secretion. The MMPs are kept inactive through an unpaired cysteine sulfhydryl group found at the C-

terminal end of their prodomain forming a cysteine-switch. Their latent state is maintained by the interaction of the free cysteine with the zinc ion bound to the catalytic domain and thus their prodomain masks the active site cleft (Springman et al., 1990). Activation requires opening of this cysteine-zinc switch by proteolytic removal of the prodomain and this is performed by other activated MMPs or serine proteases (Nagase and Woessner, Jr., 1999). Following initial cleavage, the structure of the prosegment unfolds leading to the exposure of additional sites for further cleavage by other enzymes or by autolytic processing in trans (Nagase et al., 1990). A well described activation of one MMP by another MMP activator is the activation of proMMP-2 by MT1-MMP. MT1-MMP is an activator of MMP-2 whereas both MT4-MMP and MT2-MMP are the only MT-MMP unable to activate MMP-2 (Cao et al., 1998; English et al., 2000; Miyamori et al., 2000). First, a cell surface MT1-MMP binds the N-terminal domain of TIMP-2, a tissue inhibitor of metalloproteinase 2 and is thus inhibited; the C-terminal domain of bound TIMP-2 interacts with the hemopexin domain of proMMP-2 forming a ternary complex (Zucker et al., 1998). Then, an adjacent activated MT1-MMP is recruited and activates proMMP-2 via processing. Following this first cleavage, a residual portion of the prodomain is removed by another activated MMP-2 to result in the fully active, mature form of MMP-2 (Deryugina et al., 2001). Moreover, removal of the prodomain of MT1-MMP is not essential for

its interaction with TIMP-2 and the activation of proMMP-2 (Cao et al., 1998). This mechanism of activation of proMMP-2 is shown in Figure B-3.

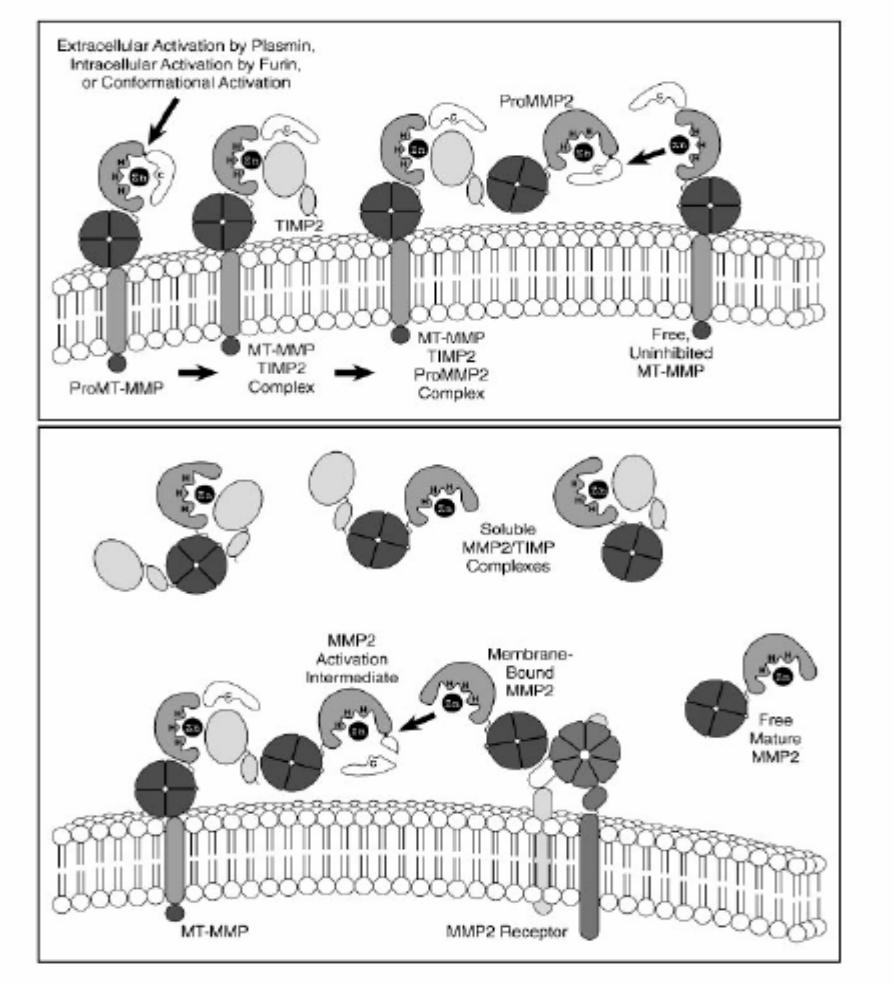

Figure B-3: Activation of MMP-2 at the cell surface.

The predominant function of the MMPs is to degrade proteins that are part of the extracellular matrix (ECM) for ECM remodelling thereby promoting cell migration. MMPs also play a role in cellular signalling and functions since they cleave cell receptors (e.g. integrins, growth factor receptors) that interact with

structural ECM components (Streuli, 1999). MMPs were shown to play a role in the regulation of cell growth since in many different MMP knockouts, there is a marked decrease in cell proliferation in tumours (Bergers et al., 2000; Coussens et al., 2000). There are three known mechanisms by which MMPs promote cancer cell proliferation. i) MMPs release cell membrane-bound precursors of various growth factors (e.g. TNF-α) (Peschon et al., 1998). ii) MMPs cleave growth factors that are sequestered by ECM proteins and thus become bioavailable (e.g. IGF-BPs) (Manes et al., 1997; Manes et al., 1999). iii) MMPs might regulate proliferative signals through cleavage of receptors that will affect ECM composition (e.g. integrins) (Agrez et al., 1994). Most of the substrates listed above and others are seen in Table B-2. Another function of the MMPs is in the regulation of apoptosis. A cell that evades apoptosis is able to survive in stressed and deprived conditions. Many MMPs have both apoptotic and anti-apoptotic roles. The MMPs that have been shown to be involved in the regulation of apoptosis are MMP-3, -7, -9 and -11. Overexpression of MMP-3 in mammary epithelial cells induces apoptosis possibly via degradation of laminin (Sympson et al., 1994; Witty et al., 1995), while MMP-7 induces apoptosis through the release of membrane-bound FASL, a promoter of cell death (Mitsiades et al., 2001; Powell et al., 1999). However, MMP-7 has a dual function as it can inhibit apoptosis by cleavage of pro-HB-EGF that will in turn stimulate apoptosis through

the ErBb4 receptor (Yu et al., 2002). MMP-11 also has both apoptotic and anti-apoptotic actions since overexpression of MMP-11 decreases apoptosis in tumour xenografts whereas cancer cells injected into MMP-11 null mice show a higher rate of apoptosis as compared to the wild-type mice (Boulay et al., 2001; Wu et al., 2001). In addition, MMP-11 and MMP-9 increase apoptosis during development although they both decrease cancer-cell apoptosis (Ishizuya-Oka et al., 2000; Vu et al., 1998). Thus, their involvement in the regulation of apoptosis is not clear cut as some possess both pro-apoptotic and anti-apoptotic roles depending on the system.

Another function of the MMPs is the regulation of angiogenesis. Angiogenesis is a process by which new blood vessels are formed within tumour tissue. MMPs were deduced to be important positive regulators of angiogenesis since endogenous and synthetic MMP inhibitors reduced tumour angiogenesis in animal models (Gatto et al., 1999; Li et al., 2001; Martin et al., 1999). In this process, the MMPs may simply act by degrading the ECM thereby allowing the endothelial cells that forms the vessels, to invade the tumour stroma. It was confirmed that cleavage of collagen type I is essential for endothelial cell invasion of the ECM and for vessel formation (Seandel et al., 2001). Some MMPs such as MMP-2, -9 and -14 are direct positive regulators of angiogenesis since in MMP-2 deficient mice, tumour angiogenesis and growth are reduced (Itoh et al., 1998).

Moreover, MMP-14 and MMP-9 null mice have impaired angiogenesis during development, supporting a role for theses MMPs in angiogenesis (Vu et al., 1998; Zhou et al., 2000). It is well known that MMPs play an important role in cancer as well as invasion and metastasis. In the metastatic process, the cancer cells need to traverse the ECM barriers which are degraded by the MMPs in order to enter the blood vessels and lymphatic extravasate and establish new proliferating colonies in other tissues. While the overexpression of TIMPs inhibits, overexpression of MMP-2, -3,-13 and -14 promotes invasion of cells through either collagen type I or matrigel (Ahonen et al., 1998; Ala-aho et al., 2002; Deryugina et al., 1997; Lochter et al., 1997). In experimental metastatic models, it was shown that downregulation of MMP-9 in cancer cells caused a reduction in the number of colonies formed in the lungs, and it is also significantly reduced in the MMP-2 and MMP-9 null mice compared to wild-type animals (Itoh et al., 1998; Itoh et al., 1999). MMPs not only play a role in cancer but also during the immune responses triggered against the cancer cells. Inflammatory reactions are an important component of human neoplasia (Coussens and Werb, 2002). The immune system is able to recognise the cancer cells as foreign and attack them although the cancer cells have developed many ways to avoid the immune response. The mechanisms by which cancer cells escape the immune surveillance involve the MMPs. Many inflammatory cells of the immune system

such as macrophages, neutrophils, natural killer cells and tumour-specific cytotoxic T lymphocytes infiltrate the tumours. Cytokine signalling through the interleukin-2 receptor-α (IL-2Rα) (see Table B-2) regulates the proliferation of T lymphocytes and MMPs, including MMP-9, which cleaves IL-2Rα thereby suppressing the proliferation of T lymphocytes (Sheu et al., 2001). In addition, TGF-β an important inhibitor of the T lymphocyte response towards tumours is activated by the MMPs (Gorelik and Flavell, 2001). It was suggested that MMP-11 inhibits chemoattraction of neutrophils and macrophages as there is an augmentation of the infiltration of tumours by these cells in MMP-11 null mice compared to wild-type animals (Boulay et al., 2001). In addition to an immune response linked with cancer cells, some cancers are also associated with chronic inflammation such as skin, breast, liver and ovary cancer (Coussens and Werb, 2002). It has been demonstrated that inflammatory cells synthesize several MMPs such as MMP-9, -12 and -14, thus these cells may stimulate cancer progression through the release of MMPs. In fact, mice that are prone to develop skin cancer have reduced tumour incidence when they are MMP-9 null (Coussens et al., 2000). Finally, the MMPs have many different functions that evolved around cell proliferation and ECM turnover.

Table B-2: List of common MMPs substrates.

MMP	1	2	3	7	8	9	10	11	12	13	14	16	18	19	26
ECM proteins:															
Aggrecan	+	+	+	+	+	+	+		+	+	+				
Collagen I	+	+	-	+	+	-			+	+	+		+	+	-
Collagen II	+				+	-				+	+				
Collagen III	+	+	+	-	+	-	+			+	+	+			
Collagen IV	-	+	+	+	-	+	+	-	+	-				+	+
Collagen V	-	+	+	-	-	+	+			-					
Collagen VI	-	-	-			-				+					
Collagen VII	+	+	+												
Collagen VIII	+														
Collagen IX	-	-	+							+					
Collagen X	+	+	+	-						+					
Collagen XI	+	+	+			+				-					

MMP	1	2	3	7	8	9	10	11	12	13	14	16	18	19	26
Collagen XIV	-		-	-		+				+					
Decorin	-	+	+	+		+									
Elastin	-	+	+	+		+	+		+						
Entactin	+	+	+	+					+		+				
Fibrilin		+	+			+			+	+	+				
Fibronectin	+	+	+	+	-	-	+		+	+	+	+		+	+
Fibulins		+		+											
Gelatin I	+	+	+	+		+	+		+	+	+			+	+
IGFBPs	+	+	+					+							
Laminin	+	+	+	+		+		-	+		+				-
Link protein	+	+	+	+		+	+								
Myelin basic	+	+	+	+		+			+						
Osteonectin		+	+	+		+				+					
Tenascin	+	+	+	+		-								+	

MMP	1	2	3	7	8	9	10	11	12	13	14	16	18	19	26
Vitronectin	+	+	+	+		+			+		+				
Other proteins:															
α 1-AC	+	+	+			-									
α 2-M	+	-	+		+	+		+	+	+	+				
α 1-PI	+	+	+	+	+	+		+	+		+				+
Casein	+	-	+	+		+	+	-		+				+	-
C1q	+	+	+		+	+				+					
E-cadherin			+	+											
Factor XII					-				+	+	+				
Fibrin	+	+	+			+					+				
Fibrinogen	+	+	+	+	+	+	+		+	+	+				
IL1α	-	-	-			-									
IL1β	+	+	+			+									
ProMMP2											+	+			

MMP	1	2	3	7	8	9	10	11	12	13	14	16	18	19	26
ProTGFβ		+				+									
ProTNFα	+	+	+	+		+			+		+				
Plasminogen		+	+	+		+			+						
Substance P		+	+		+	+									
T kininogen		-	+			-									

B.3 Identification of endogenous inhibitors of MMPs (TIMPs)

MMP activity can be abolished by the general inhibitor α2-macroglobulin and by more specific inhibitors such as the TIMPs. TIMP is a diminutive for tissue inhibitor of matrix- matalloproteinases and TIMPs are endogenous inhibitors. The human TIMP family is composed of at least four (TIMPs 1-4) secreted proteins or ECM-anchored proteins that reversibly inhibit the MMPs in a 1:1 stoichiometric ratio (Brew et al., 2000). TIMP-1, TIMP-2, and TIMP-4 are secreted proteins while TIMP-3 is bound to the extracellular matrix. The four TIMPs share between 37-51% sequence identity and have 12 conserved cysteine residues with conserved

relative spacing. These residues form 6 disulfide bridges resulting in a conserved six-loop and two domains structure. The two domains are known as the N-terminal and C-terminal domains. The N-terminal domain possesses the inhibitory activity against the MMPs and binds to their active site. In contrast, the C-terminal domain has at least two separate enzyme binding sites that interact with the hemopexin C domain and stabilise the enzyme-inhibitor complex (Murphy and Willenbrock, 1995; Willenbrock and Murphy, 1994). The C-terminal domain of TIMP is more variable among the four members and binds to the hemopexin domain of several proMMPs (proMMP-9 in the case of TIMP-1 and proMMP-2 in the case of TIMP-2 and TIMP-4), allowing for the formation of TIMP/proMMP complexes that retain an antiMMP activity. The TIMPs are capable of inhibiting all tested eukaryotic MMPs, but each exhibits specific binding to a particular gelatinase at a site distinct from the active site. They influence the activation of the pro-MMPs and act to modulate proteolysis of the ECM, especially during tissue remodelling and inflammatory processes.

TIMP-1 was initially characterized as EPA, Erythroid-Potentiating Activity, because it was stimulating the growth of human and murine erythroid progenitors (Gasson et al., 1985). Its mitogenic activity was also demonstrated *in vitro* as it is capable of mediating erythropoiesis. TIMP-1 can inhibit numerous MMPs

including MMP-1, -2, -3, -7, -8, -9, -10, -11, -12, -13 and -16 to a certain extent but not MT1-MMP. TIMP-1 is expressed constitutively showing maximal transcript levels in the bone, lung and ovary, with lesser accumulation in the heart, thymus and uterus (see Table B-3) (Leco et al., 1994; Nomura et al., 1989; Blavier et al., 1999). TIMP-1 transcripts are mainly found at site of active remodelling such as developing bone in the early mouse embryo (Flenniken and Williams, 1990). Initial studies on TIMP-1 demonstrated that it could inhibit invasion and metastasis *in vitro* by transfection of malignant cells with TIMP-1 (DeClerck et al., 1992). Moreover, metastasis to the liver is reduced in TIMP-1 overexpressing mice and is increased in mice that express a TIMP-1 antisense (Kruger et al., 1997). Likewise, ectopic overexpression of TIMP-1 in the brain of transgenic mice caused a reduction of metastasis to the brain (Kruger et al., 1998). In contrast, metastasis to the lungs was not increased in TIMP-1-deficient mice (Soloway et al., 1996). Finally, the knockout of TIMP-1 in mice causes a resistance to corneal Pseudomonas infections (Osiewicz et al., 1999). A very unique characteristic of TIMP-1, as compared to the other TIMPs, is that it can be detected in the nucleus. It was demonstrated that TIMP-1 accumulated in the nuclei of human gingival fibroblasts cells, reaching a maximum accumulation in the S phase of the cell cycle whereas TIMP-2 and -3 were not detected in the nucleus although the cells expressed their mRNAs (Zhao et al., 1998). Another study on the cellular and

subcellular localization of TIMP-1 revealed that a chimeric protein of TIMP-1 fused at its C-terminus to EGFP, enhanced green fluorescein protein, was visualized initially bound to the surface of MCF-7 breast carcinoma cells. However, TIMP-1-EGFP was also found in the nucleus of MCF-7 cells after 72 hrs in culture (Ritter et al., 1999) but the nuclear role of TIMP-1 has not been identified yet.

TIMP-2 is the most studied and understood member of the TIMP family. TIMP-2 is widely expressed throughout the body and Northern blot analysis indicated high levels of TIMP-2 mRNA in the lung, skin, reproductive organs, and brain (see Table B-3) (Blavier et al., 1999). Lower levels of expression were detected in all other organs. In contrast, liver and gastrointestinal tissues were negative for TIMP-2 with complete absence of TIMP-2 mRNA in the epithelium (Blavier and DeClerck, 1997). TIMP-2 is either found as a free form or bound to (inactive) proMMP-2 via its C-domain (Goldberg et al., 1989). TIMP-2 inhibits many MMPs such as MMP-1, -2, -3, -7, -8, -9, -10, -13, -14, -15,-16 and -19. TIMP-2 does not only inhibit MMPs, it is also required for the cell surface activation of proMMP-2 by MT1-MMP, its dominant function *in vivo* see Figure B-3 (Strongin et al., 1995). This was further supported with the TIMP-2 deficient mice that display no gross anatomical or microscopic abnormalities and retain reproductive capability despite severe impairment of proMMP-2 activation *in vivo*

and *in vitro* (Wang et al., 2000; Caterina et al., 2000). It is of note that the subtlety of the TIMP-2 deficient phenotype is not dissimilar to that of the MMP-2-deficient mouse (Itoh et al., 1997). Low to moderate levels of TIMP-2 promote the activation of proMMP-2, while higher levels inhibit its activation by saturating free active MT1-MMPs that are needed to cleave the proMMP-2 prodomain (Strongin et al., 1995). The protein levels of TIMP-2 can be regulated by the presence of type IV collagen, a substrate of MMP-2. When this substrate is present, it causes a reduction in the levels of TIMP-2 and enhances proMMP-2 activation (Maquoi et al., 2000). Furthermore, the ability of collagen to induce proMMP-2 activation on demand possibly results from the degradation of TIMP-2 since there are no changes in MMP-2, MT1-MMP, or TIMP-2 mRNA expressions or in the synthesis or activation of MT1-MMP. In addition, the C-terminal domain of TIMP-2 does not only interact with proMMP-2, but is also involved in its high-affinity binding to the cell surface since the level of TIMP-2-Δ C at the cell surface was lower than full-length TIMP-2 (Ko et al., 1997).

Human TIMP-3 shows slightly closer amino acid sequence similarity to TIMP-2 than to TIMP-1. TIMP-3 exhibits a distinctive pattern of restricted expression in adult tissues, with abundant transcripts detected in kidneys, lungs, ovary, muscle and the brain but low levels in the bones (see Table B-3) (Blavier et al., 1999), which is the richest source of TIMP-1 (Leco et al., 1994; Apte et al.,

1994). TIMP-3 is unique among the four TIMPs in that it binds tightly to the extracellular matrix (Pavloff et al., 1992; Yang and Hawkes, 1992) via its C-terminal domain. TIMP-3 is expressed in epithelial cells and is very rich in the retinal pigment epithelium. The importance of TIMP-3 in the eye is indicated by its increased expression in various degenerative retinal diseases such as Sorsby' s fundus dystrophy (SFD), an autosomal-dominant form of early retinal degeneration (Langton et al., 1998). Affected family members of SFD usually possess a missense mutation that introduce an extra cysteine residue within the C-terminal domain or a truncation of the C-terminal domain leaving a unpaired cysteine residue (Langton et al., 2000). These TIMP-3 mutant proteins dimerize via the unpaired cysteine residue and show diminished turnover, however, they retain their inhibitory activity towards MMPs and their ECM binding properties (Langton et al., 2000). TIMP-3, as the other TIMPs, is not a specific inhibitor of one MMP; it has been shown to inhibit MMP-1, -2, -3, -7, -9, -13, -14 and -15. TIMP-3 null mice showed an accelerated mammary gland involution as compared to the control depicting an anti-apoptotic role of TIMP-3 in mammary glands (Sternlicht and Werb, 2001; Fata et al., 2001). In contrast, TIMP-3-null animals develop spontaneous air space enlargement in the lung that is evident at 2 weeks after birth and progresses with the age of the animal (Leco et al., 2001). Finally, TIMP-3 expression was analysed in pregnant deciduas at various stages to verify

if it could be involved in the implantation process. TIMP-3 transcripts accumulated between day 5.5 and day 7.5, and were confined to the proximal decidual zone and there is an overlap in the expression pattern of TIMP-3 and PC5 (Leco et al., 1996; Wong et al., 2002). Moreover, there is a coordinated upregulation of both TIMP-3 and PC5 during artificial decidualization suggesting that PC5 may participate in the regulation of TIMP-3 through activation of TGF-β , activin and/ or IL-11 {Wong, 2002 149 /id;Salamonsen, 2003 247 /id}.

The last member of the family to be identified is TIMP-4. Northern blotting analysis revealed that TIMP-4 is abundant in the heart with very low levels detectable in the kidney, placenta, colon, and testis (see Table B-3) (Greene et al., 1996; Blavier et al., 1999). This unique expression pattern suggests that TIMP-4 may function in a tissue-specific fashion. TIMP-4 was shown to inhibit various MMPs such as MMP-1, -2, -3, -7, -9 and -14. TIMP-4, a close homologue of TIMP-2, also binds to proMMP-2. TIMP-4, unlike TIMP-2, does not promote proMMP-2 activation by MT1-MMP even though it interacts with proMMP-2. However, TIMP-4 like TIMP-2 binds to MT1-MMP inhibiting its autocatalytic processing (Hernandez-Barrantes et al., 2001). TIMP-4 also possesses an anti-proliferative role since overexpression of TIMP-4 in human breast cells inhibited the invasion potential of these cells *in vitro* (Wang et al., 1997). Moreover, when these cells were injected orthotopically into nude mice, TIMP-4 transfectants

showed a significant inhibition in tumour growth with a decrease by 4-10-fold in primary tumour volumes and a decrease in an axillary lymph node and lung metastasis as compared with controls (Wang et al., 1997). Since TIMP-4 is rich in the heart, a study sought to determine whether TIMP-4 induces apoptosis in cultured cardiac normal and polyomavirus transformed fibroblast cells. The data suggested that TIMP-4 controlled normal cardiac fibroblast transformation and induced apoptosis in transformed cells (Tummalapalli et al., 2001). Additional evidence for a special role of TIMP-4 in the development and function of the adult heart comes from observations of TIMP-4 overexpression in the endothelium of large vessels after balloon injury in rabbits (Dollery et al., 1999).

Table B-3: Characteristics of TIMPs 1-4

Characteristics	TIMP-1	TIMP-2	TIMP-3	TIMP-4
MR	28 kDa	21 kDa	24 kDa	22 kDa
Tissue specificity (mouse)	Bone, ovary, heart	Lung, heart, muscle, brain, skin, vessels, testis, ovary, placenta	Kidney, heart, brain, lung, ovary, placenta	Heart, brain, muscle, ovary
Inhibition of tumor invasion	+	+	+	+
Growth promoting activity	+	+	n.d.	n.d.
Inhibition of apoptosis	+	n.d.	n.d.	n.d.
Inhibition of angiogenesis	+	+	+	+

n.d. = no data.

B.4 Implication of TIMP2 in cellular proliferation

It is well known that MMPs and their inhibitors, TIMPs, are the hallmarks of cancer. MMPs are involved in several steps of cancer development whereas the TIMPs inhibit MMPs and thus are thought to inhibit cancer. It has been shown that overexpression of TIMPs by cDNA transfection in malignant cells not only inhibits local invasion but also tumour growth. As an example, overexpression of TIMP-2 markedly reduced melanoma growth in the skin of immunodeficient mice but did not prevent these highly malignant cells from spontaneously metastasizing to the lungs and lymph nodes of inoculated mice (Montgomery et al., 1994). Moreover, retroviral-mediated transduction of TIMP-2 cDNA into a limited population of tumour cells *in vivo* is sufficient to increase the accumulation of connective tissue proteins in tumour tissue, to inhibit growth and to prevent local invasion (Imren et al., 1996). There are several mechanisms that support an anti-tumour role of TIMPs. First, TIMPs have an anti-angiogenic activity either by a direct effect on endothelial cell proliferation or by their ability to downregulate the activity of MMPs required for endothelial cell migration and invasion. TIMP-1 and TIMP-2 have been purified in part from cartilage as anti-angiogenic factors

(Moses et al., 1990). TIMP-2 inhibits the growth of basic FGF-stimulated endothelial cells, which induce human microvascular cell proliferation, (Murphy et al., 1993) and inhibits tumour vascularization *in vivo* (Valente et al., 1998). Another mechanism that supports an anti-tumour activity of TIMPs is based on the increasingly recognized fact that the ECM can control essential cellular functions such as growth, differentiation, and apoptosis. It was demonstrated that TIMP-2 inhibits the growth of human melanoma cells when the cells are grown in the presence of intact fibrillar collagen (Montgomery et al., 1994). This anti-proliferative effect involves inhibition of cell-spreading, upregulation of the cyclin-dependent kinase inhibitor p27KIP-1, and blocks the cell cycle at the G1-S transition point. This growth-inhibitory effect is lost because the melanoma cells produced MMPs that degrade fibrillar collagen, but is maintained if the degradation of collagen is prevented in the presence of excess TIMP-2. Thus, by maintaining the integrity of the ECM, TIMPs maintain an indirect control over malignant cell proliferation. There is also experimental evidence suggesting that TIMPs may have MMP-independent cancer-promoting activities. In bladder and breast cancers, elevated levels of TIMP-2 in tumour tissues predict an unfavourable rather than a favourable outcome (Grignon et al., 1996; Visscher et al., 1994). It has now become clear that in addition to inhibiting the activity of MMPs, certain TIMPs have a function that either enhances proteolytic

degradation of the ECM or directly affects cell survival and growth. It is known, however, that TIMP-2 can bind to the cell surface with high affinity (Kd in the nM range), suggesting that TIMP-2 may behave as a ligand like growth factors and cytokines (Ko et al., 1997). However, the nature of a putative cell-associated "TIMP-2 receptor" has not been resolved yet. MT1-MMP could be such a "receptor" for TIMP-2, but there has been no evidence that MT1-MMP relays intracellular signals upon TIMP-2 binding and behaves as a true receptor. It was recently suggested that the receptor of TIMP-2 could be the integrin α 3β 1 since they were shown to co-immunoprecipitate together in hMVEC cells. It was demonstrated that TIMP-2 abrogates angiogenic factor-induced endothelial cell proliferation *in vitro* and angiogenesis *in vivo* independent of MMPs inhibition (Seo et al., 2003). These effects would require the α 3β 1 integrin-mediated binding of TIMP-2 to endothelial cells. Furthermore, TIMP-2 induces a decrease in total protein tyrosine phosphatase (PTP) activity associated with β 1 integrin subunits as well as dissociation of the phosphatase SHP-1 from β 1 (Seo et al., 2003). Therefore, integrin α 3β 1 could be the TIMP-2 receptor that mediates the intracellular signalling and can retain TIMP-2 at the cell surface.

MMP-2 which is known to play a critical role in tumour invasion is complexed with and inhibited by TIMP-2. Since TIMP-2 has demonstrated anti-

invasive, anti-metastatic, and anti-angiogenic activities, it has been considered as a therapeutic agent in multiple preclinical animal models of cancer. Thus, TIMP-2 could be useful to inhibit cancer metastasis (Musso et al., 1997). In a first approach, the use of recombinant TIMP-2 to prevent metastasis has been tested. Repeated intraperitoneal injections of rTIMP-2 have been shown to inhibit lung colonization of B16 melanoma cells in mice (DeClerck et al., 1992). However, the rapid *in vivo* clearance of this recombinant protein (the TIMP-2 plasma half-life is 4 min) and the amount of inhibitor required (0.9 mg/animal) have precluded this recombinant protein from any systemic use. Instead of using recombinant TIMP-2, Imren et al co-injected mice tumour cells with retroviral producer cells containing a TIMP-2 cDNA resulting in the overexpression of TIMP-2 in approximately 13% of the tumour cell population. Overexpression of TIMP-2 in these cells reduced tumour growth by 75% and blocked local invasion (Imren et al., 1996). Another group used the adenoviral transfer approach instead of the retroviral approach. They have produced an adenovirus of TIMP-2 and through systemic Ad-TIMP-2 treatment, reduced the formation of colorectal liver metastases and the growth of well-established metastases (Brand et al., 2000).

The report that TIMPs can not only block tumour invasion and metastasis, but also inhibit the growth of primary tumours has been a fundamental observation in support of the use of synthetic MMP inhibitors in cancer clinical

trials that are currently ongoing (Wojtowicz-Praga et al., 1998). Experiments testing the possibility of using TIMP as a therapeutic agent in cancer have not produced significant results; however, this is largely because of the pharmacologic properties of TIMPs and the current limitations in gene delivery. A better understanding of the dual functions of TIMPs, and in particular of the signalling pathways involved in their effect on tumour growth and survival, are also needed to further determine whether TIMP could be of potential benefit in cancer treatment.

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Preface

PC5A is an enzyme that is sorted to both the constitutive and regulated secretory pathways. This convertase was shown to cleave various substrates, but none of which are specific to PC5A. Most of the precursors processed by PC5A are also processed by furin or other convertases. In order to characterize specific substrates of PC5A, it is imperative to design specific inhibitor of each convertase to regulate their enzymatic activities. It was known that the prosegments of furin and PC7 were potent inhibitors, but not selective to their parent enzyme and we wondered if the prosegment of PC5 had similar properties.

This chapter describes the inhibitory function of the prosegment of PC5. Although it is known that the prosegments can act as inhibitors, this is the first study that tests the prosegment of PC5 both *in vitro* and *ex vivo*. For the first time, it is demonstrated that site-directed mutagenesis within the C-terminal end of full-length prosegement of PC5 do not increase the potency nor the selectivity of the inhibitor. Moreover, with the use of furin-deficient cells, FD11 cells, it was possible to test specifically the inhibitors on PC5A and furin. In these cells, the proVEGF-C substrate is not cleaved by endogenous enzymes, thus in the cells overexpressing PC5A or furin, the cleavage observed is specific to each convertase and so is the inhibition. At the beginning of this study, very little, if anything, was known about the zymogen activation of PC5A. Thus we have

examined the zymogen activation of PC5A as well as determining the residues within the prosegment that are critical for its proteolytic activity. In order to identify the residues important for the proteolytic activity of PC5A, site-directed mutagenesis were performed within the prosegment of PC5A especially around the potential secondary cleavage site. This will enable us to test whether PC5A requires cleavage at both processing sites within its prosegment for its optimal enzymatic activity. Thus, WT PC5A and various PC5A mutants were expressed in FD11 cells, furin deficient CHO-K1, and their processing activities were compared using proVEGF-C as a substrate. This work pave the road for future studies on improving the specificity of this inhibitor by further mutagenesis leading to a more powerful and selective inhibitor.

CHAPTER C

Structure-Function Analysis of the Prosegment of the Proprotein Convertase PC5A

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Running title: PC5 prosegment

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1The abbreviations used are: aa: amino acid; BFA: brefeldin A; BTMD: before trans-membrane domain; PC: proprotein convertase; SKI-1: subtilisin kexin isozyme-1; TGN: trans Golgi network; pPC5: prosegemt of PC5; ppPC5: preprosegment of PC5.

To investigate if some residues within the prosegment of PC5A are important for its optimal proteolytic function, various PC5A mutants were cellularly expressed and their processing activities were compared using proVEGF-C as a substrate. While wild type PC5A almost completely processes proVEGF-C, a prosegment deletant as well as both P1 mutants of the primary (R116A) and secondary (R84A) autocatalytic cleavage sites are inactive. The *in vitro* inhibitory potency of various decapeptides mimicking the C-terminal sequence of PC5 prosegment (pPC5) revealed that the native 107QQVVKKRTKR116 peptide is a nanomolar inhibitor, while its P6 mutant K111H is more selective towards PC5A than Furin. *In vitro* activity assays using the bacterially expressed pPC5 and its mutants revealed them to be very potent nanomolar inhibitors (IC50) and only ~ 6-fold more selective inhibitors of PC5A versus Furin. Expression of the preprosegment of PC5 (ppPC5) and its mutants in CHO-FD11 cells overexpressing proVEGF-C with either PC5A or Furin showed them to be as good inhibitors of PC5A as the serpin **α1-PDX, ppFurin or ppPACE4, but less potent towards overexpressed Furin. In conclusion, cleavages of the prosegment of PC5A at both Arg116 and Arg84 are required for PC5A cellular activity** and ppPC5 is a very potent but modestly selective cellular inhibitor of PC5A.

INTRODUCTION

Numerous secretory proteins and hormones are initially synthesized as inactive precursors that undergo post-translational processing into one or more biologically active polypeptide(s). The mammalian proprotein convertases (PCs) of the secretory pathway are calcium-dependent serine proteinases related to bacterial subtilisin. The PCs recognize various precursors and cleave at the general consensus motif (K/R)-(X)n-(K/R) ↓, where n=0, 2, 4 or 6 and X is any amino acid (Seidah and Chretien, 1999f). The PC family counts eight known members: seven dibasic-specific kexin-like convertases, Furin, PC1/3, PC2, PC4, PACE4, PC5/6 and PC7/LPC (4), and the recently discovered pyrolysin-like SKI-1/S1P that cleaves at the consensus motif (R/K)-X-(hydrophobic)-Z↓, where Z is variable (5-7)(Elagoz et al., 2002b).

PCs contain an N-terminal signal sequence, followed by a prosegment, a catalytic domain and a P-domain. In addition, PCs possess a C-terminal segment that varies between the different members. While analysis of the tissue and cellular distribution revealed that PC5 is widely expressed but enriched in certain areas such as in brain, cardiovascular system, endothelial cells and Sertoli cells (Lusson et al., 1993f;Seidah et al., 1994b)(Dong et al., 1995b), it is one of the least understood enzyme of the convertase family. Its levels are upregulated in proliferating vascular smooth muscle cells (VSMC) (Stawowy et al., 2001b) as

well as during embryo implantation (Wong et al., 2002a). PC5 exists in two different isoforms: a soluble PC5A sorted to regulated secretory granules (Lusson et al., 1993e;De Bie et al., 1996r) and a membrane-bound PC5B cycling between the trans Golgi network (TGN) and the cell surface (De Bie et al., 1996q;Xiang et al., 2000b). Active PC5A can cleave a variety of secretory precursors: pro-Mullerian inhibiting substance (Nachtigal and Ingraham, 1996c), prorenin (17), proneurotensin (18), pro-PTPμ receptor (Campan et al., 1996a), pro-cholecystokinin (20), integrin pro-α subunits (Lissitzky et al., 2000c), HIV gp160 (22), Alzheimer disease β -secretase BACE1 (Benjannet et al., 2001h), transforming growth factor TGF-β -like Lefty (24) and vascular endothelial growth factor C (VEGF-C) (Siegfried G, N.G. Seidah and A-M Khatib, submitted). Recent development on its cleavage specificity showed that, in contrast to Furin (2, 25), purified active mouse PC5A (mPC5A) cleaves *in vitro* tri- and tetra-peptides at monobasic and dibasic sites (20), in a somewhat similar fashion to PACE4 (26).

The critical role of PCs in the proteolytic maturation of multiple proprotein substrates, their implication in various pathologies (1, 27, 28)(Seidah and Chretien, 1999e) and their unidentified specific and/or redundant functions, make them attractive targets for the development of potent and selective inhibitors. The various successful approaches include: active-site-directed chloromethylketone inhibitors (29, 30), reversible peptide-based inhibitors (31-33), plant derivatives

(34), and several engineered variants of protein-based inhibitors that possess a Furin-like motif. These include α2-macroglobulin (α2-MF) (35), α1-antitrypsin (α1-AT) Portland (α1-PDX) (36-38)(Benjannet et al., 1997b;Jean et al., 1998b), proteinase inhibitor 8 (PI8) (39) and the turkey ovomucoid third domain (40). However, these effective inhibitors lack selectivity towards members of the PC family. Furthermore, both PI8 and α2-MF can inhibit many other proteases in addition to the PCs. α1-PDX was shown to inhibit all the PCs in the constitutive secretory pathway (Benjannet et al., 1997e). Recently, Tsuji et al. (41) showed that a reactive site loop variant of α1-AT (AVRR352) is ~100 fold more selective *in vitro* towards Furin and PC5 than PACE4. This indicates that a basic residue at P4 is important for the inhibition of PACE4 but not of Furin and PC5.

Previous subtilisin-, kexin- and Furin-based studies established that the prosegment could act both as an intramolecular chaperone and a potent inhibitor of its cognate enzyme (42-45). The prodomain of PCs acts as a tight binding competitive inhibitor (45, 46), while the prodomain of yeast kexin behaves as a mixed inhibitor with an IC50 of 160 nM (43). *In vitro* experiments demonstrated that the prosegment of Furin (pFurin) is 10-fold more potent towards PC5A (IC50 ~ 0.4 nM) than Furin (IC50 ~ 4 nM), whereas the prosegment of PC7 (pPC7) is a relatively selective inhibitor of its cognate enzyme (IC50 ~ 0.4 nM) (45). In addition, the prosegment of SKI-1 was also shown to specifically inhibit SKI-1 *in*

vitro but at a much lower potency (47). Finally, it was shown that *ex vivo* overexpression of the preproregions of Furin (ppFurin) and PC7 (ppPC7) resulted in potent but moderately selective inhibition of their parent enzyme (23, 45)(Benjannet et al., 2001c).

As for other convertases, autocatalytic zymogen activation of proPC5 involves cleavage at the specific primary site **KKRTKR^{116}** ↓ found at the C-terminus of the prosegment (Fig. 1A) (De Bie et al., 1996p). This primary cleavage occurs in the endoplasmic reticulum (ER) and is a prerequisite for the exit of PC5A from this compartment (De Bie et al., 1996o), as for all PCs (4, 48, 49) except PC2 (50, 51). Similar to subtilisin(52, 53), Furin (44, 54) and SKI-1 (7, 47)(Elagoz et al., 2002a), we hypothesized that: **(i)** the prosegment of PC5 (pPC5; residues 35-116) remains non-covalently associated with the active form of the enzyme and functions as an inhibitor as well as an intramolecular chaperone; **(ii)** once the complex reaches an adequate lower pH and high calcium concentrations, presumably in the TGN, the prodomain dissociates and is cleaved at an internal secondary site **HSRTIKR^{84}**↓ found in a similar position in Furin (Seidah et al., 1998).

Since no specific function of PC5A has been established yet and given that no known precursor protein is specifically processed by only PC5A and not by Furin, it is of great importance to develop a selective inhibitor of PC5A, which in

turn may help to define its function. Thus, in this study, we first defined some of the critical amino acids within the prosegment of PC5 that affect the ability of PC5A to process the substrate proVEGF-C. The data show that a PC5A isoform lacking the prosegment (PC5A-Δ pro) and Ala mutants of the P1 Arg116 or Arg84 at the primary and secondary zymogen cleavage sites, respectively, are unable to process proVEGF-C. We next assessed the inhibition of **(i)** the in-vitro pERTKR-MCA cleaving activity of PC5A and Furin using either C-terminal PC5-prosegment decapeptides, the entire pPC5 or their mutants, and **(ii)** the ex-vivo proVEGF-C processing by ppPC5 and its mutants, ppFurin, ppPACE4 and ppPC7.

EXPERIMENTAL PROCEDURES

Cellular activity and biosynthetic analysis of PC5A and its mutants- The various mutants S79R, R80A, T81R, R84A, R116A, and PC5A-Δ pro were obtained by PCR (Elagoz et al., 2002d) using the pair of oligonucleotides S6/AS17, S7/AS18, S8/AS19, S3/AS9, S5/AS3 and S9/AS20, respectively (Table I). All PCR fragments were cloned into the pCRII-TOPO TA cloning vector (Invitrogen) and sequenced completely. The amplified cDNA fragments were cloned in pIRES2-mPC5A digested with BglII. Each recombinant cDNA was transfected using Lipofectamine 2000 (Invitrogen) into CHO-FD11 cells stably expressing VEGF-C. Media were analysed by Western blot on a 12% SDS-PAGE using as the primary antibody a polyclonal anti-VEGF-C antiboby H-190 directed against the C-terminal end of VEGF-C (Santa Cruz; dilution 1:500) and as secondary antiserum anti-rabbit HRP-coupled IgGs (Life Technologies) (dilution 1:10,000). Biosynthetic analysis was performed in HK293 cells expressing either pIRES2-EGFP (control), the full-length WT PC5A or its mutants R84A and R116A. Forty-eight hours post-transfection, the cells were pulse-labeled for 4h with 250 μCi/ml [3H]Leucine (Amersham) and cell lysates and media were immunoprecipitated using a polyclonal anti PC5 antibody directed against the N-terminus of the active enzyme, i.e., aa 117-132 (dilution 1:200) (De Bie et al.,

1996n). Immunoprecipitates were then resolved by SDS-PAGE (8% Tricine gel) and autoradiographed (De Bie et al., 1996m;Benjannet et al., 1997d).

Synthesis of prosegment-derived Peptides- All peptides from Table II were synthesized with the C terminus in the amide form on a solid-phase automated peptide synthesizer (Pioneer; PE-PerSeptive Biosystems, Framingham), following the O-hexafluorophospho-[7-azabenzotriazol-1-yl]-N, N, N' , N' - tetramethuluronium (HATU)/di-isopropylethylamine (DIEA)-mediated Fmoc chemistry (56). The crude peptides were purified by RP-HPLC using an analytical Vydac C18 column with 300Å pore diameter (5 µm, 4.6 x 250 mm). Peptides were eluted with a 1%/min linear gradient (10-27%) of 0.1% (v/v) aqueous TFA/acetonitrile at a flow rate of 2 ml/min. The purified peptides were characterized by MALDI-TOF as described (45, 47, 56) and the peptide concentrations were determined by quantitative amino acids analysis.

Enzyme preparations and activity- Active enzymes were produced by infections of BSC40 cells with different recombinant vaccinia viruses of each PC (22). The media of cells infected with a soluble form of rat PC7 (VV:rPC7-BTMD) (57), soluble human Furin (VV:hFurin-BTMD) (22), mouse and human PC5A (VV:mPC5A and hPC5A) or the shed form of yeast kexin (VV:ykexin) were collected 18h post-infection and concentrated 80-fold using a centriprep YM-30 (Millipore). The concentrated media were kept at -20ºC in 40% glycerol. The

enzymatic activity of each protease was measured by their ability to cleave the fluorogenic substrate pERTKR-MCA (Peptide International). The substrate concentration added in the reaction was 5-fold the Km value of each enzyme. In the rPC7-BTMD reaction, a final concentration of 350 μM of pERTKR-MCA was used (57), for ykexin a final substrate concentration of 225 μM was used and as for hFurin-BTMD, mPC5A and hPC5A assays, 35 μM pERTKR-MCA were used (22). For each assay 1-10 μl of enzyme was added to a solution already containing 50 mM Tris-Acetate pH 7.0, 2 mM Ca2+, 0.1 mM β-mercaptoethanol and varying concentration of pERTKR-MCA in a final volume of 100 μl. A 30 min preincubation of the enzyme in the solution mix was done prior to the addition of fluorogenic substrate. Once the substrate was added, the fluorescence was measured at 0, 20, 40 and 60 min using a model LS50B (Perkin-Elmer) spectrofluorimeter.

Inhibition Assays (Ki determination)- The Ki values (nM) of the various synthetic peptides were determined using Lineweaver-Burk plots. For each assay, 6 different concentrations of substrate were used for each of the three concentrations of inhibitory peptides and the control in absence of inhibitor. The enzymes were preincubated for 30 min at room temperature with the synthetic peptides. After the incubation period, the fluorogenic substrate, pERTKR-MCA, was added at different concentrations (1.75, 3.5, 7, 17.5, 35, and 70 μM) and the

time-dependent MCA release (0, 20, 40, 60, 90, 120 and 180 min) was measured. The Ki values were calculated by plotting the results with a GraFit4 program (Erithacus Software, Ltd.) using the Lineweaver-Burk equation for competitive inhibition.

Expression and purification of bacterial mouse PC5 prosegment- The bacterial expression vector pET-24b(+) (Novagen) was cut at the 5' and 3' ends with XhoI and DraIII, respectively, to replace the stop codon by double stop codons following a hexa-His insert, using the pairs of oligonucleotides S1/AS1 (Table I). The cDNAs coding for the mPC5 prosegments (pPC5) were isolated by a three-steps PCR using Elongase (Life Technologies, Inc.) for 20 cycles. The amplification oligonucleotides used for pPC5 WT, R116A, K111H, K111I, K111L, K111P, and K111V were S2/AS2, S2/AS3, S2/AS4, S2/AS5, S2/AS6, S2/AS7 and S2/AS8 (Table I), respectively. The mutation R84A was produced by PCR using as a template the DNAs generated by two previous PCR reactions combined together. The first two PCRs that were combined used the S2/AS9 and S3/AS2 primer pairs, respectively. The second PCR reaction was performed with the pair S2/AS2. These 380 bp cDNA fragments were cloned into the pCRII-TOPO TA cloning vector (Invitrogen), for sequencing. The cDNAs were transferred into the 5' NdeI/3' NotI sites of the modified bacterial expression vector pET-24b(+), N-terminal to the hexa-histidine tag. For protein production of

these recombinants plasmids, they were transferred into host E. Coli strain called BL21 (DE3) (Novagen). Protein expression was induced by the addition of 0.4 mM IPTG for 3h at 37ºC. The cells were then harvested by centrifugation at 5,000 x g for 5 min, resuspended and homogenized in lysis buffer, as recommended (Novagen). These steps were repeated twice. Once the samples were well homogenized, they were centrifuged and the pellets were recuperated. The pellets were resuspended in solution containing 6M guanidine.HCl and purification of these pPC5s was done under these denaturing conditions on a Ni affinity column, as recommended by the manufacturer (Novagen). The eluants were then dialyzed against 50 mM sodium acetate buffer pH 6 overnight and the purity of the prosegments was determined by Coomasie staining of SDS-PAGE 14% Tricine gels. The average yield of each purified prosegment varied between 10-20 mg/L of bacterial culture. The concentrations were determined by quantitative amino acids analysis and the molecular weights were analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry on a Voyager DE-Pro instrument (PE PerSeptive Biosystems). The observed molecular masses were within 0.2% of their expected value. (WT pPC5 expected 11,684Da, obtained 11675Da; R84A pPC5 and R116A pPC5 expected 11,599Da, obtained 11,593Da and 11,599Da respectively; K111H pPC5 expected 11,694Da, obtained 11,714Da; K111L pPC5, expected 11,670Da,

obtained 11,695Da; K111P pPC5 expected 11,654Da, obtained 11,684Da; K111V pPC5 expected 11,656Da, obtained 11,671Da; K111I pPC5 expected 11,670Da, obtained 11,695Da).

Western blots using pPC5 antibodies- The bacterially produced purified native pPC5 was used to raise polyclonal antisera in rabbits. The cellular expression of the preprosegments was done by transient transfections using Effectene (Qiagen) of 6-7 x 105 HK293 cells in 60 mm dishes with a final 1.2 µg of either pIRES2-EGFP, mppPC5, rppPC7, hppFurin, hppPACE4-A or hppSKI-1. After 24h incubation at 37ºC in DMEM/10% fetal calf serum (Life Technologies, Inc.), the cells were rinsed with serum-free DMEM and incubated for another 6h with serum-free DMEM containing brefeldin A (BFA; 2.5 µ g/ml; Epicentre). The cells were washed with PBS (Phosphate Buffer Saline) and lysed with RIPA buffer as described (Benjannet et al., 2001g). The various lysates were resolved by SDS-PAGE on a 14% Tricine gel. The prosegment (pPC5) was detected by Western blotting using the pPC5 antibody (Ab:pPC5) at a 1:2500 dilution.

Stop-time inhibition assays (IC50)- The different enzymes (VV:rPC7-BTMD, VV:hFurin BTMD, VV:mPC5A, VV:ykexin) were initially preincubated for 30 min at room temperature with the different proregions at various concentrations. The proregions were initially diluted in water containing 0.1% BSA (45). The fluorogenic substrate pERTKR-MCA, was then added and the released AMC was

measured at different times over the course of 3h. The reactions were performed at saturating conditions (5 x Km value) of the fluorogenic substrate pERTKR-MCA. The Km values of the pERTKR-MCA processing are 70 μM for rPC7-BTMD, 45 μM for yKexin and 7 μM for hFurin-BTMD and mPC5A (30, 57). The IC50 values were obtained by plotting the results with the GraFit4 program.

Transfections and biosynthetic analysis of ppPC5s - The preprosegments of mPC5 (coding for residues 1-116) were amplified for 20 cycles by a three-steps PCR reaction. The primer pairs used to amplify the ppPC5 WT and R116A were S4/AS10 and S5/AS11 (Table I), respectively. All P6 ppPC5 mutants were amplified with a sense primer corresponding to a region upstream of the multiple cloning sites of pIRES2-EGFP. The ppPC5 K111H, K111I, K111L, K111P, K111V, and ppPC5-Flag (signal peptide of β -secretase BACE1-Flag epitope (Benjannet et al., 2001f) were produced using the primer pairs S4/AS12, S4/AS13, S4/AS14, S4/AS15, S4/AS16, S10/AS21 (Table I), respectively. The cDNAs of the WT, R84A and R116A ppPC5 were transferred into the EcorI site of the pIRES2-EGFP vector. The other mutants (K111H/I/L/P/V) were cloned into 5' SacI/SacII 3' sites of the pIRES2-EGFP. HK293 cells (6.5 x 105 cells) were transiently transfected using Effectene (Invitrogen) and a total of 0.6 μg of cDNAs. Two days post-transfection, the cells were washed and then pulse-incubated for 6h with 250 μCi/ml of [35S]Met (Amersham) and BFA. Cells were lysed in RIPA buffer

containing a cocktail of protease inhibitors (Roche Molecular Biochemicals). The media and cell lysates were immunoprecipitated with the polyclonal pPC5 antibody (1:250) directed against the proregion. Immunoprecipitates were resolved by SDS-PAGE on 14% Tricine gels and autoradiographed as described (Benjannet et al., 2001b).

Inhibition of VEGF-C processing ex-vivo - CHO-K1 cells were transiently transfected with hVEGF-C and either various ppPCs or α1-PDX. A total of 3 μg DNA was transfected using Lipofectamine 2000 (Invitrogen) for 0.75 x 106 cells. The CHO-FD11 cells (kindly supplied by Dr. Stephen H. Leppla; NIH), which are derived from CHO-K1 cells that are Furin-deficient, were stably transfected (Lipofectamine 2000) (Invitrogen) with either the cDNAs of pIRES2-EGFP, pIRES2-hFurin or pIRES2-mPC5A. The cells were selected with 800 μg of G418 and maintained with 400 μg. Once selected and FACS sorted for EGFP, they were further stably transfected (Lipofectamine 2000) with pcDNA3.1.Zeo-hVEGF-C. The cells expressing VEGF-C and mPC5A (FD11/ VEGF-C/PC5A) or Furin (FD11/VEGF-C/Furin) were then selected with 400 μg of Zeocin and maintained with 200 μg. The various proregions were transiently transfected in order to inhibit the processing of VEGF-C. Forty-eight hours post-transfection, the media were 6-fold concentrated using a microcon YM-10 (Millipore) and the proteins were

resolved on a 12% SDS-PAGE gel. Detection by Western blotting was done with the polyclonal anti-VEGF-C antibody (1:500).

RESULTS

Biosynthesis of PC5A and its mutants and their proteolytic activities - Biosynthetic analysis demonstrated that PC5A lacking its prosegment, PC5A-Δ pro, is not secreted from HK293 cells (Fig. 1A). In addition, wild type PC5 and its P1 mutant of the secondary processing site (R84A) are secreted as processed PC5A, while only trace amounts of proPC5A are found in the media of cells expressing the primary P1 site (R116A) mutant (Fig. 2). This indicates that zymogen processing is a requisite for the efficient exit of PC5A from the cell. We believe that the zymogen form remains primarily in the ER since it is endoH sensitive (De Bie et al., 1996l). Finally, the reported late C-terminal processing of PC5A resulting in the production of PC5-Δ C (65 kDa) (De Bie et al., 1996k) is not significantly affected in the R84A mutant since the PC5A/PC5A-Δ C ratio is relatively constant (Fig. 2). Since PC5A is capable of cleaving proVEGF-C (Siegfried G, N.G. Seidah and A-M Khatib, submitted), the latter substrate was therefore used to estimate the cellular activity of PC5A and its prosegment mutants. The ability of the PC5A mutants (Fig. 1A), P4 T81R, P5 R80A, and P6 S79R, to process proVEGF-C at the ^{222}HSIIRR↓SL^{229} site (Fig. 1B) was tested in

the Furin-deficient CHO-FD11 cells (Gordon et al., 1995b) (Fig. 3). In this cell line, expression of VEGF-C resulted mainly in the secretion of the precursor form with small amounts of processed VEGF-C (CTF). This suggests, that in this cell line endogenous proteases, other than Furin-like enzymes, can only partially process proVEGF-C, similar to gp160 (59). Extensive processing of proVEGF-C is observed when it is co-expressed with WT mPC5A (~86%) and its P6 S79R mutant (~76%; Fig. 3B). All other mutants either resulted in partial (R80A and T81R) or almost complete (R84A, R116A and PC5-Δ pro) loss of activity (Fig. 3). This indicates that P1 Arg of both primary and secondary prosegment cleavage sites are critical for enzymatic activity and that the P5 Arg and P4 Thr are also necessary for maximal proteolytic activity. In contrast, P6 Ser does not seem to play a major role, as its substitution by Arg did not significantly affect proVEGF-C processing by PC5A.

Inhibitory potency and specificity of C-terminal pPC5-derived decapeptides - Previously, we have shown that peptides as small as 10 amino acids (aa) corresponding to the C-terminal end of the proregions of Furin and PC7 were potent inhibitors of these enzymes (45, 60). In order to determine if similar peptides derived from the C-terminus of pPC5 are also potent inhibitors of PC5A, and possibly of other convertases, we synthesized a number of pPC5-derived C-terminal decapeptides. These included: WT and mutant peptides in which

conserved residues (P6 His and P4 Ile) within the processing site of integrin α chains (Lissitzky et al., 2000f) and VEGF-C, which are good PC5A substrates, were introduced. It should be noted that none of the peptides tested were cleaved by the convertases. Table II depicts their inhibitory constants (Ki) on the *in vitro* processing of the fluorogenic pERTKR-MCA substrate by mPC5A, hPC5A or soluble hFurin-BTMD (22). As compared to Furin, the selectivity and potency of the various peptides revealed that: **(1)** the native decapeptide ^{107}QQVVKKRTKR^{116} is a very potent inhibitor of mPC5A and hPC5A with Ki values of 16 and 31 nM, respectively. This peptide exhibits 6-12 fold selectivity towards PC5A as compared to Furin. **(2)** The most potent inhibitor of PC5A is the P9 Q108A peptide with a Ki of 5-6 nM, except that it is only 4-fold more selective towards Furin (Table II and Fig. 4). The worst inhibitor of PC5A is the P4 mutant R113I with a Ki of approximately 7.5 μM. **(3)** None of the 11 P6 mutants tested were more potent than Q108A but two of them, K111H and K111L, were the most selective inhibitors of PC5A (40- and 28- fold, respectively; Table II and Fig. 4). **(4)** Combination of both P6 His and P4 Ile found in the good PC5A substrates α - integrins and in proVEGF-C, resulted in a low potency inhibitor K111H/R113I (Ki ~ 1.2 μ M). **(5)** While the P6 K111L shows a good PC5A selectivity, it was surprising to find that other aliphatic residues such as Val or Ile at P6 did not. Instead, they resulted in a drastic loss of both selectivity and potency. **(6)**

Replacement of Lys at P6 by Arg that usually enhances the recognition of substrates by PCs (Seidah and Chretien, 1999d), resulted in a lower inhibitory potency, as compared to the WT and the K111H and K111L mutants. This suggests that Arg at P6 is deleterious for the inhibitory activity of the pPC5 decapeptide mimic. This result is similar to that reported for PACE4, where replacement of the P6 Leu by Arg in the serpin α 1-PDX resulted in lower inhibition (61). **(7)** The Lys at P2 seems to be important since its replacement by Thr (K115T) led to a severe loss of both potency and selectivity. Finally, all the conclusions drawn above are valid for both mouse and human PC5A, which exhibit an identical decapeptide at the C-terminus of their prosegment (Lusson et al., 1993d).

Expression of various pPC5s and antibody production. - Based on the inhibition constants obtained from the above decapeptides, we next verified if the full-length proregion of PC5 (pPC5) could be a better and/or more selective in-vitro inhibitor of PC5A. Therefore, WT pPC5 and some of its variants, namely R84A and R116A as well as the P6 mutants K111H, K111I, K111L, K111P and K111V were bacterially produced and purified (*see Experimental Procedures*). The purified WT pPC5 was used to raise a polyclonal antibody in rabbit. In order to test the antiserum selectivity, we expressed the preprosegments of PC5, Furin, PC7, PACE4, and SKI-1 in HK293 cells treated with brefeldin A (BFA), in order to

retain them in the ER (63) and maximize their levels, and analyzed their immunoreactivity by Western blot (Fig. 5). It is clear that the antiserum is highly selective, as it recognizes only pPC5 in HK293 cell extracts (8.9 kDa, derived from ppPC5) and the purified protein from a bacterial extract (10.2 kDa). The difference in molecular masses is due to the 17 aa C-terminal extension, which includes a hexa-histidine tag, of the bacterial pPC5 antigen (*see legend and Experimental Procedures*). Furthermore, this antiserum recognizes the intracellular 8.9 kDa pPC5 that is generated by the zymogen processing of the full-length enzyme in CHO-FD11 cells in absence or presence of BFA (Fig. 5).

Comparative in vitro inhibition efficacies of pPC5s - The IC50 values of the various bacterially produced pPC5s on the *in vitro* processing of the fluorogenic substrate pERTKR-MCA by mPC5A are presented in Table III. Only the R116A and the K111L are much less active inhibitors of PC5A *in vitro* and the apparent rank order of potency is K111H > WT > [K111V ~ K111I] > [R84A ~ K111P] > K111L > R116A. Based on these and similar data with other enzymes, the inhibitory IC50 values of pPC5 and its mutants on the *in vitro* activity of mPC5A, hFurin-BTMD, rPC7-BTMD and soluble ykexin (using equal starting pERTKR-MCA cleavage activities of each enzyme) are presented in Table III. In general, all tested pPC5s were potent nM inhibitors of PC5A and were more selective for this enzyme. Furthermore, in agreement with a previous report on pFurin and

pPC7 (45) pPC5s were more potent that their corresponding decapeptides and showed IC50 values that are at least 20-fold lower (*not shown*). The most potent ones were the WT and the P6 mutants K111H, K111V and K111I (IC50 < 10 nM), while the K111P mutant is as selective as the WT but ~3-fold less potent. Curiously, no strict correlation could be established between the potency and selectivity of the decapeptides and those of the corresponding pPC5s. For example, the pPC5 K111L mutant was ~ 11-fold less active than WT pPC5 (IC50 ~71 nM) and only 2-fold selective for PC5A, whereas the corresponding K111L decapeptide was almost as potent as the WT peptide and 40-fold more selective (Table II and Fig. 4). This suggests that residues N-terminal to the selected decapeptide of pPC5 may also affect significantly the inhibitory and selectivity properties of the prosegment. The various prosegments were also very potent inhibitors of hFurin and rPC7 with IC50 in the nM range. The best inhibitor of mPC5A, hFurin and rPC7 is also the K111H mutant with an IC50 of ~ 4, 21 and 23 nM, respectively. All pPC5s tested failed to potently inhibit ykexin (IC50 in the μ M range; Table III). Finally, the P1 mutant R116A exhibits a ~ 40-fold lower potency (IC50 ~ 267 nM), while the R84A one is only 3-fold less potent (IC50 ~ 18 nM) than WT pPC5.

Biosynthetic fate of the cellularly expressed ppPC5 and its mutants - We first tested the integrity and total level of the various prosegment constructs

following their expression in the easily transfected HK293 cells that were pulse-labeled for 6h with [35S]Met in the presence of BFA, ensuring their retention in the ER. The preprosegments (ppPCs) were cloned into the pIRES2-EGFP vector with their own signal peptides (45). As shown in Fig. 6A, immunoprecipitations with the pPC5 antibody revealed a similar expression level for all ppPC5 constructs. Furthermore, WT ppPC5, or its R84A and R116A derivatives were effectively processed by the signal peptidase, since sequencing of the [3H]Val 8.9 kDa form revealed Val at positions 2 and 9 confirming that the sequence starts at Arg35 of mPC5A (*not shown*), as for the whole enzyme (De Bie et al., 1996j). Analysis of the media revealed the secretion of a smaller fragment (~3 kDa) (Fig. 7B). Since the R84A mutant is similarly processed as the other forms, this suggests that processing of pPC5 into the secreted ~3 kDa form does not seem to require Arg^{84}, and may thus be performed by another enzyme. In contrast, the removal of the signal peptide was practically abolished in a construct connecting a β -secretase signal peptide-FLAG (Benjannet et al., 2001a) to the N-terminus of the wild type pPC5 sequence (pFpPC5). This signal peptide ↓ FLAG motif was previously shown to be efficiently cleaved in the ER when connected to β -secretase resulting in an N-terminally flagged-BACE1 (Benjannet et al., 2001j). However, when connected to pPC5, it either does not enter the ER or resists cleavage, explaining the absence of prosegment in the

medium (Fig. 6B). A similar observation of an incomplete signal peptide removal was also reported for the cellularly expressed ppFurin and ppPC7 (45).

Inhibition of cellular proVEGF-C processing - We next compared the ability of the various ppPC5s, ppFurin, ppPACE4, ppPC7 and α 1-PDX to inhibit the cellular processing of proVEGF-C. These analyses were performed in wild type CHO-K1 cells (Fig. 7) and in its derivative the Furin-negative CHO-FD11 cells (Fig. 8). The latter were made to stably express both proVEGF-C with either mPC5A (FD11/VEGF-C/PC5A; Fig. 8A) or hFurin (FD11/VEGF-C/Furin; Fig. 8B). As control, we analyzed the fluorescence level of EGFP of each transfected cell pool by FACS, which indicated equivalent transfection efficiencies (*not shown*). In the parental CHO-K1 cells, we note that WT and R84A ppPC5 are equivalent to α 1-PDX and ppFurin as inhibitors of proVEGF-C processing, while ppPACE4 and ppPC7 were less effective (Fig. 7). Since CHO-K1 cells contain mRNAs coding for Furin and other endogenous PCs (59), we could not tell which PC is mainly responsible for the processing of proVEGF-C in these cells, and to what extent each convertase is blocked by the above inhibitors. In order to compare the cellular inhibition capacity of each ppPC on either PC5A or Furin, we opted to analyze the above inhibitors in either FD11/VEGF-C/PC5A (Fig. 8A) or FD11/VEGF-C/Furin (Fig. 8B) cells. We used the CHO-FD11 cells since the data show that in these cells proVEGF-C is practically not processed. Stable co-

expression of PC5A (Fig. 8A) or Furin (Fig. 8B) with proVEGF-C resulted in ~50% or 100% processing, respectively (pIRES lanes) thus the inhibitions achieved by the various ppPCs are directly observed on either PC5A or Furin. In the FD11/VEGF-C/PC5A cells α 1-PDX and most prosegments tested are ≥ 80% inhibitory. Exceptions include ~55% inhibition by ppPC5-K111L and no inhibition by either ppPC5-R116A, which agrees with the *in vitro* data (Table III; Fig. 7), or ppPC7 (Fig. 8A). In the FD11/VEGF-C/Furin cells, α 1-PDX and ppFurin are ~31-39% inhibitory, whereas ppPACE4 (~23%), ppPC7 (~18%) and the ppPC5s (< 14%) are relatively weak inhibitors, and ppPC5-R116A is non-inhibitory (Fig. 8B). These data support the concept that most ppPC5s tested best inhibit PC5A as compared to Furin *ex vivo*.

DISCUSSION

Previous work on subtilisin (42) and subsequently on Furin (54) demonstrated the presence of a primary site found at the C-terminus of the prosegment, which when cleaved in the ER generates a tight binding complex between the prosegment and the enzyme. This generally inactive complex requires a secondary processing event within a conserved region of the prosegment (Seidah et al., 1998), an event thought to be favored within the acidic environment of the TGN (54). Therefore, we first mutated the P1 Arg at the

primary site of PC5A into Ala (R116A) and demonstrated that this resulted in an uncleavable proPC5A zymogen, mostly blocked in the ER and barely secreted even in an overexpression system (Fig. 2), and unable to cleave proVEGF-C (Fig. 4). These data extend the notion that primary cleavage of the prosegment is a prerequisite for PC5 to exit from the ER (De Bie et al., 1996i), and puts this enzyme in the same category as Furin (44), PC1 (50), PACE4 (48), and PC7 (57), but not PC2 (50, 51, 64). Alignment of the various PC-prosegments (Seidah et al., 1998) suggested that Arg^{84} occupies the P1 position of the secondary processing site of proPC5A, within the sequence ^{79}SRTIKR^{84}↓. In support for the requirement of a P1 Arg at this site for zymogen activation, the mutant R84A is normally secreted with a molecular mass similar to that of the WT PC5A (Fig. 2), but is unable to process proVEGF-C (Fig. 3). This is reminiscent of the phenotype of the equivalent R75A Furin mutant (mutation of P1 of the secondary cleavage site) that traffics normally but is inactive (54). A possible explanation is that while the primary site processing occurred, the secondary one may not have occured, resulting in the permanent association of the enzyme with its inhibitory prosegment. In agreement with this hypothesis, using the pPC5 antibody we observed a co-immunoprecipitation of a ~8.9 kDa [3H]Leu polypeptide with the processed ~105 kDa PC5A-R84A but not with the wild type nor the R116A mutant (*not shown*).

Since the inactive PC5A-R84A (Fig. 3) is C-terminally processed to its 65 kDa form similar to the WT enzyme (Fig. 2), this suggests that this cleavage is not autocatalytic as was originally suspected (De Bie et al., 1996h), but rather implicates another enzyme that is yet to be defined (N. Nour and N.G. Seidah, in preparation). With respect to the other secondary site mutants, the data revealed that P6 Ser is not critical, but that P5 Arg^{80} and P4 Thr^{81} seem to play prominent roles, since their replacement by Ala significantly reduced proVEGF-C processing (Fig. 3). In that context, it is interesting to note that in Furin mutation of the corresponding secondary processing site P4 Val^{72} into Arg resulted in a mostly unfolded, unprocessed inactive enzyme that remains in the ER (54). Finally, as was observed for Furin (54), kexin (43) and SKI-1 (Elagoz et al., 2002e), PC5A-Δ pro remained in the ER (*not shown*), was not secreted (Fig. 2) and was inactive (Fig. 3).

We demonstrated that a 10 aa peptide corresponding to the C-terminus of pPC5 is a potent *in vitro* inhibitor of PC5A and Furin with Ki values of ~16 and ~190 nM, respectively (Table II). Thus, as originally observed for PC7 and Furin (45), synthetic prosegment decapeptides are potent inhibitors of more than one convertase, and are poorly selective (45). A similar conclusion was recently reached with dodecapeptides mimicking the C-terminus of the wild type prosegment of each of the seven known PCs (65). In an effort to improve the

potency and/or selectivity of the inhibitory propeptides we targeted the P6, P4 and P2 amino acids (Table II), which are known to be critical for Furin (2). Among others, decapeptides containing P6 His or Arg and P4 Ile were tested as they are found in PC5A-specific substrates (Nachtigal and Ingraham, 1996b;Campan et al., 1996c;Lissitzky et al., 2000e) and a Q108A mutant was chosen since Gln at P9 is conserved in all PC-prosegments (Seidah et al., 1998). Compilation of our results revealed that although selectivity towards PC5A as compared to Furin can be improved, especially for the P6 K111H and K111L mutants, the potency of these inhibitors is at best in the same range as the WT sequence (Fig. 4; Table II). Interestingly, while the P9 Q108A mutant is the most potent inhibitor, it is not selective at all (Fig. 4; Table II).

In parallel, we introduced these mutations in bacterially expressed full length prosegments in the hope of obtaining more selective, but still highly potent inhibitors (45). Based on their IC50 values, the entire WT prosegment was ~6-fold more selective towards PC5A than Furin and none of the mutant prosegments exhibited a better selectivity *in vitro* (Table III). In addition, there was an overall absence of correlation between the results obtained with pPC5s and synthetic decapeptides (compare Tables II and III). The structure and/or additional interactions offered by the entire pPC5s, as compared to decapeptides, may explain the differences between these two types of inhibitors. In this context,

it was recently shown by NMR spectroscopy that in solution the prosegment of mouse PC1 adopts a well ordered structure that is similar to bacterial subtilases (66), whereas a 24-mer pPC7 C-terminal peptide adopts a helical structure (60).

We next extended these data towards inhibition of cellular proVEGF-C processing by WT or mutant ppPC5s, ppPACE4, ppFurin, ppPC7 or α1-PDX. In native CHO-K1 cells, all tested ppPCs inhibit proVEGF-C processing (Fig. 7) except for ppPC5-R116A (*not shown*). In the FD11/VEGF-C/PC5A cells, ppPC5s inhibit proVEGF-C processing by > 80%, except ppPC5-K111L (~55% inhibition) and ppPC7 (no inhibition; Fig. 8A). Interestingly, both ppPC5-K111L and ppPC7 (67) contain a P6 Leu that may hinder their PC5-directed inhibitory properties. It was recently reported that *in vitro* pPC7 could inhibit PC5A with a Ki of 0.1 nM and was more selective towards PC5A than its cognate enzyme PC7 (65). However, our previous (45) and present data do not agree with this conclusion. Indeed, while the PC5A-directed proVEGF-C processing was not inhibited by ppPC7 in the FD11/VEGF-C/PC5A cells, it was blocked by ppFurin, ppPACE4 and ppPC5, as well as by α1-PDX (Fig. 8A). In FD11/VEGF-C/Furin cells, no significant inhibition of proVEGF-C processing by any of the prosegments was observed and only ~30-40% inhibition was achieved by α1-PDX and ppFurin (Fig. 8B). This is likely due to the high Furin activity in these overexpressing cells, since in the parental CHO-K1 cells these inhibitors were quite effective on the endogenous convertases (Fig. 7). Thus, at high Furin levels α1-PDX, and ppFurin are much better inhibitors than ppPC5, while the latter and ppPACE4 are similarly effective on lower endogenous Furin levels (compare Figs. 7 and 8B).

In conclusion, this work dealt with the zymogen processing of PC5A and the data showed that while the primary site mutant R116A remains intracellularly as an inactive and unprocessed zymogen, the secondary site mutant R84A is equally inactive although it is normally processed and secreted as a complex with its full length prosegment. Our results also effectively demonstrated that while the wild type prosegments of the convertases are potent *ex vivo* inhibitors of their cognate enzyme, they lack specificity and should not be used as a diagnostic tool to identify the type of convertase involved in a given dibasic or monobasic processing reaction. However, they could potentially be used to inhibit a pool of convertases that may be implicated in pathological situations such as in tumor development and metastasis, as was originally reported for α1-PDX (Khatib et al., 2001a). It is hoped that pharmacological use of PC-inhibitors including those presented above and novel ones such as polyarginines (70) will be more exploited in the future as novel tools in pathologies clearly implicating one or more convertase(s) (28).

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TABLE AND FIGURE LEGENDS

Table I. *Sequence of oligonucleotides used for PC5 constructs*

The indicated sense (S) and antisense (AS) oligonucleotides were use in pairs (S/AS) in PCR reactions, as indicated in EXPERIMENTAL PROCEDURES.

Table II. *Inhibitory potency of pPC5 C-terminal decapeptides*

The indicated enzymes were preincubated for 30 min at pH 7 with each decapeptide, the pERTKR-AMC substrate was then added at different concentrations and the time-dependent MCA release was measured. The Ki values were calculated using a GrapFit4 program for competitive inhibition.

Table III. *Inhibitory potency of pPC5 and its mutants*

Bacterially produced pPC5s were preincubated with the indicated enzymes for 30 min at pH 7, the pERTKR-AMC substrate was then added and the released AMC measured at different times over 3 h. The IC50 values were obtained using a GrapFit4 program. The values in parentheses represent the selectivity ratio with respect to mPC5A.

Fig. 1. **Schematic representation of full-length mouse PC5A and human proVEGF-C**. The structure of PC5A includes a signal peptide (SP) followed by a prosegment, a catalytic domain, a P-domain in addition to the C-terminal cysteine-rich domain. The selected underlined residues within the prosegment were mutated in this study. The PC-processing site (HSIIRR) is depicted in the proVEGF-C structure. The potential N-glycosylation sites are emphasized by and elevated circle.

Fig. 2. **Biosynthetic analysis of PC5A and its primary and secondary site mutants.** HK293 cells were transfected either with a recombinant pIRES2 vector expressing PC5A-WT, -R84A, -R116A or PC5A-Δ pro. The cells were pulse-labeled for 4h with [3H]Leucine. Cell lysates and media were immunoprecipitated with the anti-PC5-MAP antibody directed at the N-terminus of the catalytic subunit (De Bie et al., 1996g), thus recognizing the ~115 kDa proPC5A, ~105 kDa mature PC5A and the ~65 kDa PC5A-ΔC.

Fig. 3. **Processing of proVEGF-C by mPC5A and its mutants in CHO FD11 cells.** [A] Western blot comparing the processing of stably expressed proVEGF-C by either the empty pIRES2-EGFP vector (pIRES) or its recombinants encoding mPC5A and mutants of its P1 (primary and secondary sites), secondary site P4,

P5 and P6 (S79R, R80A, T81R, R84A, R116A) and Δ pro. [B] Histogram representing the % cleavage of proVEGF-C by mPC5A and its mutants as estimated by quantitative analysis (Image Quant). The percentages were obtained from the ratio of VEGF-C/ (proVEGF-C + VEGF-C).

Fig. 4. **Graphical representation of the comparative inhibitory potency (on mPC5A; line graph) and selectivity (vs Furin; histogram) of each PC5-derived prosegment decapeptide** (*see also* Table II).

Fig. 5. **pPC5 polyclonal antibody specificity**. The polyclonal antibody generated was obtained against the purified, bacterially produced mouse pPC5. HK293 and CHO-FD11 cells were transiently transfected with different preprosegment (ppPCs) or full-length PC5A, respectively. The next day, the cells were incubated for 6h in the presence (+) or absence (-) of BFA. The cell lysates were resolved by SDS-PAGE and the migration of immunoreactive prosegment of PC5 (8.9 kDa) compared to the bacterially produced pPC5 (10.2 kDa) by Western blot using the Ab:pPC5 antibody (1:2500). Note that the bacterial construct has a C-terminal extension of 17 residues that includes the hexa-histidine tag (DYDLSHAAALEHHHHHH), explaining its slower migration (10.2 kDa) as compared to its cellularly-derived form (8.9 kDa).

Fig. 6. **Biosynthetic analysis of ppPC5s.** [A] HK293 cells were transfected with either the empty vector (pIRES), or the WT, R84A, K111H, K111I, K111L, K111P, K111V or R116A ppPC5s. The cells were pulse-labeled for 6h with [35S]Met in the presence of BFA. Cell lysates were immunoprecipitated with the anti-pPC5 antibody and the immunoprecipitates resolved by SDS-PAGE on a 14% Tricine gel. [B] HK293 cells were transfected with either the empty vector (pIRES), WT, R84A, R116A ppPC5s or ppPC5-Flag (BACE1 SP-FLAG-pPC5). The cells were pulse-labeled for 6h with [35S]Met. Cell lysates and media were immunoprecipitated with the Ab:pPC5 and the proteins resolved by SDS-PAGE on a 14% Tricine gel. Note that the secreted pPC5 (~3 kDa; cleaved pPC5) is smaller than the cellular form (8.9 kDa), and that the signal peptide is not removed from the BACE1 SP-FLAG-pPC5 construct.

Fig. 7. **Inhibition of proVEGF-C processing in CHO-K1 cells.** Western blot analysis of the parental CHO-K1 cells transiently co-expressing proVEGF-C and either empty vector (pIRES), WT ppPC5, R84A ppPC5, α1-PDX, ppPC7, ppPACE4 or ppFurin. The media were resolved by SDS-PAGE on a 12% Glycine gel and revealed with anti-VEGF-C antibody. The estimated % inhibitions are shown at the bottom of the gel.

Fig. 8. **Inhibition of proVEFG-C processing in FD11 cells overexpressing PC5A or Furin.** Western blot analysis of the media of CHO-FD11 cells overexpressing VEFG-C and [A] FD11/VEGF-C/PC5A cells or [B] FD11/VEGF-C/Furin cells transiently transfected with either the empty vector (pIRES), WT, R84A, K111H, K111I, K111L, K111P, K111V or R116A ppPC5s as well as ppfurin, ppPACE4, ppPC7 or α1-PDX. Proteins were resolved by SDS-PAGE on a 12% Glycine gel and revealed with the anti-VEGF-C H-190 antibody.

Table I. *Sense (S) and Antisense (AS) oligonucleotides utilized in the various constructions*

Primers	Sense (S)	Antisense (AS)
S1/AS1	CTCGAGCACCACCACCACCACC ACTAATAAGATCCG	TGGCCCACTACGTGAACC
S2/AS2	ACATATGCGCGTCTACACCAAC CACTGG	TGCGGCCGCATGGCTGAGGTCATAATC
S3/AS3	GACCATTAAA*GC*GTCTGTTCTCT CGAG	TGCGGCCGCATGGCTGAGGTCATAATCC*GC*CTT GGTTCT
S4/AS4	AAGCTTGGGACCATGGACTGGG ACTGGGGGAACCGC	TGCGGCCGCATGGCTGAGGTCATAATCCCTCTT GGTTCTTTT*GTG*CACCAC
S5/AS5	AGAACCAAG*GCG*GATTATGAC	TGCGGCCGCATGGCTGAGGTCATAATCCCTCTT GGTTCTTTT*GAT*CACCAC
S6/AS6	CACTTCTACCAT*C*GTAGGACC	TGCGGCCGCATGGCTGAGGTCATAATCCCTCTT GGTTCTTTT*GAG*CACCAC
S7/AS7	CTACCATAGT*GC*GACCATTAAAA GG	TGCGGCCGCATGGCTGAGGTCATAATCCCTCTT GGTTCTTTT*TGG*CACCAC
S8/AS8	CATAGTAGGA*GG*ATTAAAAGGT CTG	TGCGGCCGCATGGCTGAGGTCATAATCCCTCTT GGTTCTTTT*CAC*CACCAC
S9/AS9	TGCCGGACGGATTATGACCTCA GCCATG	CTCGAGAGAACAGAC*GC*TTTAATGGTC
S10/AS 10	GCGCGCCGCGTCTACACCAACC ACTGG	GGGCCCTCATTAGTCATAATCCCTCTTGGTTCTT TT
AS11		GGGCCCTCATTAGTCATAATCC*GC*CTTGGTTCTT TT
AS12		CCGCGGAGATCTTCAGTCATAATCCCTCTTGGTT CTTTT*GTG*CACCAC
AS13		CCGCGGAGATCTTCAGTCATAATCCCTCTTGGTT

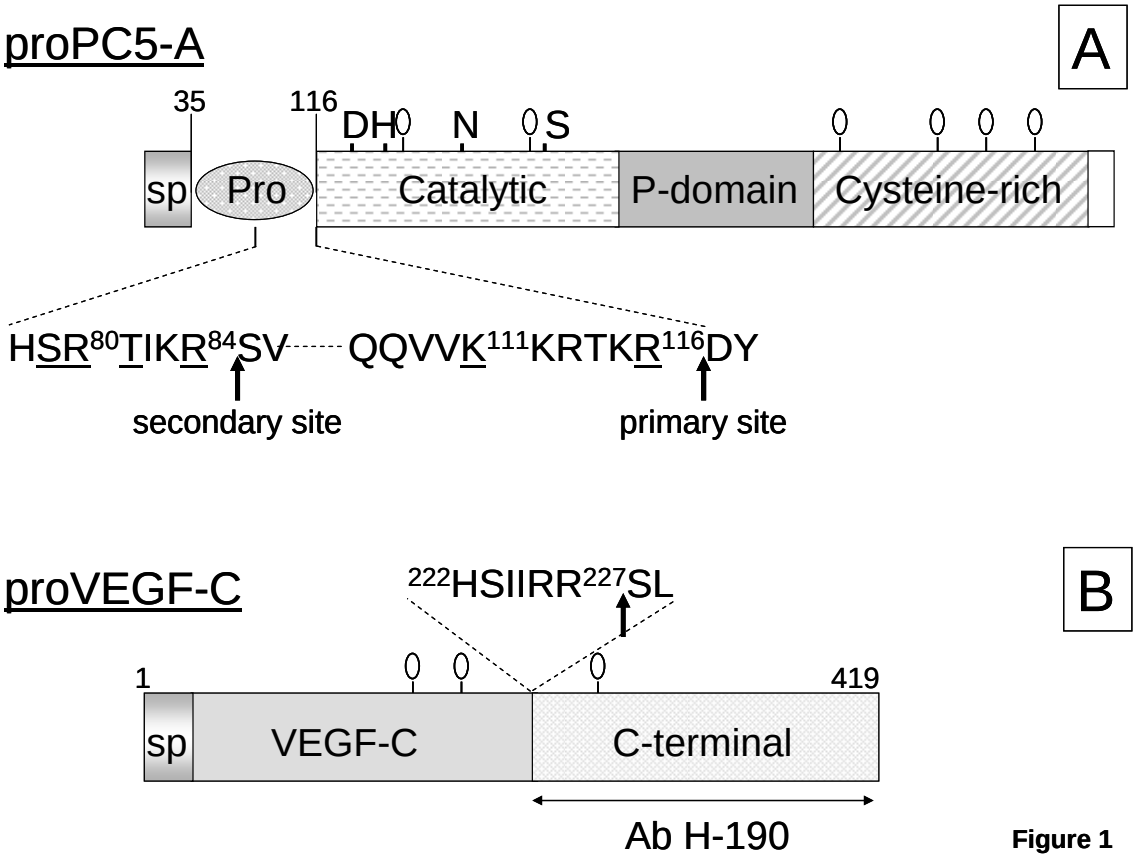
		CTTTT*GAT*CACCAC
AS14		CCGCGGAGATCTTCAGTCATAATCCCTCTTGGTT CTTTT*GAG*CACCAC
AS15		CCGCGGAGATCTTCAGTCATAATCCCTCTTGGTT CTTTT*TGG*CACCAC
AS16		CCGCGGAGATCTTCAGTCATAATCCCTCTTGGTT CTTTT*CAC*CACCAC
AS17		CCTTTTAATGGTC*GC*ACTATGGTAG
AS18		CCTTTTAATGGTC*GC*ACTATGGTAG
AS19		CAGACCTTTTAAT*CC*TCCTACTATG
AS20		GTCATAATCCGTCCGGCATACCGGGAG
AS21		*CCGCGG*TCAGTCATAATCCCTCTTGGTTCT

Table II. Inhibitory potency of proPC5 C-terminal decapeptides

Inhibitor	Peptide sequence	Ki (nM)		
		mPC5A	hPC5A	hFurin
WT	**^{107}QQVVKKRTKR^{116}**	**16±1**	**31±4**	**190±20**
Q108**A**	Q**A**VVKKRTKR	5.3±0.1	6.6±0.2	21±4
K111**H**	QQVV**H**KRTKR	12±2	22±5	330±40
K111**L**	QQVV**L**KRTKR	24±4	22±3	950±120
K111**R**	QQVV**R**KRTKR	63±12	60±16	160±14
K111**P**	QQVV**P**KRTKR	84±17	88±18	100±10
K111**Q**	QQVV**Q**KRTKR	130±19	120±16	2020±250
K111**A**	QQVV**A**KRTKR	140±16	110±13	960±120
K111**V**	QQVV**V**KRTKR	190±20	240±23	1100±100
K111**I**	QQVV**I**KRTKR	380±50	406±40	1680±90
K111**S**	QQVV**S**KRTKR	410±62	440±38	3030±380
K115**T**	QQVVKKRT**T**R	970±190	1000±380	6900±1800
K111**W**	QQVV**W**KRTKR	1080±180	1000±140	6710±780
K111**H**/R113**I**	QQVV**H**K**I**TKR	1200±270	910±200	3900±800
K111**E**	QQVV**E**KRTKR	2300±790	2300±790	14000±5900
R113**I**	QQVVKK**I**TKR	7300±3300	7500±2000	8000±2500

Table III. Inhibitory potency of pPC5 and its mutants

	IC_{50} (nM)			
Prosegment	mPC5A	hFurin	rPC7	yKexin
WT	6.5±0.1 (1)	41±2 (6)	47±2 (7)	1550±60 (240)
K111H	4.4±0.4 (1)	21±3 (5)	23±1 (5)	1730±65 (390)
K111V	8.5±0.1 (1)	30±3 (3.5)	37±2 (4)	1530±160 (180)
K111I	9.3±0.8 (1)	42±2 (5)	44±1 (5)	1550±211 (167)
R84A	17±1 (1)	43±1 (2.5)	44±2 (2.5)	6580±3200 (370)
K111P	18±1 (1)	126±13 (7)	134±7 (8)	1370±89 (75)
K111L	71±6 (1)	148±4 (2)	131±2 (1.8)	3330±196 (50)
R116A	258±18 (1)	419±21 (1.6)	541±14 (2)	2350±903 (10)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35
116
DH
N
S
sp
Pro
Catalytic
P-domain
Cysteine-rich
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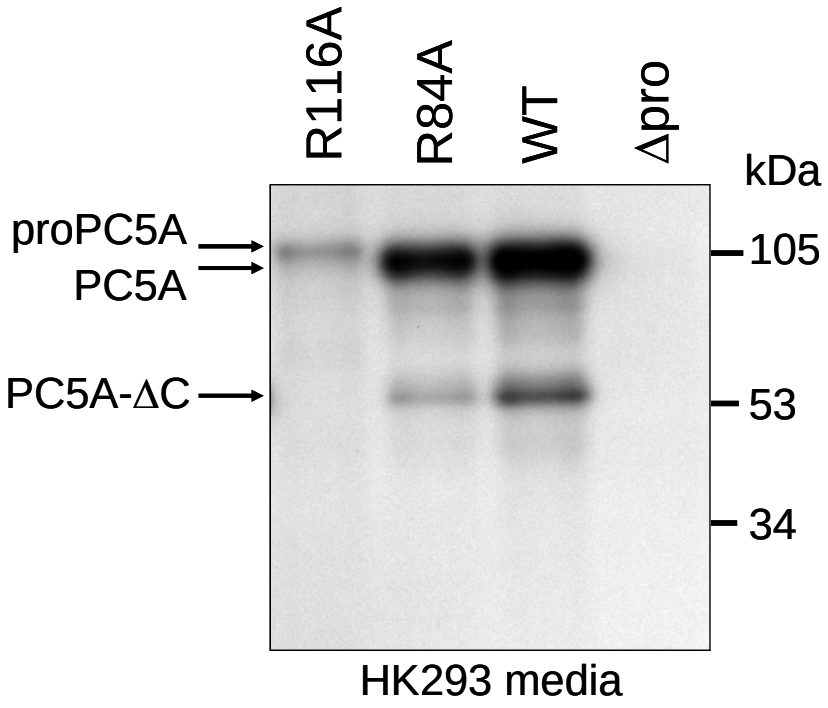
QQVVK111KRTKR116DY
secondary site
primary site
proVEGF-C
B
222HSIIRR227SL
1
419
sp
VEGF-C
C-terminal
Ab H-190

Figure 1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WT
Δpro
kDa
proPC5A
PC5A
PC5A-ΔC
105
53
34
HK293 media

Figure 2

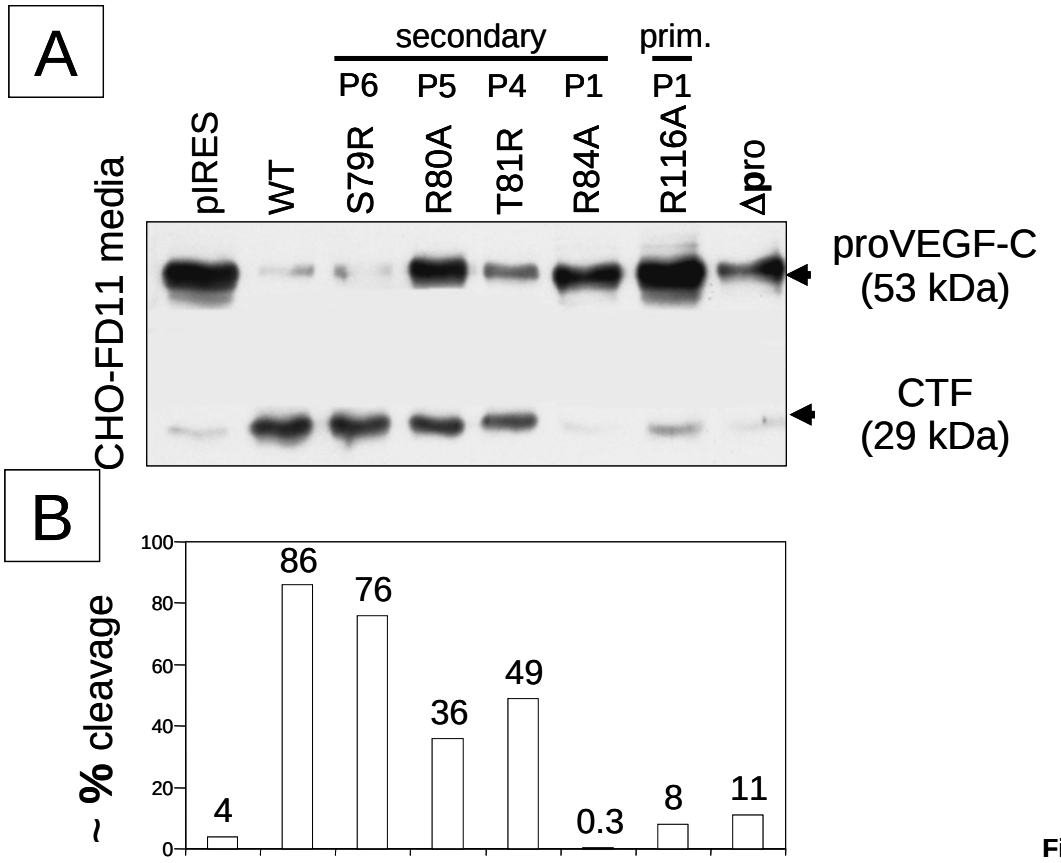
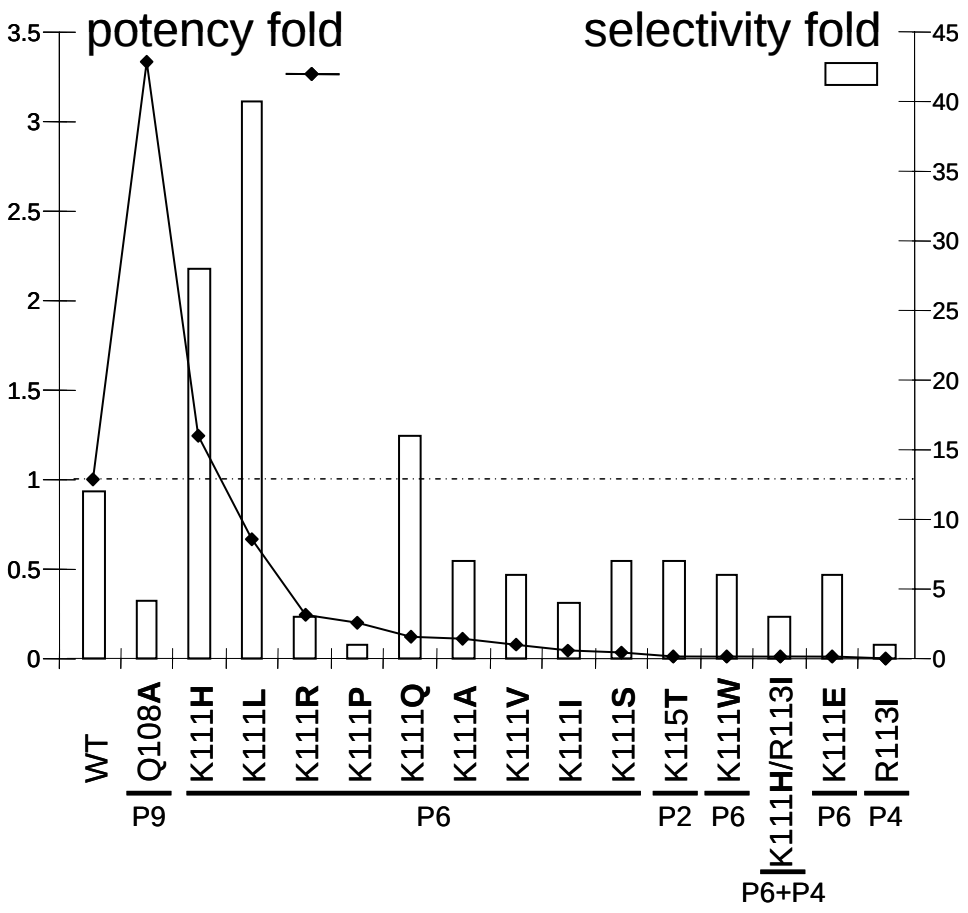

A
secondary
prim.
P6
P5
P4
P1
P1
pIRES
WT
S79R
R80A
T81R
R84A
R116A
Δpro
CHO-FD11 media
proVEGF-C
(53 kDa)
CTF
(29 kDa)
B
~ % cleavage
100
80
60
40
20
0
4
86
76
36
49
0.3
8
11

Figure 3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3
2.5
2
1.5
1
0.5
0
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40
35
30
25
20
15
10
5
0
WT
Q108A
K111H
K111L
K111R
K111P
K111Q
K111A
K111V
K111I
K111S
K115T
K111W
K111H/R113I
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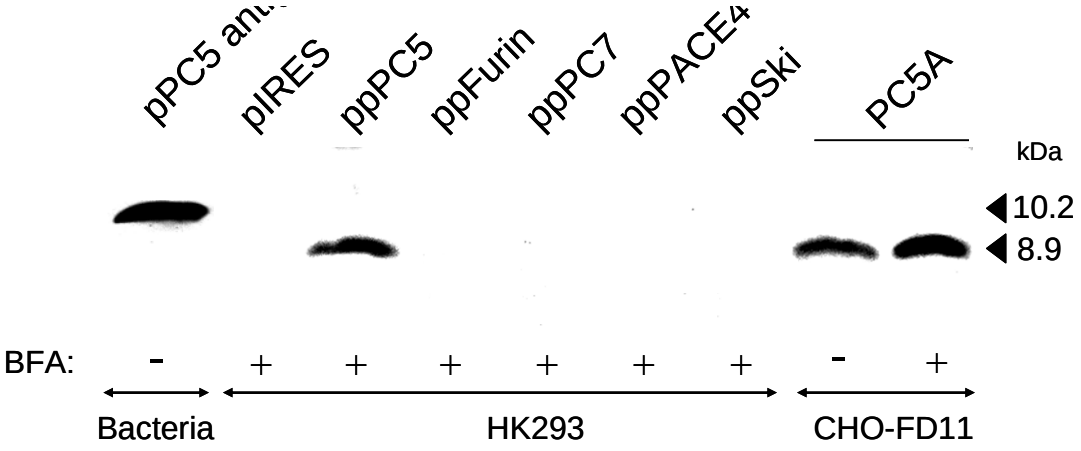
P2
P6
P6
P4
P6+P4

Figure 4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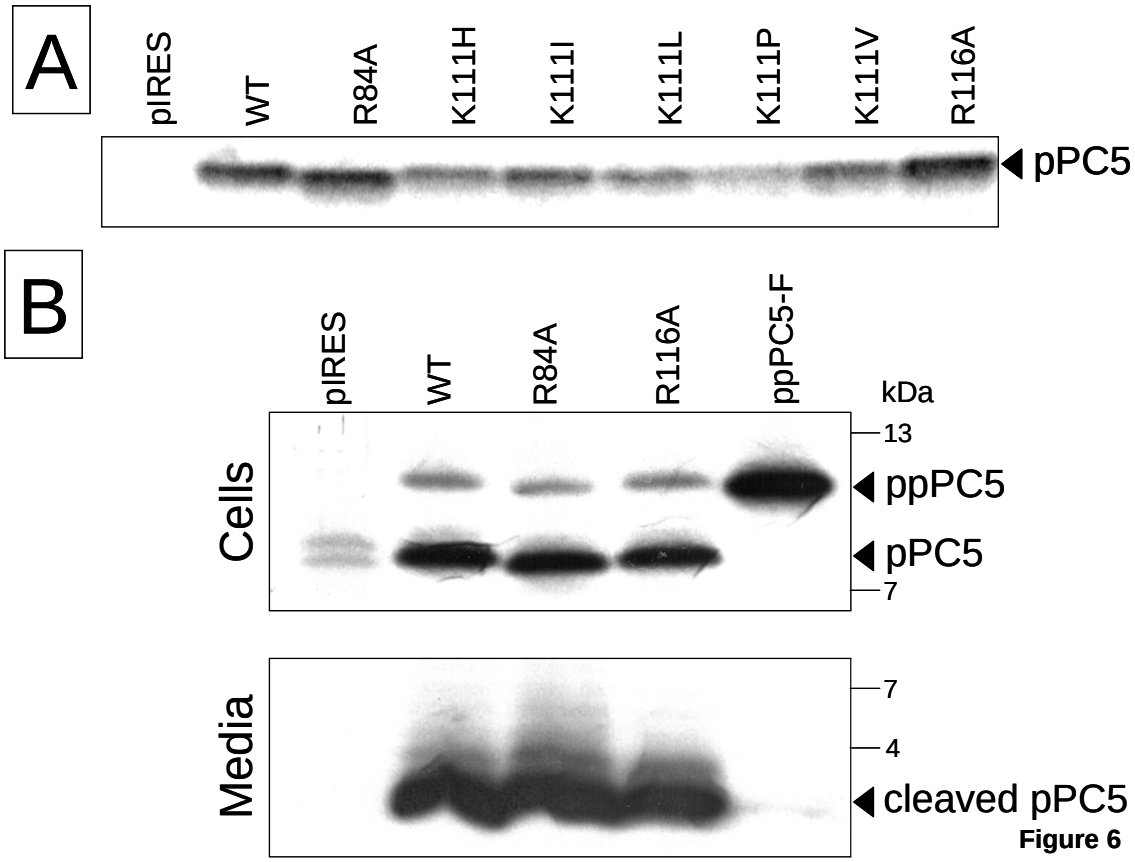
ppPC5
ppFurin
ppPC7
ppPACE4
ppSki
PC5A
kDa
10.2
8.9
BFA: - + + + + + + - +
Bacteria
HK293
CHO-FD11

Figure 5


A
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WT
R84A
K111H
K111I
K111L
K111P
K111V
R116A
pPC5
B
pIRES
WT
R84A
R116A
ppPC5-F
kDa
Cells
13
ppPC5
pPC5
7
Media
7
4
cleaved pPC5

Figure 6

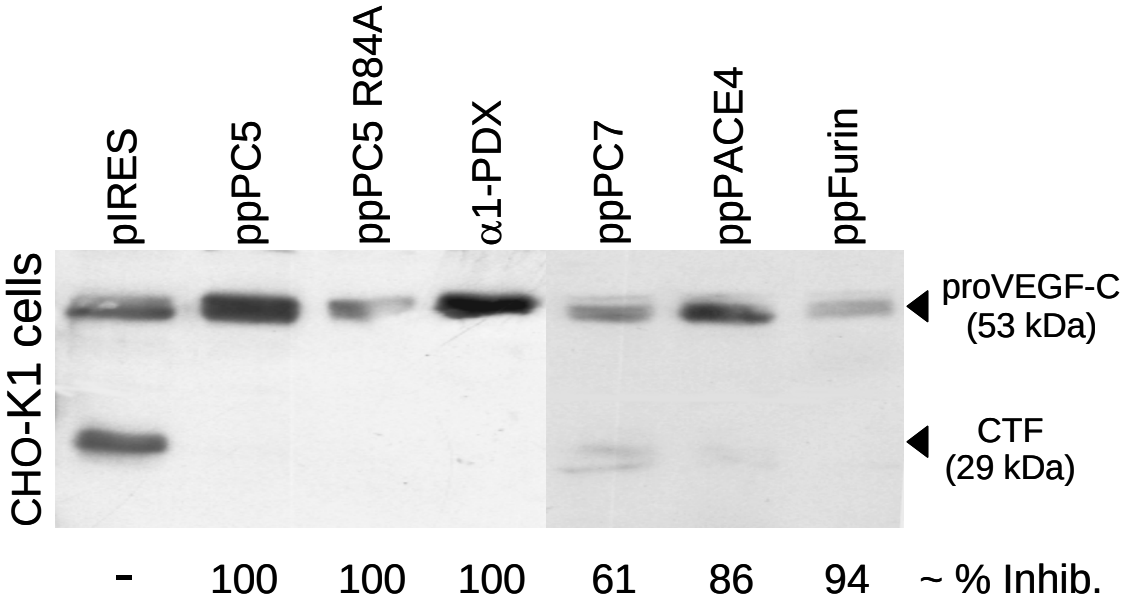
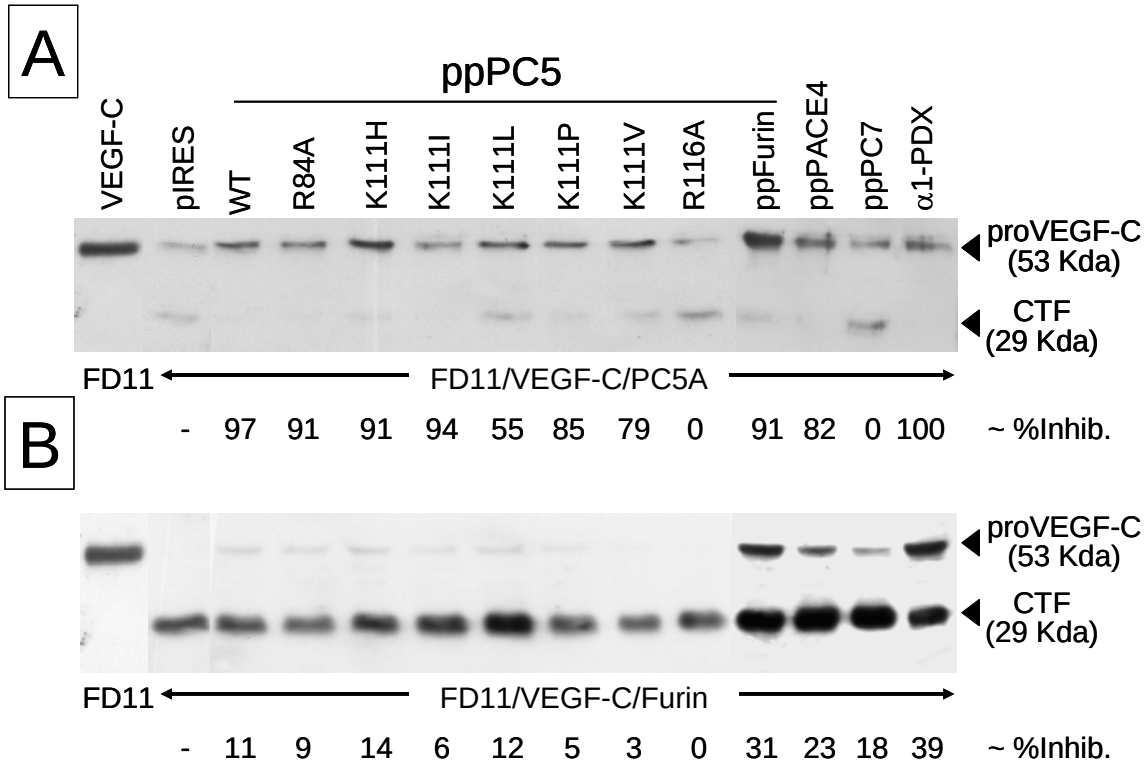

pIRES
ppPC5
ppPC5 R84A
α1-PDX
ppPC7
ppPACE4
ppFurin
CHO-K1 cells
proVEGF-C
(53 kDa)
CTF
(29 kDa)
-
100
100
100
61
86
94
~ % Inhib.

Figure 7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ppPC5
VEGF-C
pIRES
WT
R84A
K111H
K111I
K111L
K111P
K111V
R116A
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ppPACE4
ppPC7
α1-PDX
proVEGF-C
(53 Kda)
CTF
(29 Kda)
FD11
FD11/VEGF-C/PC5A
B
- 97 91 91 94 55 85 79 0 91 82 0 100 ~ %Inhib.
proVEGF-C
(53 Kda)
CTF
(29 Kda)
FD11
FD11/VEGF-C/Furin
- 11 9 14 6 12 5 3 0 31 23 18 39 ~ %Inhib.

Figure 8

Preface

This chapter describes a study on the function of another structural domain of PC5A, namely the cysteine-rich domain (CRD). Four members of the convertase family; furin, PACE4, PC5A and PC5B exhibit a CRD at their C-terminus. The significance of this cysteine-rich domain is unclear as removal of the C-terminus of furin does not affect its proteolytic activity. Thus, it was of interest to investigate the role of the CRD of PC5A and also to rationalize its observed cleavage. An earlier research has shown that PACE4 can be found in the extracellular matrix component of cells and that it can bind heparin via a cationic stretch found within its CRD. Moreover, PC5A but not furin is also capable of binding heparin. It was suggested that the CRD could confer resistance to enzymatic proteolysis, allow the enzyme to access plasma membrane proteins or protein assemblies possibly involved in cell-cell interactions or in cell growth regulation.

In order to identify the specific role of the CRD of PC5A, we have designed cDNAs including full-length PC5A, the PC5A without its CRD (PC5-Δ C) and the complete CRD with the signal peptide (CRD-PC5). It was the first attempt to express a domain rich in cysteines that would be well folded and secreted. Indeed, these CRD proteins were folded and secreted. These constructs were used to determine if the CRD of PC5A affects the subcellular localization of the

enzyme by immunofluorescence analysis. Moreover, the same constructs were employed to verify if the CRD is also involved in protein interaction by co-immunoprecipitation experiments. We wanted to determine which type of protease is involved in the processing of the C-terminus of PC5A encompassing its CRD. Thus, various protease inhibitors were tested to identify which type of protease is implicated in this processing. In addition, the C-terminal processing of PC5A may affect its proteolytic activity against a substrate. Finally, the potential antiproliferative role of PC5A was tested by its overexpression or its downregulation using a siRNA in cells and the overexpression would lead to a reduction in the cellular proliferation rate. Finally, this study also investigated the level of expression of PC5 in lung tumours as compared to the adjacent normal tissues, which will further support the fact that PC5A possesses a potential antiproliferative role.

CHAPTER D

TIMP-2 Mediated Cell Surface Localization of the Convertase PC5 through the Cysteine-Rich Domain: Role in Cellular Proliferation

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Summary

The convertases PC5, PACE4 and furin contain a C-terminal cysteine-rich domain (CRD) of unknown function. We demonstrate that full length PC5A can activate the cell surface matrix metalloprotease proMT1-MMP into MT1-MMP, which in turn cleaves the CRD of PC5A. Confocal and biochemical analyses revealed that the CRD is essential for cell surface localization of PC5A and that it co-localizes and co-immunoprecipitates with the tissue inhibitor of metalloproteinases-2 (TIMP-2). The plasma membrane localization of PC5A in

TIMP-2 null fibroblasts was only observed upon co-expression with TIMP-2. Thus, cell surface immobilized TIMP-2 can retain PC5A via its CRD. Moreover both PC5A and TIMP-2 exhibit anti-proliferative activity, and PC5-derived siRNA treatment of HT1080 cells reversed this effect. Finally, in various cancers the mRNA expression of PC5 and TIMP-2 are positively correlated, supporting a role of cell-surface PC5 in cellular proliferation.

Running title: TIMP-2 binds the cysteine-rich domain of PC5 and PACE4

Introduction

Post-translational processing of numerous secretory proteins to generate biologically active moieties is accomplished by the proprotein convertases (PCs), which are serine proteinases related to the bacterial enzyme subtilisin and yeast kexin. These proteinases perform critical functions in a variety of physiological and pathological processes. There are 7 known basic amino acid (aa)-specific PC family members that cleave various secretory precursors following basic residues: furin, PC1/3, PC2, PC4, PACE4, PC5/6 and PC7 (Seidah and Chretien, 1999c). Recently, two other non-basic aa-specific convertases implicated in cholesterol metabolism have been identified, namely SKI-1/S1P (Seidah and Prat, 2002) and NARC-1/PCSK9 (Abifadel et al., 2003b;Seidah et al., 2003b). All basic aa-specific convertases contain the same N-terminal organization starting with a signal peptide, followed by a prodomain, catalytic and a β-barrel P-domain, while the C-terminal architecture is specific to each convertase (Seidah and Chretien, 1999b). Following the P-domain, three of the basic-aa-specific convertases, furin, PACE4 and PC5 contain a Cysteine-Rich Domain (CRD) (Seidah and Chretien, 1999a). PC5 is the only member of this convertase family that exists as two isoforms: soluble PC5A and membrane-bound PC5B, the latter having an extended C-terminal CRD (Nakagawa et al., 1993c). PC5A is sorted to both the constitutive and regulated secretory pathways, whereas PC5B is localized only within the

constitutive secretory pathway (De Bie et al., 1996f), mainly in a Golgi compartment communicating with endosomes (Xiang et al., 2000a). The CRD of PC5A contains four N-linked glycosylation sites and 44 cysteine residues arranged in 5 tandem repeats with the consensus motif (Lusson et al., 1993c): Cys-Xaa2-Cys-Xaa3-Cys-Xaa5-7-Cys-Xaa2-Cys-Xaa8-15-Cys-Xaa3-Cys-Xaa9-16. This 5-tandem repeats motif is conserved between frog and mammalian PC5A (Gangnon et al., 2003a). PACE4 also possesses 5-tandem repeats within its CRD, while furin exhibits only two shortened repeats (Nakagawa et al., 1993d). So far, the function of the CRD of each PC is unknown, though it was suggested that secreted PC5A and PACE4, but not soluble furin, could bind heparin within the extracellular matrix (ECM), likely via a cationic stretch of amino acids within their CRD (Tsuji et al., 2003a). Finally, in the regulated corticotroph AtT20 cells, it was previously observed that the membrane-bound PC5B (~210 kDa) can be shed into the medium (~170 kDa) and that both PC5A and PC5B are processed at their C-termini prior to and/or during secretion to produce a shorter PC5-Δ C secreted form (~65 kDa) lacking the CRD (De Bie et al., 1996e).

Although the *in vivo* functions of PC5 are not well delineated, it was shown to be implicated in the processing of a number of cell surface precursors such as adhesion molecules including integrin α-chains (Lissitzky et al., 2000b;Bergeron et al., 2003a;Stawowy et al., 2004a), the neural adhesion protein L1 (Kalus et al.,

2003b), TGF-β like proteins (Nachtigal and Ingraham, 1996a;Ulloa et al., 2001b;Stawowy et al., 2003), a receptor protein tyrosine phosphatase RPTPμ (Campan et al., 1996d), and membrane-bound metalloproteinases such as ADAM-17 (Srour et al., 2003) and possibly the membrane type-1 matrix metalloproteinase MT1-MMP (Yana and Weiss, 2000h). The latter plays an essential role in extracellular matrix remodeling and signaling (Tam et al., 2004b) after the activation of its zymogen proMT1-MMP by a furin-like convertase (Yana and Weiss, 2000e).

The degradation of ECM proteins is usually performed by matrix metalloproteinases (MMPs). This family of proteinases is composed of 23 members (Sternlicht and Werb, 2001b;Overall and Lopez-Otin, 2002). MMPs are not only involved in ECM remodeling, they can also regulate the function of an increasing number of important signaling proteases through limited proteolysis (McQuibban et al., 2000;Tam et al., 2004a). The activity of MMPs is regulated by specific endogenous inhibitors known as tissue inhibitors of metalloproteinases (TIMPs), four of which are known (TIMP-1, -2, -3 and -4) (Jiang et al., 2002;Baker et al., 2002). The activation of proMMP-2 is mediated via MT1-MMP at the cell surface and it involves the formation of a ternary complex that requires TIMP-2. At the plasma membrane, the N-terminal inhibitory domain of TIMP-2 binds to the catalytic site of MT1-MMP, thus inactivating this metalloprotease. The hemopexin

C-domain of the soluble proMMP-2 interacts with this binary complex by specifically binding to the C-terminal domain of TIMP-2 (Overall et al., 1999a;Kai et al., 2002b), thus forming a ternary complex at the plasma membrane. The prodomain of proMMP-2 is then cleaved and activated into MMP-2 by a second MT1-MMP molecule (Butler et al., 1998;Zucker et al., 1998b). Although at low concentrations of TIMP-2, its proMMP-2 activation effects outweigh its inhibitory actions, at high concentrations, TIMP-2 is an inhibitor of MMP-2, thereby preventing tumor cell invasion and metastasis. However, it was recently shown that TIMP-2 is also able to inhibit endothelial cell proliferation (antiangiogenic role) through a mechanism that is independent of MMP-2, but implicates its binding to integrin α 3β 1 (Seo et al., 2003f).

The aims of the present study center on the elucidation of the functional importance of the CRD of PC5A, and include: **(1)** the rationalization of the PC5A CRD excision and its relation to MT1-MMP activation; **(2)** the cell surface localization of PC5A and the implication of its CRD in this process; **(3)** the role of TIMP-2 in these processes; and **(4)** the importance of PC5 in proliferation both *ex vivo* and in solid tumors. To accomplish these objectives, we used recombinant wild type PC5A, TIMP-2 and MT1-MMP and various deletions thereof, and/or trafficking mutants, as well as cellular, immunocytochemical and transcriptional analyses. We found that TIMP-2 mediates the cell surface localization of PC5A

through binding of its CRD, thus revealing a new non-inhibitory role for this multipotent inhibitor. Moreover, PC5A also exhibits an anti-proliferative activity in cell lines, similar to its partner TIMP-2. Finally, using quantitative RT-PCR (qRT-PCR) on biopsies of solid human lung tumors as compared to their adjacent normal tissues, as well by bioinformatics analyses, we provide evidence that PC5 and TIMP-2 mRNA levels are similarly regulated.

Results

Excision of the CRD via C-Terminal Processing of PC5A and PC5B

Constitutively secreting COS-1 cells were transiently transfected with either an empty pIRES2-EGFP vector, or the same construct expressing a recombinant soluble PC5A or membrane-bound PC5B (Figure 1A). The conditioned media and cell extracts were analyzed by Western blotting using an N-terminal PC5-specific antibody (De Bie et al., 1996d). In cell lysates, PC5B is found in two forms, the full-length membrane-bound PC5B (FL-PC5B; ~210 kDa) and a shorter form (sPC5B; ~170 kDa), also found in the medium, together with a ~65 kDa product, both likely representing shed forms (Figure 1A). On the other hand, mostly mature full-length PC5A (FL-PC5A; ~110 kDa) was detected in the cell extract, a form also found in the medium together with a significant amount of a

C-terminally processed ~65 kDa product (Figure 1A). The ~110 and ~65 kDa PC5A-derived proteins were previously observed in the regulated AtT20 cells (De Bie et al., 1996c), and are now detected in transfected COS-1 (Figure 1A), HK293 (Figure 2A), and CHO (Figure 2B) cells suggesting that such C-terminal truncation of PC5A is widespread and is not restricted to regulated cells.

A recent study revealed that the inactive, secreted proPC5A-R84A mutant was still processed into the ~65 kDa form, implying that the C-terminal truncation of PC5A is not autocatalytic (Nour et al., 2003a). In order to identify the cleavage site, we analyzed in COS-1 cells the processing of various point mutants of PC5A around the suspected processing site (within aa 613-623; Figures 1B, C). Since all constructs contained a C-terminal V5-epitope, we were able to visualize the ~50 kDa C-terminal fragment (PC5A-CT; Figure 1C). While the Y619P mutant is not cleaved at all, processing of the K613A, R616A and R618A mutants is significantly reduced (>70%). Furthermore, the S620P mutant did not have a significant effect on processing, whereas both the R621A and the E623A mutants are ~2-fold better cleaved that the wild type (WT) enzyme (Figure 1C). This experiment suggested that Tyr_{619} is critical for cleavage. This was further confirmed by the N-terminal sequencing of the ^{3}H-Tyr labeled ~50 kDa C-terminal fragment obtained from the well processed R621A mutant, revealing Tyr residues at positions 10 and 15 (*not shown*; in bold in Figure 1B).

MT1-MMP Can Excise the CRD of PC5

In order to characterize the type of candidate protease(s) responsible for the processing of PC5 at its C-terminus, we tested various classes of protease inhibitors, including the general PC-inhibitor α 1-PDX (Figure 2A) (Benjannet et al., 1997a;Jean et al., 1998a) and metalloprotease inhibitors (Figure 2C). In HK293 cells the C-terminal processing of both WT PC5A and its R621A mutant were inhibited by co-transfection of α 1-PDX (Figure 2A). However, further work demonstrated that PCs are not directly implicated in this C-terminal processing event. Accordingly, we analyzed the fate of PC5 and its R621A mutant in either CHO cells or in furin-deficient CHO-FD11 cells in the presence or absence of exogenous furin (Gordon et al., 1995a). Thus, while PC5A and its R621A mutant are processed in CHO cells, they are not significantly cleaved in CHO-FD11 and CHO-FD11 + furin cells (Figure 2B), suggesting that furin is not directly involved in this processing. Similarly, expression of each of the other PCs (PC5A, PACE4 or PC7) in FD11 cells did not affect this C-terminal cleavage (*not shown*). These results argue against the direct implication of PCs in the C-terminal processing of PC5A, and the inhibition by α1-PDX rather suggests a role of PCs in the activation of a cognate proteinase(s). Since PCs are known to activate a number of metalloproteinases such as ADAMs and MT-MMPs, it was hypothesized that activated MT1-MMP may be involved in the cleavage of PC5 in a reciprocal processing reaction. Hence,

we tested the possible inhibitory effect of various metalloprotease inhibitors in HK293 cells (Figure 2C). Accordingly, the metal chelators EDTA, EGTA and the MMP inhibitor GM6001 (Elagoz et al., 2002f) are effective blockers of PC5-CT cleavage, while the ADAM17 inhibitor TAPI, the angiotensin converting enzyme inhibitor captopril and the thermolysin and neutral endopeptidase-24.11 inhibitor phosphoramidon are not (Figure 2C). Thus, this directed us towards the likely involvement of PC-activated MMPs, including MT1-MMP and MT2-MMP, in the generation of PC5A-CT. This hypothesis was tested in COS-1 cells that endogenously do not express MT1-MMP (Yana and Weiss, 2000c). Although both membrane-bound metalloproteinases MT1-MMP and MT2-MMP contain a furin-susceptible activation site (Sternlicht and Werb, 2001b), only MT1-MMP markedly enhanced the excision of the CRD PC5A (Figure 2D).

We next showed that while furin (Yana and Weiss, 2000a) and full length PC5A can process proMT1-MMP into MT1-MMP, PC5-Δ C, lacking the CRD, is unable to do so, since the % processing is similar to that observed in the control (CTL) empty vector-transfected cells (Figure 2E). Thus, the CRD is critical for the ability of PC5A to process proMT1-MMP and its removal by MT1-MMP would in turn affect the specific proteolytic activity of PC5A-Δ C.

TIMP-2 Interacts with PC5A Via the CRD

Since furin and MT1-MMP co-localize at the cell surface of renal cells (Mayer et al., 2003) and as we (Figure 2E) and others (Yana and Weiss, 2000b) showed that both furin and PC5A process MT1-MMP, we tested the cell surface localization of PC5A and PC5A-ΔC. In addition, since TIMP-2 was shown to be associated with MT1-MMP in the context of proMMP-2 activation (Overall et al., 1999b;Kai et al., 2002a), we also investigated whether the TIMP-2/MT1-MMP complex regulates the excision of the CRD of PC5. Accordingly, we used a cellular missorting technique previously applied to the β3-integrin (Conesa et al., 2003d), whereby the C-termini of the soluble TIMP-2 and the ectodomain of MT1-MMP were fused to a lysosomal/endosomal targeting sequence consisting of the Lamp1 transmembrane-cytosolic tail, herein named TIMP-2-LP (Figures 3A) and MT1-MMP-LP (Figures 2D,E). According to this dominant negative technique, partners of TIMP-2 or MT1-MMP would be expected to cycle to the cell surface and be dragged to the endosomal/lysosomal compartments for degradation (Conesa et al., 2003c). Both TIMP-2 and TIMP-2-LP are well expressed (Figure 3A). Amazingly, in contrast to MT1-MMP-LP (Figure 2D) and TIMP-2 (Figure 3B), expression of TIMP-2-LP (Figure 3B) led to the virtual elimination of secreted full length PC5A. On the other hand, co-expression of TIMP-2-LP with PC5-Δ C (lacking the CRD) had no effect on its secretion level (Figure 3B). This was the first indication that TIMP-2 may possibly interact with the CRD of PC5A.

Further confirmation of this model was obtained by co-expression of TIMP-2-LP with a construct comprising the PC5A signal peptide directly fused to the CRD (herein called PC5A-CRD; see Figure 5A). Thus, while PC5A-CRD is well folded and secreted, co-expression of TIMP-2-LP led to a significant reduction of the secreted protein (Figures 3B,C). As a specificity test, we compared the effect of TIMP-2 and TIMP-2-LP on the secretion level of the CRDs of PC5A and its closest homologue PACE4. As a control, we also tested the recently described novel convertase NARC-1 because it exhibits a different CRD at its C-terminus and possesses a unique cleavage specificity (Seidah et al., 2003a). The data showed that for the full length PC5A and the CRDs of both PC5A and PACE4, their secretion levels were reduced upon co-expression of TIMP-2-LP but not TIMP-2, while the secretion of full length NARC-1 was not affected (Figure 3C).

An independent verification of the potential interaction between PC5A and TIMP-2 was obtained from co-immunoprecipitation experiments of PC5A with either endogenous or co-expressed TIMP-2 and TIMP-2-LP in HT1080 cells. We first observed that in cell extracts and conditioned media overexpressed FL-PC5A co-immunoprecipitates with endogenous TIMP-2 (CTL/FL-PC5A in Figure 4A). Equivalent data were also obtained when both partners are co-expressed, i.e., PC5A with either TIMP-2 or TIMP-2-LP (Figure 4A).

In an effort to define the region within the CRD critical for its interaction with TIMP-2, we attempted to test whether (i) the loss of the granule sorting domain of PC5A, corresponding to its last C-terminal 38 aa, or (ii) sulfation of the CRD (De Bie et al., 1996b) could affect the binding of PC5A to TIMP-2. Our data revealed that PC5A-Δ38 (lacking the C-terminal 38 aa) (De Bie et al., 1996a) was equally immunoprecipitable with TIMP-2 and TIMP-2-LP as the full length PC5A (*not shown*). Similarly, we found that inhibition of PC5A Tyr-sulfation by sodium chlorate did not affect the ability of the CRD to bind TIMP-2. Therefore, we concluded that neither the C-terminal 38 aa nor sulfation of its CRD is required for the binding of PC5A to TIMP-2.

In order to probe the specificity of this TIMP-2/PC5A interaction, we tested the ability of ^{35}S-Met/Cys pulse-labeled CRD-PC5A, CRD-PACE4 and CRD-furin to co-immunoprecipitate with TIMP-2 or TIMP-2-LP using a TIMP-2 specific antibody (Figure 4B). Such interaction was observed only with CRD-PC5A and CRD-PACE4 but not with CRD-furin (Figure 4B). With the caveat that different expression vectors were used, the data seem to suggest that a small fraction of the total cellular overexpressed TIMP-2 is bound to the CRDs. This indicates that PC5A and PACE4, but not furin, possess a similar TIMP-2-binding motif within their CRDs, in accordance with their identical number of cysteine repeats, quite distinct from the two shortened repeats of furin (Nakagawa et al., 1993e).

PC5A and TIMP-2 Co-Localize at the Cell Surface

Cell surface immunofluorescence was performed on COS-1 cells that reportedly express TIMP-2 but are deficient in endogenous MT1-MMP (Pavlaki et al., 2002). Accordingly, expression of FL-PC5A, PC5A-Δ C or PC5A-CRD (Figure 5A) in COS-1 cells demonstrated that only those constructs containing the CRD were detectable at the cell surface (Figure 5B). Since our polyclonal human TIMP-2 antibody did not detect the endogenous protein (CTL in Figure 5), we co-transfected TIMP-2 with V5-labeled recombinant PC5A constructs (Figure 5C). Cell surface labeling was first performed using an anti-TIMP-2 antibody and a secondary antibody coupled to fluorolink Cy5 (blue color). In all cases overexpressed TIMP-2 was detected at the cell surface, even though these cells lack MT1-MMP. This suggested that TIMP-2 can bind other plasma membrane proteins in COS-1 cells, e.g., $\alpha 3\beta 1$ (Seo et al., 2003e), or other membrane-type matrix metalloproteases (Sternlicht and Werb, 2001b). To test whether PC5A via its CRD colocalizes with cell surface TIMP-2, we co-expressed TIMP-2 with various PC5A constructs (Figure 5C). Immunolocalization of PC5A expression was obtained using an anti-V5 antibody followed by the addition of a secondary antibody conjugated to Alexa Fluor555 (red color). Confocal cell surface analysis revealed that FL-PC5A and PC5A-CRD co-localize extensively with TIMP-2,

whereas PC5A-ΔC was not detectable even in the presence of excess TIMP-2 (Figure 5C).

Since integrin α 3β 1 binds TIMP-2 (Seo et al., 2003d) and furin/PC5 can process integrin α-chains such as α3 (Lehmann et al., 1996b), we tested the co-immunoprecipitation of FL-PC5A and PC5A-ΔC with endogenous integrin α 3 in HT1080 cells in the presence or absence of co-expressed TIMP-2. Results showed that only FL-PC5A co-immunoprecipitates with α3β1 and not PC5A-ΔC. In contrast, FL-PC5A did not co-immunoprecipitate with α3 when co-expressed with TIMP-2 indicating that both FL-PC5A and TIMP-2 possibly compete for a similar α 3 binding site (Figure S1).

TIMP-2 is Required Cell Surface Localization of PC5A

TIMP-2 null cells and TIMP-2 null cells overexpressing MT1-MMP (Morrison et al., 2001a) are useful tools to probe the critical importance of the expression of TIMP-2 for the cell surface localization of PC5A. Both cell types were transiently transfected with FL-PC5A with or without TIMP-2. Transfected cells were treated with concavalin A (ConA) in order to enhance the cell surface localization of TIMP-2 (Morrison et al., 2001b). Immunofluorescence analysis revealed that only in the presence of TIMP-2 could PC5A localize to the cell surface (Figure 6). Interestingly, PC5A was mostly found at the surface of cells that showed a high TIMP-2 labeling and revealed an uneven punctate staining, indicating that the cell

surface localization of PC5A may depend on the presence of optimal amounts of TIMP-2 and/or specific lipid-protein complexes.

PC5 Acts as a Suppressor of Cellular Proliferation and is Down Regulated in Certain Tumors

Zymography analysis of HT1080 cells overexpressing PC5A and/or TIMP-2 revealed that: **(i)** PC5A does not compete with endogenous MMP-2 for TIMP-2 binding, **(ii)** it does not co-immunoprecipitate with MMP-2, and **(iii)** the processing of proMMP-2 into MMP-2 is not affected by the presence of PC5A (*not shown*). These results suggested that although PC5A interacts with TIMP-2 it does not affect the role of the latter in MMP-2 activation. Since it was recently shown that TIMP-2 inhibits cellular proliferation (Hoegy et al., 2001) and that this anti-proliferative role may be mediated through a newly characterized TIMP-2 receptor, namely integrin α 3β 1 (Seo et al., 2003c), we were curious to verify if PC5A can affect cellular proliferation. The effect of PC5A on the proliferation of human prostate cancer PC3 cells was initially tested by Western blotting of the level of proliferating cell nuclear antigen (PCNA), an accepted marker of cellular proliferation (Stawowy et al., 2001c). Although the level of PCNA was not affected upon expression of PC5A-ΔC, it is significantly reduced (~2 fold) when FL-PC5A is present (Figure 7A). Thus, FL-PC5A suppresses cellular proliferation and its CRD is critical for this inhibitory activity. As a corollary, we assessed the PCNA levels of

transfected HT1080 cells with either FL-PC5A or with a siRNA against PC5 that resulted in a ~50% reduction of its overall mRNA levels (as assessed by quantitative qRT-PCR, *not shown*). Cells treated with PC5-siRNA exhibited significantly increased (~2 fold) PCNA levels, as compared to cells transfected with a control siRNA (Figure 7B). An independent confirmation of the anti-proliferative effect of PC5A was obtained from the rate of [^{3}H]-thymidine incorporation, revealing that only FL-PC5A overexpression, but not PC5A-ΔC, results in a ~2-fold decrease in radiolabel incorporation into HT1080 cells (Figure 7C). We conclude that PC5A can act as a suppressor of cellular proliferation, possibly via its interaction with the antiproliferative TIMP-2/α3β1 complex (Seo et al., 2003b).

In order to further elucidate the importance of PC5 and its correlation to TIMP-2 in cellular proliferation, we tested by qRT-PCR their mRNA expression in human lung tumors (T) (Mbikay et al., 1997b) as compared to adjacent normal (N) tissues where PC5 (Lusson et al., 1993b;Seidah et al., 1994b) and TIMP-2 (Blavier and DeClerck, 1997b) are highly expressed. In one paired sample (N1/T1) of unclassified lung carcinoma we observed that PC5 and TIMP-2 mRNA levels are similarly reduced by ~65-70% (Table 1). Low levels of both mRNAs (~50-85% lower) are also observed in two other unpaired adenocarcinoma lung tumors (T2* and T3*) as compared to a normal lung tissue (N2*). These data indicate that in a subset of lung tumors, PC5 and TIMP-2 mRNA expression may be downregulated as compared to normal tissue. Similar analyses were also

performed to measure the mRNA levels of furin, SKI-1 and NARC-1 (Table 1). The data revealed that while the expression SKI-1 and NARC-1 is relatively invariable in the above tissues, furin increases ~3-5 folds in the unpaired tumors. However, in another paired adenocarcinoma (N4/T4) we observed a ~2-fold increase in PC5 mRNA levels without much change in TIMP-2, SKI-1 or furin. Notably, in this particular tumor the mRNA level of NARC-1 is upregulated 8-fold. Since this is a limited set of samples, we also examined the correlation between the expression of PC5 and TIMP-2 using the publicly available bioinformatics infrastructure for cancer genomics research Oncomine 2.0 (http://www.oncomine.org/). This facility consists of a large number of cancer microarray data sets and a correlation module to identify genes that are co-regulated with a gene of interest in a selected cancer type. Using this tool, we were able to identify two cancer types where PC5 and TIMP-2 expression were coordinately dowregulated, namely in breast cancer (Perou et al., 2000) and cutaneous B-cell lymphoma (Storz et al., 2003).

Discussion

The present study demonstrated that PC5A cleaves and likely activates cell-surface membrane-bound metalloproteinases such as MT1-MMP. On the other hand, PC5A is itself processed by a metalloproteinase, e.g., MT1-MMP, resulting

in the excision of its CRD. This intriguing observation led to the hypothesis that the intracellular activation of proMT1-MMP into MT1-MMP by a proprotein convertase, such as PC5A, may be followed by the loss of the CRD of the cognate convertase, possibly at the cell surface. This may thus modulate the substrate recognition by PC5A at the cell-surface and/or regulate its extracellular activity. To further probe the proximity of the cell-surface localized MT1-MMP to its substrate PC5A; we tested the possibility that the secreted MT1-MMP inhibitor TIMP-2 could recruit PC5A to the cell-surface. Accordingly, we used a cell-based missorting approach (Conesa et al., 2003b), as well as co-immunoprecipitation and co-localization immunocytochemical techniques. The data demonstrate that TIMP-2 binds PC5A through its CRD and that cell-surface immobilized TIMP-2 is required for the plasma membrane co-localization of PC5A with TIMP-2 (Figures 3-6). It is possible that the level of the cell-surface heterodimer is regulated via processing of the CRD by a metalloprotease such as MT1-MMP, resulting in the release of PC5-□C. This would therefore lead to a decrease in the anti-proliferative activity of full length PC5A (Figure 7; Table 1).

Earlier studies demonstrated that the majority of protein precursors cleaved by the soluble/secreted PC5A are membrane-bound proteins, e.g., adhesion molecules including integrin α-chains (Lehmann et al., 1996a;Lissitzky et al., 2000d;Stawowy et al., 2004b) and the neural adhesion protein L1 (Kalus et al., 2003c), as well as TGFβ-like precursors (Dubois et al., 2001a;Ulloa et al., 2001c),

resulting in active αβ heterodimers. TIMP-2 was recently shown to bind α3β1 and to signal an anti-proliferative response through this receptor (Seo et al., 2003a). Since both TIMP-2 and PC5A co-immunoprecipitate with endogenous α3, it is possible that the TIMP-2/PC5A complex plays a role in the observed inhibition of angiogenesis by TIMP-2 (Seo et al., 2003h). This finding is consistent with the high expression level of PC5A in endothelial cells lining blood vessels (Beaubien et al., 1995) and its reported transcriptional regulation though contact inhibition (Campan et al., 1996e).

Prior to this work, the function of the CRD of PC5, PACE4 and furin was unknown. It was suggested that the CRD of the convertases could allow them to access plasma membrane precursors (Oliva, Jr. et al., 2000a), interact with the extracellular matrix (Tsuji et al., 2003b), affect cell growth and or localization (Lusson et al., 1993a), as well as enhance the stability of the convertase PC5B (Wang et al., 2004b). From this study, we demonstrated that the CRD of the soluble PC5A targets this enzyme to the plasma membrane. Moreover, we showed that in contrast to furin and NARC-1, the CRD of PC5A and PACE4 can specifically interact with TIMP-2. Therefore, we concluded that the TIMP-2/CRD complex formation of PC5A or PACE4 likely implicates a presently undefined TIMP-2-recognition motif, not contained within the CRD of furin and NARC-1. Whether other TIMPs (e.g., TIMP1, 3 and 4) also interact with the CRD of PC5A is yet to be determined, especially in view of the fact that the mRNA levels of

TIMP-3 and PC5A are coordinately regulated, and both are co-expressed during placental embryonic implantation and decidualization (Rancourt and Rancourt, 1997a;Wong et al., 2002d) and are possibly involved in the cell-surface processing of zona pellucida domain proteins (Jovine et al., 2004). In addition, PC5A can process TGFβ-like (usually anti-proliferative) proteins (Dubois et al., 2001b;Ulloa et al., 2001d) and active TGFβ regulates the expression of TIMP-3 (Leco et al., 1994a).

The crystal structure of TIMP-2 revealed it to contain two domains, an N-terminal and a negatively charged C-terminal domain linked by a single Glycine residue (Fernandez-Catalan et al., 1998;Morgunova et al., 2002a). While the inhibitory N-terminal domain binds to the active sites of MT1-MMP, the C-terminal domain interacts with the hemopexin domain of proMMP-2. We do not know at present which domain of TIMP-2 binds the CRD of PC5A and PACE4. Interestingly, it was reported that the contact surface in the complex of TIMP-2 and proMMP-2 implicates two areas of hydrophobic interactions surrounded by electrostatic contacts (Morgunova et al., 2002b). In proMMP-2, this cluster is composed of Ala^{583}, Tyr^{607}, Leu^{609}, Val^{619} and Phe^{621}. Surprisingly, alignment of this cluster with the CRD of PC5A, revealed that the proMMP-2 sequence ^{607}YYLKLE(N-glycosylation)QS^{615} closely resembles the PC5A sequence ^{661}YYYKLK(N-glycosylation)NT^{669}. The latter motif is located 42 residues C-

terminal to the identified processing site of PC5A occurring at Tyr^{619} (Figure 1B) and that results in the CRD release. It is thus tempting to speculate that it may be the C-terminal domain of TIMP-2 that binds the CRD of PC5A.

The conservation of the Cys motif within the CRD of PC5A and PACE4 was also found in non-convertase proteins such as in the epidermal growth factor receptor (Ward et al., 1995b) and in a novel ECM protein known as Fras1 (McGregor et al., 2003a). Furthermore, blasting the PC5A CRD sequence (using NCBI blastp program) resulted in a perfect alignment of the Cys residues with those of the Neu proto-oncoprotein, also known as the receptor protein-tyrosine kinase erbB-2 (accession # AY116182). All these proteins are localized at the plasma membrane and are implicated in development and/or in cellular growth. A recent study on the function of the second CRD of the EGFR demonstrated its importance for the targeting of this receptor to caveolae/rafts at the plasma membrane (Yamabhai and Anderson, 2002a). These studies are in accord with the role presently ascribed to the CRD of PC5A as a critical domain for the plasma membrane localization of this enzyme. The observed punctate labeling of the cell surface PC5A (Figures 5,6) resembles that of the cell surface distribution of caveolae/rafts domains (Puyraimond et al., 2001b).

The interaction of the CRD of PC5 with TIMP-2 and the recently reported antiproliferative activity of TIMP-2 (Seo et al., 2003i) suggested that PC5 could

play a role in cellular proliferation. Our data revealed that overexpression of PC5A or downregulation of PC5 expression led to a decrease or increase in cell proliferation, respectively (Figure 7). We therefore investigated the possible downregulation of PC5 expression in tumors. We previously reported that human breast cancer cells rarely express PC5 (2/30 tumors express this enzyme), whereas most express furin and PACE4 (Cheng et al., 1997a). In the present work, we investigated the expression of PC5 mRNAs in human lung tumors as compared to adjacent healthy tissues. The limited data available revealed that while SKI-1 and NARC-1 mRNA levels remain mostly unchanged, both PC5 and TIMP-2 are downregulated in 3 of the 4 tumors studied (Table 1). A previous study revealed that furin and especially PACE4 were upregulated in these same lung tumor tissues (Mbikay et al., 1997b). In agreement with these findings, □-array analysis recently showed that PACE4, but not PC5, is upregulated 14-fold in a murine mammary tumor cell model for growth and metastasis (Yang et al., 2004). Thus, it seems that while both PACE4 and PC5 can bind TIMP-2 via their CRD, they do not follow the same regulatory pathways and they present distinct but complementary expression in various tissues such as in the brain and the pituitary gland (Dong et al., 1995a).

Browsing through the publicly available data from the NCI60 Cancer Micro array Project (http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch) for convertase expression in tumors, revealed that in non-small cell lung and colon

carcinomas the mRNA levels of PC5 were also reduced or unchanged as compared to adjacent healthy tissues, whereas those of PACE4 are either unchanged or increased. In contrast, furin seems to be upregulated in most cancers. Moreover, using the Oncomine 2.0 tool (http://www.oncomine.org/) we found that in breast cancer and cutaneous B-cell lymphoma PC5 and TIMP-2 expression were coordinately dowregulated. However, in thyroid tumors (Huang et al., 2001) and astrocytomas (Khatua et al., 2003) PC5 mRNA levels better correlate with those of TIMP-1, while in brain tumors PACE4 levels correlate with those of TIMP-3 (van den et al., 2003). It was also observed that the lack of TIMP-2 expression was an unfavorable prognostic survival factor in synovial sarcoma (Benassi et al., 2001), and reduced TIMP-2 levels are seen in ovarian carcinoma cells, possibly marking the acquisition of a metastatic phenotype (Davidson et al., 2001). Since both TIMP-2 and PC5 are binding partners and seem to be similarly regulated in some tumors, this suggests that the measurement of PC5 levels could also be useful as a prognostic marker to define tumor aggressiveness and risk of metastatic events.

In conclusion, the data presented in this work reveals a new function for the CRD of the convertases PC5 and PACE4 and provides a link between serine subtilase-like enzymes and the metalloproteinases through TIMP-2 and possibly other TIMPs. This may also lead the way towards the future identification of other

interacting molecules with the CRDs of furin and NARC-1. The extension of these results towards the realm of pharmacological relevance may lead to the development of specific comparative assays for the relative levels of PC5 and PACE4 to TIMPs in relation to pathologies such as tumor cell proliferation and metastasis.

Experimental Procedures

Vectors Constructs

Mouse PC5A was cloned into EcorI/AgeI digested pIRES2-egfp vector (Clonetech) with a C-terminal V5-tag. Mutagenesis was done by PCR using the pIRES2-PC5A-V5 cDNA template to generate the K613A, R616A, R618A, Y619P, S620P, R621A and E623A mutants. The pIRES2-PC5A-Δ C-V5 (aa 1-612) construct was generated by PCR using the primers:5' GGGCGGTAGGCGTGTACGGTGG/

3' ACCGGTGGGAAACTCGTTGG

TTGGGGAG. The pIRES2-PC5A-CRD-V5 was also generated by PCR using the following primers: 5' GGGCGGTAGGCGTGTACGGTGG/ 3' TGGCTGTACCGTC

CGGCATACCGGGAG and 5' TGCCGGACGGTACAGCCATACTCCCCAAC/ 3' CTTCGGCCAGTAACGTTAGGGG, respectively. The primers used to generate the final cDNA in the second PCR reaction were: 5' GGGCGGTAGGCGTGTACGGTGG/ 3' CTTCGGCCAGTAACGTTAGGGG. The pIRES2-PACE4-CRD-V5 was generated as explained above for pIRES2-PC5A-CRD-V5 except that different primers were used. The first PCR reactions

were done with these primers: 5' GGGCGGTAGGCGTGTACGGTGG/ 3' GGTGTGGTACGGGTGCTCGGAGCAGGC and 5' CTGCCTGCCGCCTGCTCCGAGCACCCG / 3' CTTCGGCCAGTAACGTTAG GGG using as cDNA template pIRES2-PACE4-V5. The following PCR reaction to produce the final cDNA used the primers: 5' GGGCGGTAGGCGTGTACGGTGG/ 3' CTTCGGCCAGTAACGTTAGGGG. Human TIMP-2 was cloned into the digested Ecor1/Sma1 phcmv3 vector. The TIMP-2-Lamp1 protein (TIMP-2-LP) was obtained by fusion of the N-terminal human TIMP-2 to the transmembrane-cytosolic tail (TM-CT) of human Lamp1, a lysosomal protein (Figure 3A), as described (Conesa et al., 2003a).

Cell Lines and Transfections

COS-1, HT1080 and HK293 cell lines were grown in DMEM medium with 10% fetal bovine serum (FBS), whereas PC3, CHO-K1 and FD11 cells were grown in F12K medium with 10% FBS. All cell lines were transfected with Lipofectamine 2000 (Invitrogen) using a 2:1 ratio to cDNA. The HK293 cells were transfected with Effectene (Qiagen) at a 10:1 ratio of effectene:cDNA. TIMP-2 null cells were maintained as previously reported (Morrison et al., 2001c). These cells were transfected using the calcium phosphate precipitation technique as described in the commercially available protocol (BD Biosciences).

Protease Inhibitors and Microsequencing

24h post transfection, HK293 cells were treated for 6 hours with different chelators and protease inhibitors in serum-free media. EDTA and EGTA (Sigma) were used at a final concentration of 2mM while GM6001 (Chemicon) was employed at 25 μ M. Captopril was used at a final concentration of 0.1mM whereas TAPI and Phosphoramidon (Roche Molecular Biochemicals) were used at 10μ M final. Microsequencing of the ^{3}H-Tyr labeled R621A-PC5A was performed as described (Benjannet et al., 2001d)

Antibodies and Immunoprecipitations

Detection by Western blotting of PC5A and PC5B was done using a primary polyclonal rabbit anti-PC5 antibody directed against the N-terminus of active PC5 (1:2000) (Nour et al., 2003c). The secondary antibody used was an anti-rabbit IgG coupled to HRP (Sigma 1:10000). The various truncated forms of PC5A and the different PC5A mutants were detected by western blot employing a monoclonal mouse anti-V5-HRP antibody (Ab:V5; 1:5000 Invitrogen). The MT1-MMP protein was detected using a rabbit anti-MT1-MMP antibody (Ab:MT1-MMP; 1:2000, generous gift from Weiss S.J.). The actin levels were detected with the use of a rabbit anti-actin antibody (1:1000 Sigma). The proliferation experiments

looking at the PCNA levels used a mouse anti-NCL-PCNA antibody (1:200 Novocastra). The secondary antibody used was an anti-mouse IgG coupled to HRP (Sigma 1:10000). The immunoprecipitations were done on both the condition media and the cell lysates. Cell lysis was performed in radioimmune precipitation assay buffer containing a cocktail of mixed protease inhibitors as previously described (Benjannet et al., 2001i). The IgGs used for immunoprecipitations were the rabbit polyclonal anti-TIMP-2 antibody (1:200), Ab:V5 (1:500) and Ab:α 3 (Chemicon 1:200). Incubations with antibodies were done overnight at 4° C and those of the Protein A/G PLUS-agarose (Santa Cruz Biotechnology) were performed to 2-3h at 4 ° C. The immunoprecipitations were used for certain Western blots and biosynthesis experiments.

Western Blot, Biosynthetic Labeling and Zymography

For Western blotting, the cells were washed with serum-free media 24h post-transfection and incubated with serum-free media for the remaining 24h. Following 48h, the media were collected and cells were lysed with radioimmune precipitation assay buffer containing a cocktail of mixed protease inhibitors as previously described (Benjannet et al., 2001e). Proteins from the media and 30µ g of proteins from cell extracts were resolved on 8% SDS-PAGE gel. Biosynthesis were performed 24h post-transfection, the cells were washed and

pulse-labeled for 4 hours with 250 μ Ci/ml of ^{35}S-Met/Cys in RPMI medium containing 0.01% dialysed serum. After the pulse, the media were recovered and the cells lysed as mentioned previously and the immunoprecipitated proteins were resolved on a 10% SDS-PAGE gel. The zymography experiments were performed on serum-free media of HT1080 samples collected 48h post-transfection. The proteins were resolved on a 10% acrylamide gel containing 1mg/ml of gelatin (Sigma), the gel washed twice for 30 min with a 2.5% triton solution and incubated overnight at 37° C in a solution of 50mM Tris pH=8.0 containing 2mM calcium and 0.02% sodium azide. Coomassie blue staining revealed the bands.

Cell Surface Immunofluorescence

COS-1 and TIMP-2 null cells were coated on microscope cover glasses No.0 and transfected the following day. 48h post-transfection the cells were washed 3X with 1X PBS and fixed with 3% paraformaldehyde for 1h at 4° C. After fixation, cells were washed 3X with PBS and incubated for 1h with 5% normal goat serum (blocking buffer) at 4° C. The cells were then incubated overnight at 4° C with monoclonal mouse anti-v5 antibody (1:200) with or without polyclonal rabbit anti-TIMP-2 antibody (1:200) in blocking solution. After the incubation, the primary antibodies were removed and cells were washed 4X with PBS and incubated at

37˚ C for 60 minutes with goat anti-rabbit IgG Cy5-conjugated (1:1000 Amersham) and with donkey anti-mouse IgG Alexa Fluor®555-conjugated (10 µ g/ml Molecular Probes). Once secondary antibodies removed, the cells were washed 4X with PBS. Confocal immunofluorescence analyses were performed and all steps were done in non-permeable conditions.

siRNA and Proliferation Assay

siRNA against human PC5A were designed and four siRNA were mixed and tested in HT1080 cells as well as the non-silencing fluorescein-labeled control (Qiagen). siRNA1 AACTACGATGCTCTGGCAAGT, siRNA2 AATGATGCAAGCAACGAGAAC, siRNA3 AATCACTCCATGGAAGGATTC, siRNA4 AACAGACGACTATGGCACA

GA and the control AATTCTCCGAACGTGTCACGT. The siRNA were introduced in cells using RNAiFect Transfection Reagent (1:3 ratio; Qiagen). The endogenous levels of PC5A mRNA were measured using quantitative qRT-PCR techniques. 6h after the transfections in HT1080 and PC3 cells, the cells were washed with serum-free medium and incubated overnight with serum-free medium. 48h post transfection the cells were recuperated and lysed as described above. Western blot was done on the cell extracts to verify the level of PCNA, actin and PC5. The thymidine incorporation assay was performed in 96 wells

plate as described previously elsewhere (Khatib et al., 2001b). The HT1080 cells were serum-starved 6h post-transfection and incubated for the next 48h. For the last 6h of incubation, 1µCi/ml of 3H-methyl-thymidine (Amersham) was added, and cells were harvested onto glass-fiber filters using a cell harvester (Pharmacia, Wallac Oy, Turku, Finland), and radioactivity was counted. Results were expressed as fold increase/decrease as compared to the cells transfected with the empty vector.

Quantitative RT-PCR

The mRNAs were extracted by (Mbikay et al., 1997b). These mRNA were used for quantification of PC5, TIMP-2, SKI-1, NARC-1 and furin by qRT-PCR technique. For each experiment, 2 PCR reactions were performed in triplicate: normalizing ribosomal protein S14 and the other for the gene of interest. The reactions were done in QuantiTec SYBR green PCR master mix (Qiagen). The primers were:hS14: sense GGCAGACCGAGATGAATCCTCA; antisense CAGGTCCAGGGGTCTTGGTCC. hPC5: sense TGACCACTCTTCAGAGAATGGATAC, antisense GAGATACCCACTA GGGCAGC. hTIMP-2: sense GAAGAAGAGCCTGAACCAC, antisense CGTTGATGT

TCTTCTCTGTG. hfurin: sense CTCACCCTGTCCTATAATCG, antisense ATCCCAGGAATGAGTTGTC, hSKI-1: sense CAAGTGAGGCCCTTGTCC, antisense CTCCCAGGAAGGCAAAGAC and hNARC-1: sense ATCCACGCTTCCTGCTGC, antisense CACGGTCACCTGCTCCTG. The PCR program used was previously described (Dubuc et al., 2004)

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Figure legends

Figure 1. C-terminal cleavage of PC5 and the effect of various mutations on this processing. (A) Western Blot (antibody: N-terminal of active PC5) of the SDS-PAGE-resolved conditioned media and lysates of COS-1 cells expressing either the empty vector pIRES2-EGFP (pIR), or recombinant PC5A and PC5B. (B) Schematic representation of PC5A and the identified C-terminal cleavage site. The recognition sites of the two antibodies used (Ab:PC5 and Ab:V5) are depicted. (C) Western Blot of conditioned media of COS-1 cells expressing the different mutants of PC5A using an anti-V5 antibody. The % of processing of each mutant into the PC5A-CT form was obtained by Image Quant analysis.

Figure 2. The effect of protease inhibitors and overexpression of proteases on the C-terminal cleavage of PC5A. (A) V5-tagged WT PC5A and the R621A mutant were expressed with or without α 1-PDX in HK 293 cells. Western blots were done on conditioned media using the Ab:V5. (B) Ab:V5-Western blot analysis of the conditioned media of CHO-K1 cells, FD11 and FD11-furin (stably overexpressing furin) expressing WT PC5A or its R621A mutant. (C) Ab:V5-Western blot analysis of the media of HK293 cells transfected with WT PC5A and incubated with different protease inhibitors. (D) The effect of MT1-MMP, MT1-

MMP-LP and MT2-MMP on the C-terminal cleavage of PC5A in COS-1 cells. Samples from media were resolved on SDS-PAGE and the PC5A forms were detected using the anti-V5 antibody. The MT1-MMP-LP form is a fusion protein in which the C-terminal transmembrane domain-cytosolic tail of MT1-MMP was replaced by that of Lamp1. (E) COS-1 cells transfected with the cDNA of MT1-MMP or MT1-MMP-LP with either the empty vector, FL-PC5A, PC5A-Δ C or FL furin. Equal amounts of proteins were resolved by SDS-PAGE for each sample and the Western blots (upper panel) were done using an anti MT1-MMP antibody (Yana and Weiss, 2000d). In the lower panel, the V5-levels of FL-PC5A and PC5A-Δ C were compared.

Figure 3 . Effects of TIMP-2 and TIMP-2-LP on the levels of secreted convertases. (A) Schematic representation of TIMP-2 and TIMP-2-LP and their similar expression in HK293 cells, as measured by their cellular ^{35}S-Met/Cys immunoprecipitation levels. Western blots (Ab:V5) of the media and cell extracts of COS-1 cells expressing various forms of (B) PC5A and (C) CRD-PC5, CRD-PACE4 and NARC-1 in the presence or absence of TIMP-2 or TIMP-2-LP.

Figure 4. Co-immunoprecipitations of TIMP-2 with PC5A or the CRDs of PC5, PACE4 and furin. (A) HT1080 cells were transfected with FL-PC5A in the

presence or absence of TIMP-2 or TIMP-2-LP. The media and the cell extracts were immunoprecipitated using Ab:TIMP-2 and resolved by SDS-PAGE and then revealed with Ab:V5. (B) HK293 cells were transfected with either the CRD of PC5A, PACE4 or furin in the presence or absence of TIMP-2 or TIMP-2-LP. The cells were pulse-labeled with ^{35}S-Met/Cys and the cell extracts were immunoprecipitated with Ab:TIMP-2 and resolved by SDS-PAGE.

Figure 5. Cell surface colocalisation of TIMP-2 and FL PC5A. (A) Schematic representation of the 3 constructs used for the confocal analysis. (B) Cell surface labeling of COS-1 cells expressing FL-PC5A, PC5A-Δ C or PC5A-CRD. (C) Cell surface immunofluorescences of COS-1 cells transfected with empty vectors alone, TIMP-2 and the empty vector (pIRES2- EGFP), FL-PC5A, PC5A-Δ C or PC5A-CRD. The green-EGFP fluorescence is a control of transfection while the blue labeling and the red labeling are against TIMP-2 and the various forms of PC5A respectively.

Figure 6. PC5A requires TIMP-2 at the cell surface for its anchorage. TIMP-2 null fibroblast cells were transfected with the empty vectors or with FL-PC5A in the presence or absence of TIMP-2 and then treated with concavalin A overnight

(Morrison et al., 2001d). Cell surface labeling was performed using Ab:V5 (red labeling) and Ab:TIMP-2 (blue labeling).

Figure 7. PC5A can suppress cell proliferation. PCNA Western blot (Ab:NCL-PCNA) of (A) PC3 cell extracts expressing either the empty vector, FL-PC5A or PC5A-Δ C, and (B) HT1080 cell extracts expressing either the empty vector, siRNA against human PC5A or full length PC5A. The levels of PC5A were detected using Ab:V5. (C) Thymidine incorporation cell proliferation assay was performed on HT1080 cells overexpressing either the empty vector, FL PC5A or PC5A-Δ C (n=5). The [^{3}H]Thymidine was added for the final 6 h of incubation, and radioactivity was measured as previously described (Khatib et al., 2001c).

Figure S1. PC5A co-immunoprecipitates with integrin α 3. HT1080 cell extracts expressing either FL-PC5A or PC5-Δ C with or without TIMP-2 were immunoprecipitated with Ab:α 3. The proteins were resolved by SDS-PAGE and Western blotted with Ab:V5. The levels of expressed PC5 were compared by Western blot.

Table 1. Detection of the mRNA levels of convertases and TIMP-2 in lung tumors and in the normal tissues by qRT-PCR. *refers to unpaired tissues.

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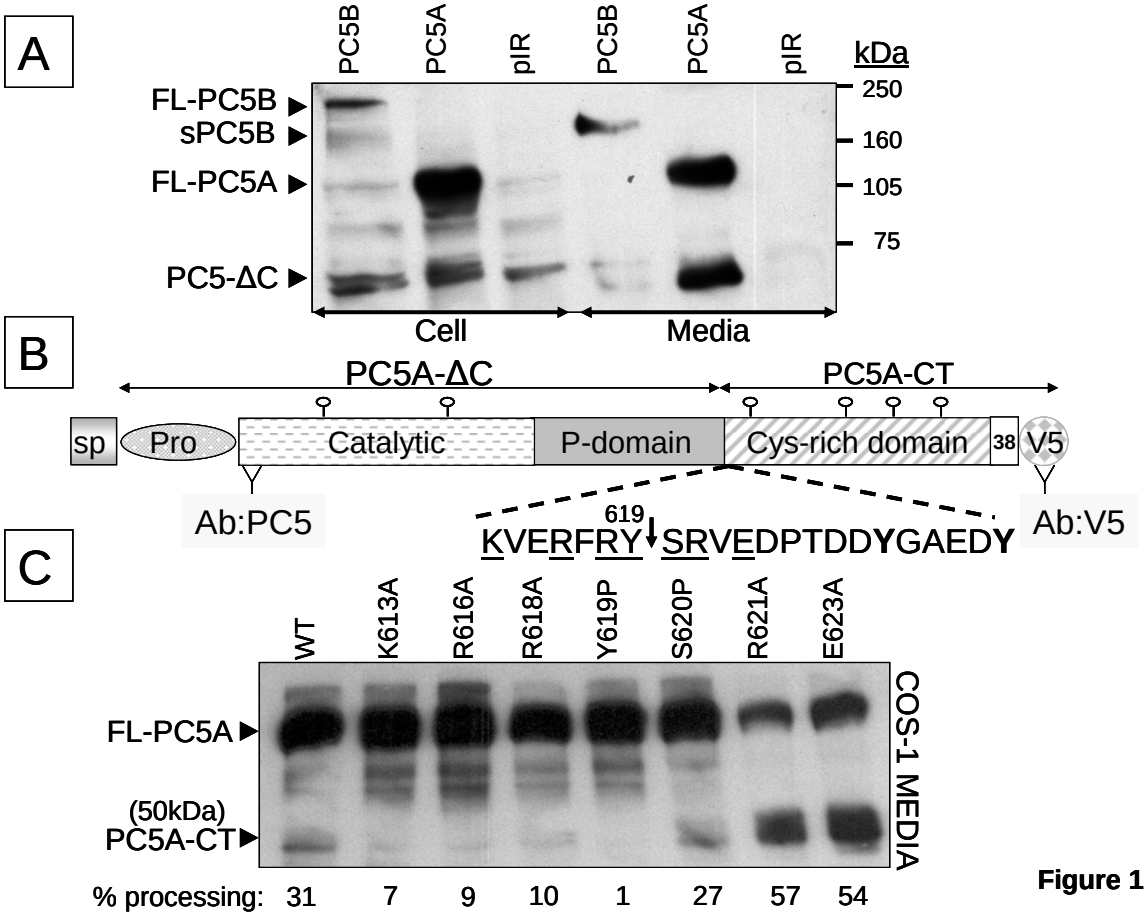
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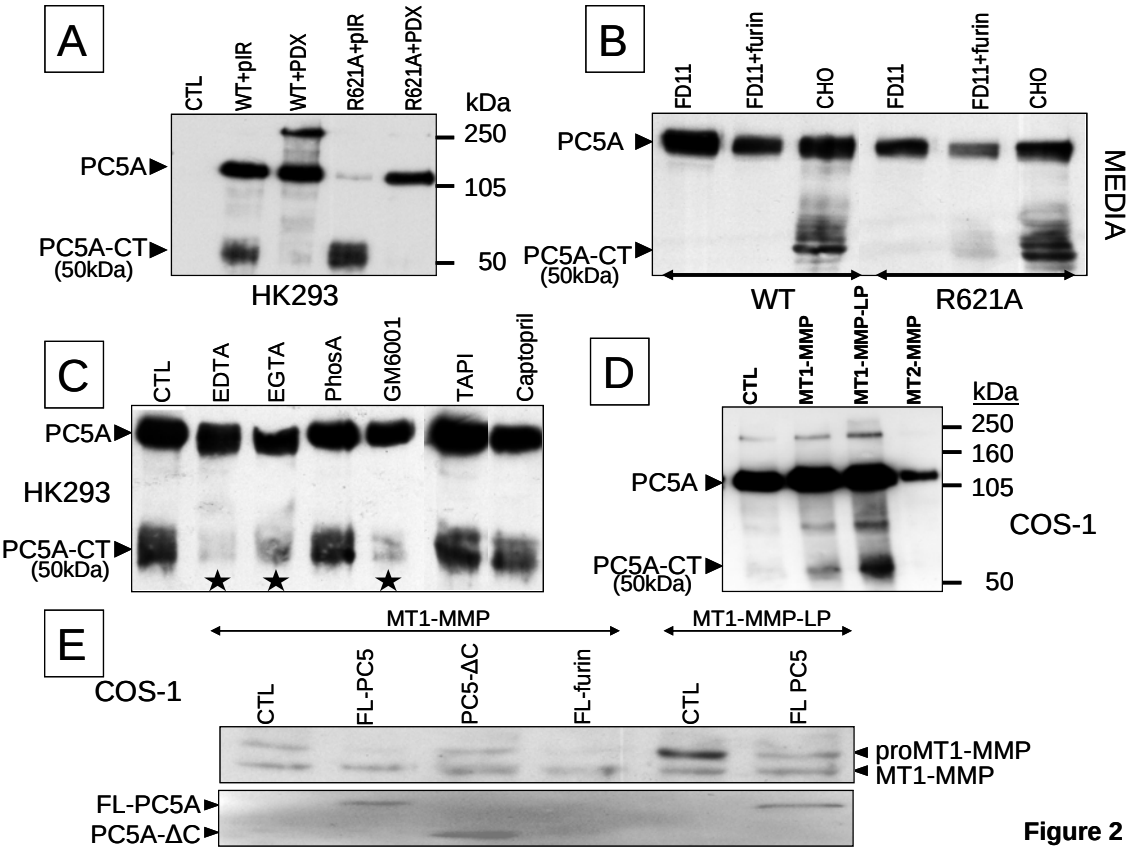
PC5B
PC5A
pIR
PC5B
PC5A
pIR
kDa
FL-PC5B
sPC5B
FL-PC5A
PC5-ΔC
250
160
105
75
Cell
Media
B
PC5A-ΔC
PC5A-CT
sp
Pro
Catalytic
P-domain
Cys-rich domain
38
V5
Ab:PC5
Ab:V5
619
KVERFRY↓SRVEDPTDDYGAEDY
C
WT
K613A
R616A
R618A
Y619P
S620P
R621A
E623A
FL-PC5A
(50kDa)
PC5A-CT
COS-1 MEDIA
% processing: 31 7 9 10 1 27 57 54

Figure 1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WT+PDX
R621A+pIR
R621A+PDX
kDa
250
105
50
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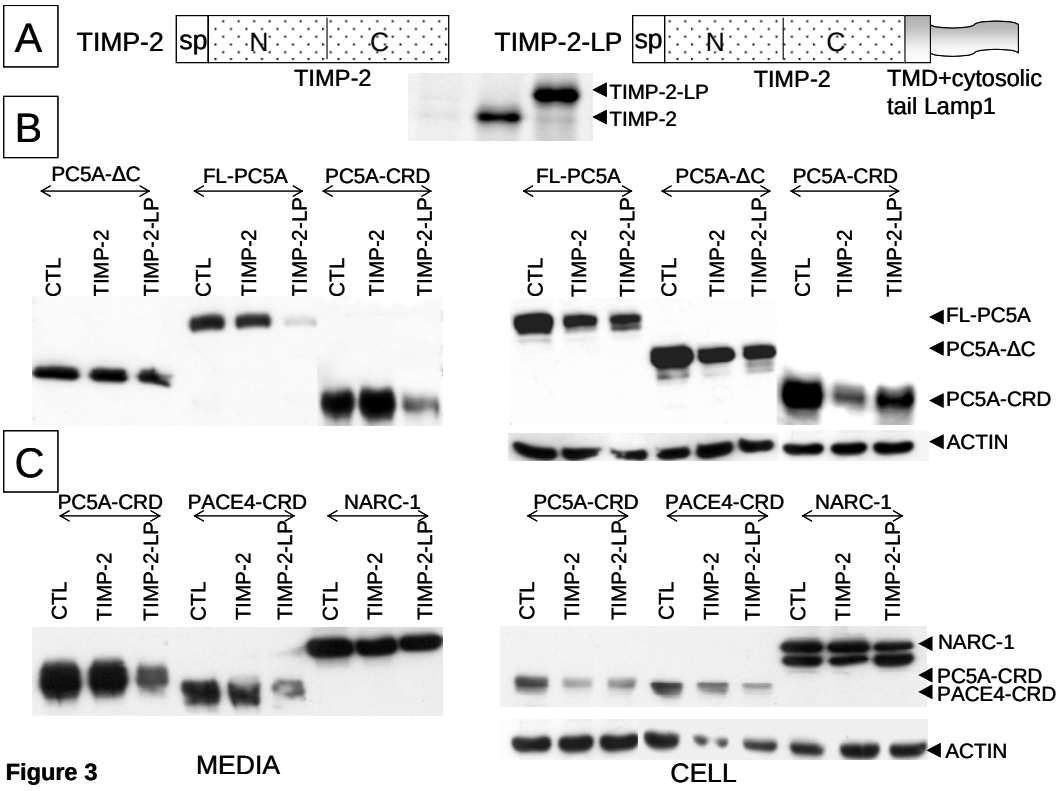
PC5A-CT
(50kDa)
HK293
B
FD11
FD11+furin
CHO
FD11
FD11+furin
CHO
PC5A
PC5A-CT
(50kDa)
MEDIA
WT
R621A
C
CTL
EDTA
EGTA
PhosA
GM6001
TAPI
Captopril
PC5A
HK293
PC5A-CT
(50kDa)
D
CTL
MT1-MMP
MT1-MMP-LP
MT2-MMP
kDa
250
160
105
50
PC5A
PC5A-CT
(50kDa)
COS-1
E
COS-1
MT1-MMP
MT1-MMP-LP
CTL
FL-PC5
PC5-ΔC
FL-furin
CTL
FL PC5
proMT1-MMP
MT1-MMP
FL-PC5A
PC5A-ΔC

Figure 2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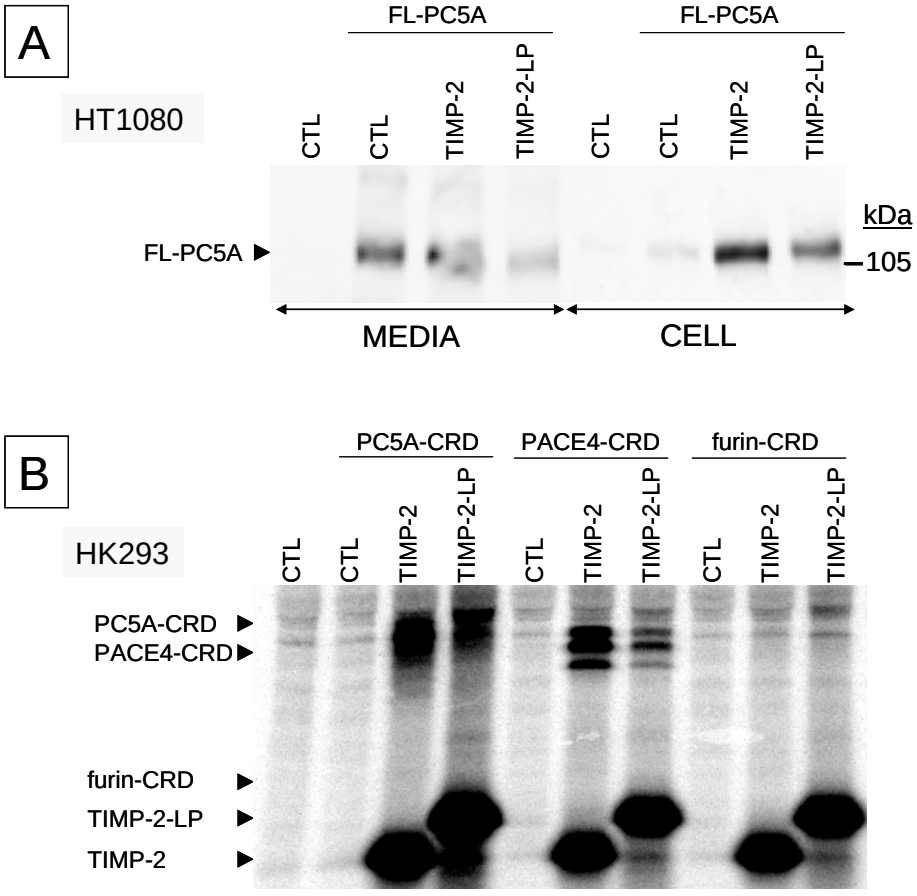
TIMP-2
sp
N
C
TIMP-2
TIMP-2-LP
sp
N
C
TIMP-2
TMD+cytosolic tail Lamp1
TIMP-2-LP
TIMP-2
B
PC5A-ΔC
FL-PC5A
PC5A-CRD
CTL
TIMP-2
TIMP-2-LP
FL-PC5A
PC5A-ΔC
PC5A-CRD
ACTIN
C
PC5A-CRD
PACE4-CRD
NARC-1
NARC-1
PC5A-CRD
PACE4-CRD
ACTIN
MEDIA
CELL

Figure 3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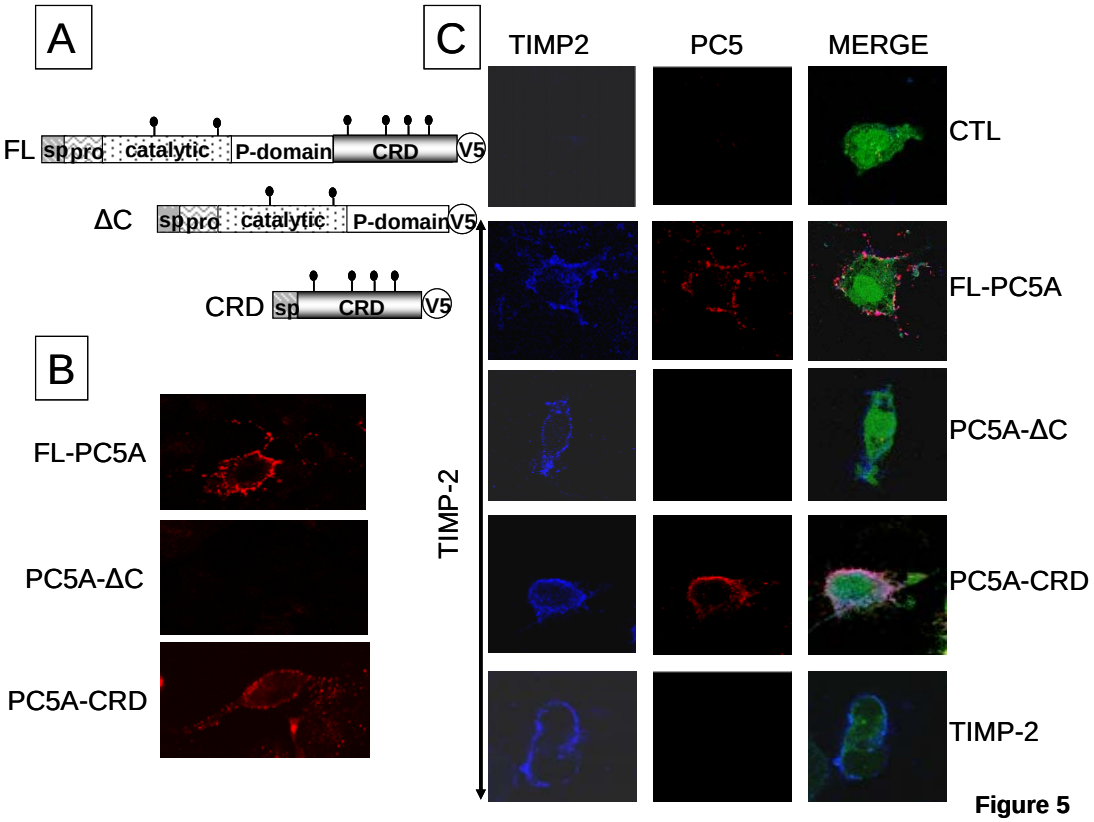
CTL
CTL
TIMP-2
TIMP-2-LP
CTL
CTL
TIMP-2
TIMP-2-LP
kDa
FL-PC5A
105
MEDIA
CELL
B
HK293
PC5A-CRD
PACE4-CRD
furin-CRD
PC5A-CRD
PACE4-CRD
furin-CRD
CTL
CTL
TIMP-2
TIMP-2-LP
CTL
TIMP-2
TIMP-2-LP
CTL
TIMP-2
TIMP-2-LP
PC5A-CRD
PACE4-CRD
furin-CRD
TIMP-2-LP
TIMP-2

Figure 4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sp
pro
catalytic
P-domain
CRD
V5
ΔC
CRD
B
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PC5A-ΔC
PC5A-CRD
C
TIMP2
PC5
MERGE
CTL
FL-PC5A
PC5A-ΔC
PC5A-CRD
TIMP-2
TIMP-2

Figure 5

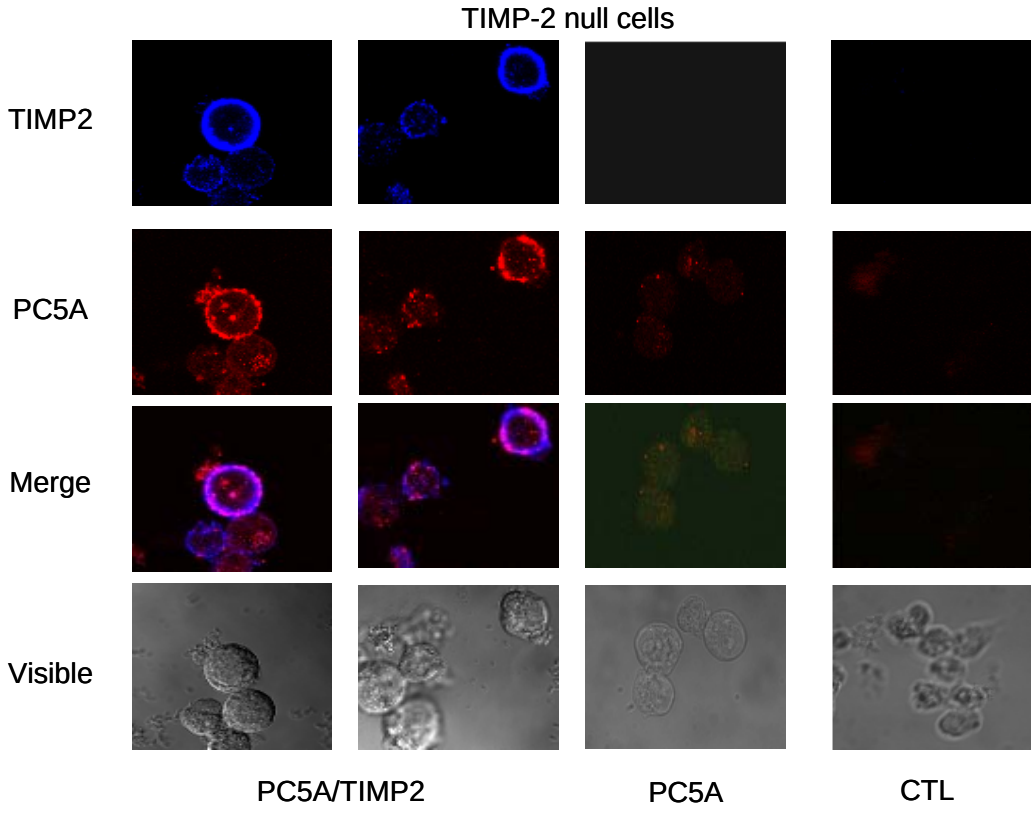
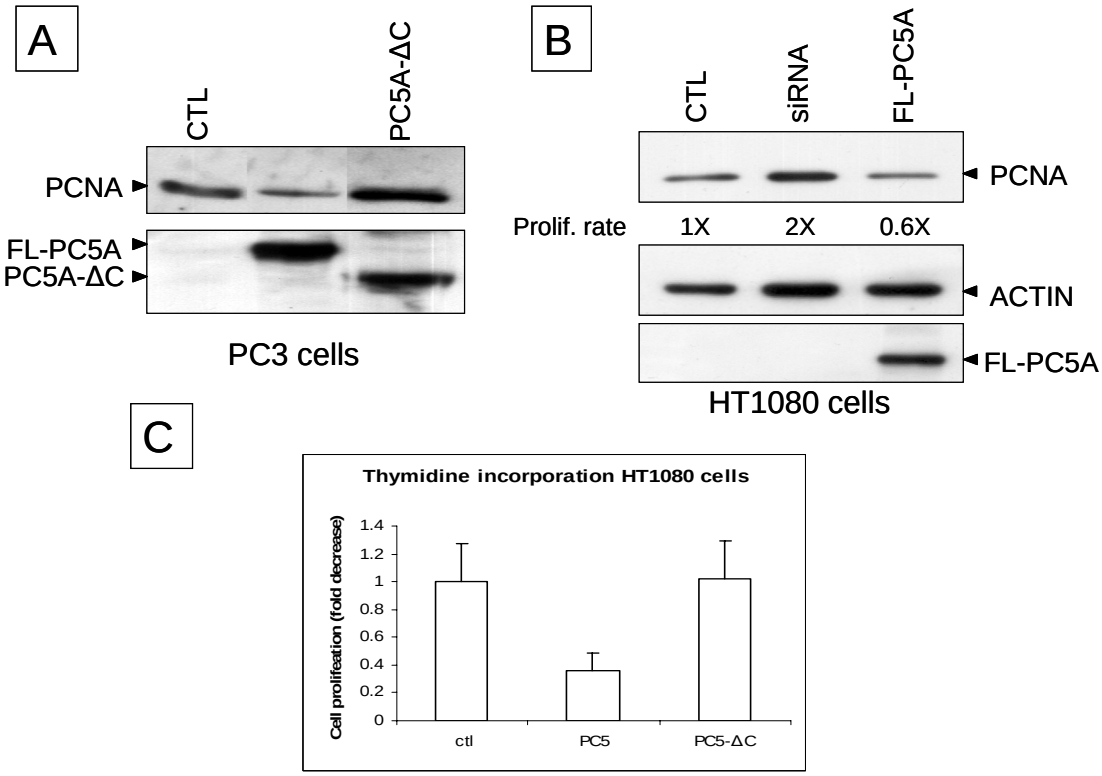

TIMP-2 null cells
TIMP2
PC5A
Merge
Visible
PC5A/TIMP2
PC5A
CTL

Figure 6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CTL
PC5A-ΔC
PCNA
FL-PC5A
PC5A-ΔC
PC3 cells
B
CTL
siRNA
FL-PC5A
PCNA
Prolif. rate
1X
2X
0.6X
ACTIN
FL-PC5A
HT1080 cells
C
Thymidine incorporation HT1080 cells
Cell prolifeation (fold decrease)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ctl
PC5
PC5-ΔC

Figure 7

	Lung						
	N1	T1	N2*	T2*	T3*	N4	T4
PC5	1.9 ± 0.3	0.6 ± 0.0	2.3 ± 0.2	0.5 ± 0.1	1.1 ± 0.0	2.9 ± 0.1	5.9 ± 0.4
TIMP2	43 ± 0.0	16 ± 0.1	36 ± 3	5 ± 0.9	9 ± 0.5	46 ± 5.8	43 ± 2.3
Furin	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	0.9 ± 0.2	0.6 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
SKI-1	0.03 ± 0.01	0.03 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
NARC	1.3 ± 0.1	1.6 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	0.9 ± 0.1	0.1 ± 0.0	0.8 ± 0.05

Table 1

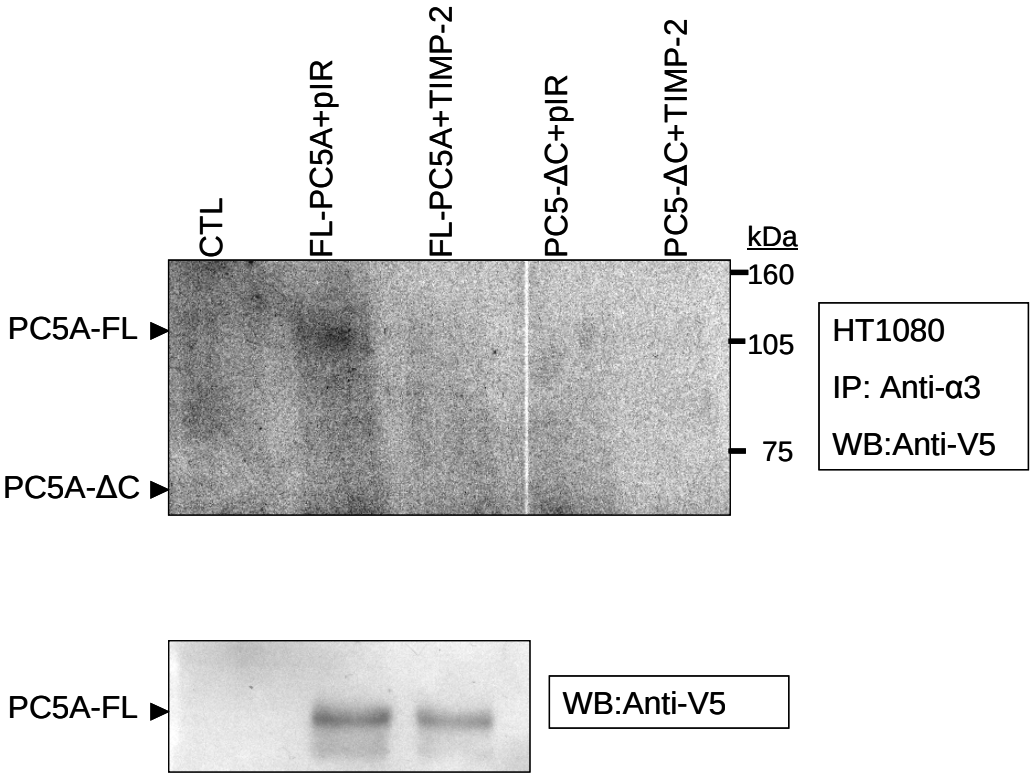
CTL
FL-PC5A+pIR
FL-PC5A+TIMP-2
PC5-ΔC+pIR
PC5-ΔC+TIMP-2
kDa
160
105
75
PC5A-FL
PC5A-ΔC
HT1080
IP: Anti-α3
WB:Anti-V5
PC5A-FL
WB:Anti-V5

Figure S1

CHAPTER E

General Conclusion and claims to original research

Conclusions

The identification of the mammalian proprotein convertase family, which resides in the secretory pathway, enabled us to understand the proteolytic activation of various polypeptides. Based on their similar structures, it is clear that PCs have related functions. The N-terminal domains are conserved between the family members while the C-terminal domains are specific to each convertase. The unique function of each convertase is most likely due to its distinct C-terminal structure. Thus, it was of interest to investigate the inhibitory activity of the prosegment of PC5 and to characterize the function of the cysteine-rich domain of PC5A.

Previous studies on subtilisin (Fu et al., 2000) , furin (Zhong et al., 1999), PC7 (Zhong et al., 1999) and PC1(Boudreault et al., 1998) and now PC5 (this work) have demonstrated that their prosegments can act as inhibitors in trans. They are potent inhibitors, but lack selectivity towards their cognate enzymes as they can inhibit other PCs almost as well. Alignment of the PC prodomains shows low levels of overall homology, but the most conserved region is found at their C-terminus. Since the C-terminus of the prodomain is essential for enzymatic inhibition (Anderson et al., 1997; Boudreault et al., 1998; Nour et al., 2003; Zhong et al., 1999),

peptides derived from the C-terminus of pPC5 were synthesized. Mutations within the decapeptides did not increase the potency and specificity of inhibition. (Fugere et al., 2002; Nour et al., 2003). The lack of specificity of the peptides could also be due to the absence of an extended N-terminal sequence as the full-length propeptides are better inhibitors than their peptide counterparts. In this work, mutagenesis was performed not only on the decapeptides, but also on the full-length prosegment of PC5 around the P6 residue and this did not change the specificity and effectiveness. Thus, the next step would be to perform an alanine scan of the entire prodomain to verify if a higher specificity of inhibition could be achieved by a residue found other than of the C-terminus.

Recently, the endogenous inhibitors of both PC1 and PC2 have been characterized, namely proSAAS (Fricker et al., 2000) and the 7B2 protein respectively (Van Horssen et al., 1995). Why would only these two convertases possess an endogenous inhibitor and not the other PCs? Since each convertase displays a specific tissue expression and performs unique functions it would be natural to think that each PC would possess a specific endogenous inhibitor. These two convertases primarily reside within the secretory granules of cells containing a regulated secretory

pathway; PC5A (De, I et al., 1996) and PACE4 (Mains et al., 1997) were also detected in granules. Moreover, both proSAAS and 7B2 are similar to the granin family, which exhibits a neuroendocrine distribution and is present in the regulated secretory pathway. Thus, if only the proteases that are sorted through the regulated pathway have an endogenous inhibitor than PC5A and PACE4 should also have their own endogenous inhibitor. Since the convertase and its inhibitor have a similar tissue distribution and are co-regulated (Jeannotte et al., 1997), treating a cell line that expresses PC5 with an mRNA silencing technique (siRNA against PC5) would therefore result in a decrease of the possible endogenous inhibitor. Then the total cell extracts of both the untreated and treated cells could be compared by mass spectrometry, or μ RNA analysis, which will permit the identification of potential endogenous inhibitor(s).

The C-terminal region of the convertases is where most structural diversity is observed between the members. Removal of the C-terminal domain that follows the conserved P-domain does not affect the proteolytic activity of furin (Hatsuzawa et al., 1992), PC7 (Munzer et al., 1997), PACE4 (Taniguchi et al., 2002) or PC5B (Wang et al., 2004). Only PACE4, furin and the two PC5 isoforms: PC5A and PC5B possess a cysteine-rich domain (CRD) at their C-terminus directly after the P-domain.

It was shown that PACE4 was detected in the extracellular material fraction of cells overexpressing it and that it was able to tightly bind to heparin via a cationic stretch found within its CRD. Similarly, PC5A is also able to bind to heparin, whereas soluble furin does not (Tsuji et al., 2003). This domain had never been studied prior to this work. From this study, the CRD of PC5A is involved in the localisation of the enzyme at the cell surface likely via its interaction with TIMP-2, an endogenous inhibitor of the MMPs. Since we have shown that the CRD of PACE4 and not the CRD of furin also binds TIMP-2, it would be of interest to verify if PACE4 can also be detected at the cell surface and if it is via its CRD. Moreover, swapping the CRD of furin by the CRD of PC5A would direct the furin-PC5 chimera to the cell surface and this chimera would also interact with TIMP-2. The CRD of PC5A could be fused to an immunoglobulin chain and this chimera would be detectable at the cell surface as it was done to demonstrate that the C-terminus of PC1 contains a granule sorting signal (Jutras et al., 2000). A similar experiment was performed to show that the C-terminal domain of PC2 contained a sorting signal to the granule. The C-terminus of furin was replaced by the C-terminus of PC2 and the furin-PC2 chimera was indeed targeted to the granule demonstrating that there is a sorting signal within the C-terminus of PC2 (Creemers et al., 1996).

Moreover, is the interaction of the CRD of PC5A specific to TIMP-2 or can it binds to other TIMPs? Co-immunoprecipitation experiments of the CRD of PC5A with the four TIMPs will determine if the interaction with TIMP-2 is specific. The interaction of PC5A with the TIMPs may be tissue specific. Recently, PC5 has been found to be co-expressed in embryo-proximal decidua in association with TIMP-3 and exhibits a great degree of temporal and spatial overlap with TIMP-3 gene expression (Wong et al., 2002). Their expressions were both induced during the decidual cell response using an *in vivo* model of artificial decidualization. In this model, it would be of interest to verify if PC5A can also interact with TIMP-3 and if it is through its CRD. Immunofluorescence analysis would also reveal if PC5A can be detected at the plasma membrane of the decidua cells. Since TIMP-3 null mice exist, it would be easy to transfer some fibroblast cells in culture and verify if TIMP-3 is necessary for the sorting of PC5A to the cell surface. The CRD of PACE4 is similar to the CRD of PC5A and we showed that they both bind to TIMP-2, as such the CRD of PACE-4 may also interact with other TIMPs.

It is the first time that the cysteine-rich domain of a convertase is expressed as a single independent domain. The CRD of PC5A, PACE4

and furin were well expressed, folded and secreted. The first crystal structure of a convertase obtained was of furin comprising only the catalytic and P domains (Henrich et al., 2003). The C-terminal domain encompassing the CRD, the transmembrane domain and cytosolic tail were removed. Since the CRD is well folded, its structure could be determined by crystallography. It would be interesting to compare the structure of the CRD of PC5A in the presence or absence of TIMP-2. It would reveal new insights on how the two proteins interact together and which domain of TIMP-2 is involved in this binding (C- or N-terminal domain). If the crystal of the CRD is easily obtained, then the structure of full-length PC5A could be elucidated thus, explaining the substrate specificity of the enzyme and the functional aspects of certain domains.

Alignment of the cysteine-rich domain of PC5A with the NCBI database showed that the first non-convertase protein that has the most similar CRD is EGFR. All the cysteines within the CRD of PC5A align perfectly with the cysteines of the CRD of EGFR. Interestingly, the second cysteine-rich domain of EGFR contains targeting information for the caveolae/rafts as it is expressed in the same fractions as caveolin-1, a membrane protein of caveolae. Deletion of the CRD causes the missorting

of the receptor while introduction of this domain within the LDL receptor directed this chimera to the caveolae/rafts (Yamabhai and Anderson, 2002). Thus, the CRD of the EGFR receptor possesses targeting information as in the CRD of PC5A. It would be interesting to verify if PC5A is also targeted to the caveolae/rafts of the plasma membrane through its CRD by isolating the plasma membrane and verifying if PC5A is found in the same fractions as caveolin-1. Furthermore, the cells overexpressing PC5A could be treated with methyl-β-cyclodextran to deplete the membrane of cholesterol and this would result in membranes containing lower levels of PC5A found in rafts compared to the untreated cells. It is also possible that PC5A does not interact directly with the caveolae/rafts and that such an interaction might be via its interaction with TIMP-2. It was observed by immunogold electron microscopy that MT1-MMP, TIMP-2, MMP-2 and the integrin α 3β 1 had a pericellular patches staining that colocalized with caveolin-1 (Puyraimond et al., 2001). The cell surface localization of PC5A displays a similar pattern of staining to caveolin-1 (Figures D.5-6), which is a punctate labeling of the cell periphery forming patches. Thus, colocalisation and co-immunoprecipitation experiments of PC5A and caveolin-1 will indicate if PC5A is indeed found in caveolae/rafts.

The caveolae/rafts are thought to exist in most cell types, although they are most abundant at the plasma membrane of endothelial cells, epithelial cells, fibroblasts and smooth muscle cells (Parton and Simons, 1995). PC5A is also rich in smooth muscle cells (Stawowy et al., 2001), endothelial cells (Campan et al., 1996) and epithelial cells (Rancourt and Rancourt, 1997). Caveolins are membrane proteins of caveolae that interact with integrins, linking them to tyrosine kinase FYN. These proteins are involved in essential cellular functions including signal transduction, lipid metabolism, cellular growth control and apoptosis. Since PC5A is found in the same cell types as the caveolae and that it interacts (Figure D.8) and processed integrins α -chains (Bergeron et al., 2003; Lissitzky et al., 2000), it suggests that PC5A may be found within caveolae. Moreover, we suggest that PC5A may be acting as a tumor suppressor as it is downregulated lung cancers. When looking at PC5A in a survey of gene expression in a panel of 60 NCI cancer cell lines exhibiting patterns related to their tissue of origin (http://genome-www5.stanford.edu/cgi-bin/source), we see that PC5A is mainly downregulated in various cancer cell lines while in others the expression is either unchanged or slightly augmented. The other proteins that have a similar pattern of expression

as PC5A in these cell lines are caveolin-1 and caveolin-2. Therefore, it suggests that PC5 and the caveolins may have comparable function(s) in these cell lines as their genes are similarly regulated. It is suggested that caveolin-1 may function as a tumor suppressor because overexpression of caveolin-1 in MCF-7 human breast cancer cells resulted in a decrease in cellular proliferation compared to the control (Hino et al., 2003). Likewise, it was shown to be downregulated in various types of cancer including small cell lung cancer (Sunaga et al., 2004), prostate cancer (Pflug et al., 1999) and sarcomas (Wiechen et al., 2001). This anti-proliferative function was further demonstrated in caveolin-1 null mice as they are dramatically more susceptible to carcinogen-induced tumorigenesis, as they develop skin tumours at an increased rate. (Capozza et al., 2003). Therefore, combining the expression of both caveolin-1 and PC5A would synergistically enhance their anti-proliferative roles and thus diminish cell proliferation and cancer development.

The null animal model of PC5A will facilitate the understanding of this convertase' s biological function. Functional inactivation of PC5B results in embryonic lethality at E10.5-11.5, although no details are available yet (Taylor et al., 2003). In our group, preliminary results on the knockout of

PC5A in mice indicate that its inactivation is also lethal. This lethality possibly occurs during implantation of the embryo since we now know that PC5A' s expression is upregulated during implantation and thus, the loss of the enzyme would result in improper implantation and lethality. It was recently described that inhibition of PC5 using an antisense morpholino approach during early pregnancies *in vivo* and *in vitro* blocked decidualization and embryo implantation (Nie et al., 2004; Okada et al., 2004; Salamonsen et al., 2003). Since the knockout mice model of PC5A seems lethal, the conditional knockout of PC5A is also underway; it will help identify the role of this convertase in specific tissue where it is abundant such as in the intestine, the lungs and reproductive system. Once the conditional null mice are obtained, it would be interesting to induce tumors in the null tissues and then add PC5A or TIMP-2 by adenovirus gene transfer to see if it behaves as a tumour suppressor. In addition, PC5A in combination with TIMP-2 would synergistically enhance their anti-proliferative activities and decrease tumour development more rapidly. Since the phenotype of TIMP-2 null mice is only a defect in MMP-2 activity and that we have shown that PC5A can activate MT1-MMP, we could verify if there are changes in MMPs proteolytic activities in PC5A conditional knockout tissues. In addition, rescue experiments could be

performed in the conditional tissues by overexpressing either FL PC5A or PC5-Δ C to verify if the CRD is necessary for any biological activities of the enzyme. Moreover, cells derived from the null PC5A tissues could be cultured and experiments could be performed to verify what substrates or inhibitors are affected by the loss of PC5A by mass spectrometry.

Finally, it is clear that the prodomain of PC5 as that of the other PCs and the subtilisin BNP' , acts as an effective inhibitor of the parent enzyme as well as a chaperone. However, the prosegments do not display any selectivity and it would be of interest to define the residues within the prosegment that would increase the selectivity of these inhibitors. The cysteine-rich domain of PC5A plays a role in the localization of the enzyme in addition to interacting with the secreted TIMP-2. This interaction with TIMP-2 and the cell surface localization of PC5A may indicate a possible proteolytic function of PC5A at the surface. Since PC5A may possess an anti-proliferative function, it could possibly be used as a therapeutic agent in cancer. Therefore, these new studies on the structure-function of PC5A contributed to improving our understanding of the biological function of this convertase and may lead to more clinical applications.

Claims to original research

In the present work, we concentrate on the characterization of two structural elements part of the proprotein convertase PC5A. The first study identified the inhibitory and chaperone functions of the prodomain. While the other study revealed a new function of the cysteine-rich domain of PC5A, which is for the cell surface localisation of the enzyme and its interaction with TIMP-2.

Claims pertaining to research elaborated in Chapter C:

- We are the first to demonstrate that mutation at P1 of the primary cleavage site of the prosegment of PC5A results in an enzyme that is not activated and is secreted as a zymogen in trace amounts. However, despite mutation at P1 of the secondary cleavage site within the prosegment, the enzyme is still well processed and is secreted as mature PC5A. These two mutants as well as a prosegment deletion completely blocked their proteolytic activity on the processing of proVEGF-C.
- We have examined the inhibitory potency of decapeptides derived from the C-terminus of the prosegment of PC5. Most of the

peptides were potent inhibitors (low nM), but not very selective towards PC5A as compared to furin. Introduction of a lysine at P6 increased the selectivity of the peptide inhibitor towards PC5A.

- *In vitro*, the bacterially expressed WT pPC5 is a potent inhibitor and the introduction of point mutations did not increase the potency or the selectivity. *Ex vivo*, ppPC5 and ppfurin completely blocked the processing of proVEGF-C in CHO-K1 cells and were as good as α 1-PDX.
- *Ex vivo*, the pPC5 and most of its mutants as well as the pfurin, pPACE4 and α 1-PDX were efficient at inhibiting the processing of proVEGF-C by PC5A. Only the mutant at P1 of the primary cleavage site had no inhibitory effect. The same inhibitors were tested on the processing of proVEGF-C by furin and only pfurin and α 1-PDX displayed inhibition.
- We also produced a polyclonal antibody towards the pPC5 that is specific. Using this antibody we showed that the prosegments of PC5 expressed in mammalian cells are found degraded in smaller fragments in the conditioned media.

Claims pertaining to research elaborated in Chapter D:

- We confirmed that both PC5A and PC5B are found in a shorter form truncated at their C-terminus prior to the cysteine-rich domain, called PC5-Δ C, in the media of cells devoid of a regulated secretory pathway. We introduced mutations in the potential cleavage site area to observe that the tyrosine 619 is critical for this cleavage to occur.
- Our studies are the first to define the potential protease involved in the truncation of PC5. Various inhibitors were tested and revealed that α 1-PDX, EDTA and GM6001, a matrix-metalloproteinase inhibitor, were the best at blocking the truncation of PC5A. Since the overexpression of other PCs did not increase this cleavage, it suggests that a MMP type is involved as it can be activated by furin and inhibited by α 1-PDX and that overexpression of MT1-MMP resulted in an increase in PC5A cleavage.
- We are the first to demonstrate that PC5A can activate proMT1-MMP into MT1-MMP and that PC5-Δ C, lacking the CRD, is not able to process proMT1-MMP. Thus, C-terminal cleavage may regulate the proteolytic activity of PC5A.
- We are the first to express the cysteine-rich domain of a convertase as a single independent domain in mammalian cells. The proteins

are well folded and well secreted. We have shown that both the CRD of PC5A and PACE4 co-immunoprecipitate with TIMP-2 while the CRD of furin did not. Moreover, PC5A can only co-immunoprecipitate with TIMP-2 when its CRD is present.

- Our studies are the first to establish a role for the CRD of PC5A as it is required for the cell surface localisation of the enzyme. Without the CRD, the enzyme is not detectable at the plasma membrane. Furthermore, we have demonstrated that PC5A is found at the cell surface through its interaction with TIMP-2 via its CRD. PC5A failed to be observed at the surface of cells deficient in TIMP-2, but was found at the surface when TIMP-2 was co-expressed.
- Finally, we have shown for the first time that a PC can regulate cell proliferation. Overexpression of PC5A in PC3 and in HT1080 cells resulted in a decrease in cellular proliferation whereas the cells treated with a siRNA against PC5 increased the proliferation rate. This anti-proliferative role of PC5A is also seen in a downregulation of its mRNA levels in lung tumour tissues compared to the normal adjacent tissues.

Claims pertaining to both Chapter C and D:

These two studies have enabled us to establish for the first time the functional relevance of the prosegment with its two cleavage sites and the cysteine-rich domain of PC5A. These two domains are important for the proper targeting of the enzyme to its subcellular compartment and for its proper proteolytic activation.

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