Substrate Specificity of Widely Expressed Subtilisin-like Proprotein Convertases

by

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Thesis submitted to the Faculty of the University of Michigan –Flint in partial fulfillment of the requirements for the degree of Master of Science in Biology

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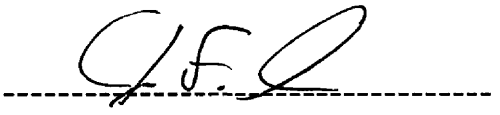

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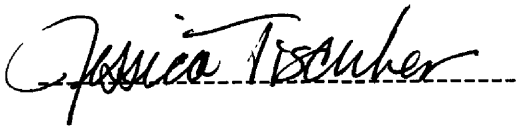

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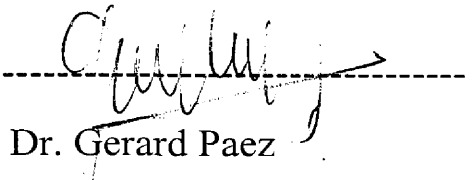

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Abstract

Limited endoproteolysis plays a key role in the activation and maturation of proteins that traverse the secretory pathway. Subtilisin-like Proprotein Convertases (SPCs) are a family of eukaryotic endoprotease that carry out limited endoproteolysis in the secretory pathway. SPCs activate a diverse array of proproteins like clotting factors, enzymes, hormones, and also proteins involved in infectious and non-infectious diseases. SPCs activate proteins by recognizing a cleavage sequence generally described as N-Arg-X-X-Arg-C (with each amino acid designated as N-P4-P3-P2-P1-C). However, each enzyme has its unique substrate preferences, which likely impart particular physiological and pathological roles. We examined the substrate preferences of widely expressed SPCs (furin, PACE4, PC5, and PC7) at the P2 position of substrates, using both qualitative and quantitative analysis. We found that all the enzymes show a similar pattern of substrate preferences, but at the same time they also have unique characteristics. Furin at pH 6.0 showed a broader range of substrate preferences than at pH 7.0. PACE4 showed stringent substrate specificity at P2 position. PC7 was less efficient over the entire range of substrates. To understand which amino acids might make PACE4 different from furin, we

examined the S2 subsite (amino acids in the enzyme interacting with the P2 position in substrates) of PACE4 by creating three mutants called E94D, D90T, DM. The results of D90T suggest that aspartic acid at position 90 of PACE4 helps mediate P2 sequence requirements. Qualitative results did not necessarily agree with the quantitative results likely because of overall change in the structural conformation of the substrate proteins.

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Introduction

Discovery of Subtilisin-like Proprotein Convertases

In organisms from yeast to mammals, a broad spectrum of biologically important peptides and proteins are synthesized as higher molecular mass precursors. These are post-translationally converted into biologically active forms by cleavage, typically at sites formed by doublets or clusters of basic amino acids. This limited endoproteolysis of precursor proteins is a common mechanism for production of functional proteins and peptides that traverse the secretory pathway (1-3). Endoproteases are the enzymes that hydrolyze peptide and protein substrates at these specific internal peptide bonds (4).

The Kex2 protease [Kex2p; also referred to as kexin (EC 3.4.21.61)] of *Saccharomyces cerevisiae* was the first of the eukaryotic secretory system endoproteases to be discovered. Kex2p is a Ca2+-dependent, neutral serine protease with a subtilisin-like catalytic domain that is involved in processing pro-α-mating factor and pro-killer toxin (5-6). Kex2p is a membrane-bound glycoprotein localized in Trans Golgi Network (TGN) (5).

The first mammalian homolog of Kex2p was discovered by Fuller et al. in 1989 by a search of sequence databases. The gene identified was designated *fur,* which got its name from c-***f***es/*fps* **u**pstream **r**egion. *Fur* coded for a protein, named Furin, highly similar to Kex2p in the subtilisin-like catalytic domain and

its flanking regions (7). Furin is now known to be one member of a class of related endoproteases.

Subtilisin like Proprotein Convertases (SPC's)

PCR-based strategies have identified additional members of the mammalian subtilisin/Kex2p family, namely PC1/PC3 (Prohormone or Proprotein Convertase 1), PC2, PC4, PC5/PC6, PC7/PC8/SPC7/LPC (Lymphoma Proprotein Convertase) and PACE4 (Paired Basic Amino Acid Cleaving Enzyme 4). Two more distantly related endoproteases, called SKI-1/SIP (Subtilisin Kexin isoenzyme-1/ site-1 protease) and NARC-1/ PCSK9 (Neural apoptosis-regulated convertase-1/Proprotein convertase subtilisin-Kexin like-9) have also been discovered (8). Because of their subtilisin-like catalytic domain, these proteases are collectively called Subtilisin-like Proprotein Convertases, or SPCs (4, 7, 9, and 10). Kex2p counterparts have also been identified in many non-mammalian eukaryotes (7).

Tissue Distribution

On the basis of their tissue distribution the mammalian proprotein convertases can be classified into two groups. Furin, PACE4, PC5/PC6, LPC/PC7/PC8/SPC7 and SKI-1/SIP are expressed in a broad range of tissues and cell lines. Expression of PC2, PC1/PC3, PC4 and NARC-1/PCSK9 is tissue specific (7, 11). Among these enzymes, PACE4, PC4 and PC5/PC6 have isoforms that are generated via alternative splicing of the same primary transcripts. PC6

occurs in two isoforms; PC6A, which has been found in a variety of tissues and cell lines, and PC6B, which has a much larger cysteine-rich region than PC6A and has been found mainly in intestine (12).

Structural Organization

The structural organization of the SPCs appears to be highly similar and contains common features. One common feature of all these enzymes is that they are synthesized as zymogens, with multiple basic residues often seen at the presumed maturation cleavage site. Typically, the propeptide is autocatalytically removed through cleavage at the aforementioned multibasic site during maturation of the convertases. The catalytic domain of proprotein convertases typically extends over 330 amino acids and is highly conserved among all members of the family. The active-site residues Asp, His and Ser are the catalytic triad and are universally conserved. An Asn residue, which stabilizes the oxyanion hole in the reaction transitional state, is present at corresponding positions in all members except for PC2, where the Asn residue is replaced by an Asp. The sequences flanking these residues are highly conserved as well.

Following the catalytic domain is a 140-amino-acid region that has been referred to as the `Homo B', `P' or `middle' domain. This domain is also well conserved among eukaryotic convertases, including Kex2p, but is absent in bacterial subtilisins. A transmembrane domain is also seen in some SPC's (Furin, PC5 and PC7). Towards the C-terminus, furin, PACE4 and PC5/PC6 A and B

have a Cys-rich domain of unknown function. Overall, PACE4 and PC4 are very similar to furin, while PC1, PC2 and PC7 are increasingly different from furin (7, 13). Figure 1 shows the structural organization of most SPC's.

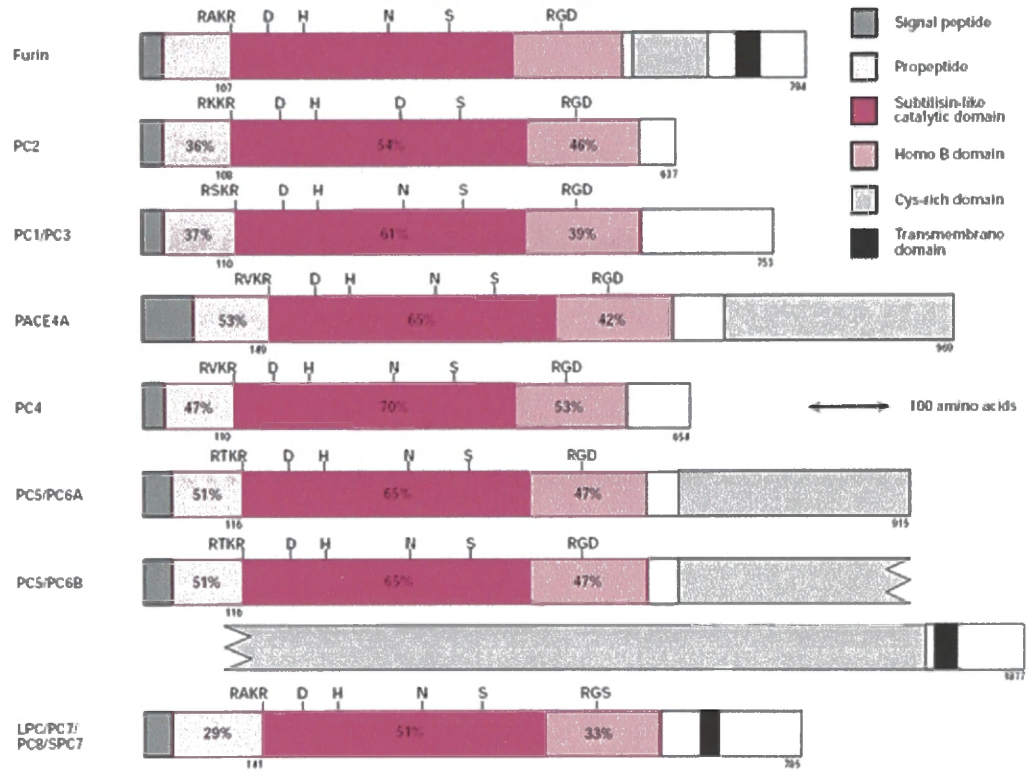

Figure 1: Schematic representation of SPC's. For each region, the percentage of amino acid identity with furin is shown. Also, the catalytic residues (Asp, His, Ser, and Asn or Asp) and the Arg-Gly-Asp (Ser) (Recognition sequence for integrins) sequences are shown (7). The more distantly related members of the family (SKI-1 and PCSK9) are not included.

Activation and Localization

SPC's are initially synthesized as zymogens. An initial autocatalytic processing event in the endoplasmic reticulum (ER) (all SPCs except for PC2) or in immature secretory granules (PC2) generates a heterodimer of the inhibitory prosegment and the rest of the molecule (prosegment-SPC). The heterodimer exits

the ER and sorts to specific subcellular compartments, where the full catalytic activity is acquired, usually following a second autocatalytic cleavage of the prosegment. This liberates the active enzyme from the inhibitory prosegment. This exquisitely well-orchestrated zymogen activation mechanism ensures that the convertase is only active at intracellular site(s) where the conditions of pH and calcium are optimal for the in *trans* cleavage of specific substrates. Only the mature enzyme is assumed to be capable of processing substrates; therefore, removal of the pro-domain seems to be essential for acquiring substrate cleavage activity (14).

Furin, PC5 and PC7 have transmembrane domains and are localized in the *trans*-Golgi network (TGN). Furin is a trans membrane protein with a type I topology. Furin is initially synthesized as a pro-furin zymogen, which is rapidly converted into active furin by intermolecular, auto-cleavage of the propeptide at the Arg-Ala-Lys-Arg site (residues104-107). The autocatalytic cleavage of the propeptide appears to occur in the endoplasmic reticulum (ER). Propeptide cleavage is a prerequisite for transport of furin molecules out of the ER, but propeptide cleavage is not sufficient for the activation of furin. Even after its cleavage in the ER, the propeptide remains associated with the furin active site and functions as a potent autoinhibitor of the endoprotease. The propeptide release requires a second cleavage at the Arg-Gly-Val-Thr-Lys-Arg site (residues 70-75) in the middle of the propeptide. This cleavage is dependent upon the acidic pH in the Golgi apparatus. Furin ultimately cycles between the TGN and the cell

surface. Furin shows its protease activity in a broad pH range of 6.0 to 8.5, with a peak at 7.0 (7). However, the kinetics of the initial proprptide cleavage in the ER (pH 7.0) and the second cleavage in the Golgi apparatus (pH 6.0) suggest that furin can have different activity at different pHs. This could have implications for the substrate specificity of TGN-associated versus secreted forms of furin.

ProPACE4 (approximately 106 KDa) is cleaved intracellularly before the secretion of PACE4 (approximately 97 K Da). PACE4 seems to be activated in the TGN and/or at the cell surface (15). The neuroendocrine-specific convertases, PC2 and PC1/PC3, are mainly localized in secretory granules. PC5/PC6A has also been reported to be localized to secretory granules (7). Pro-PC7 is synthesized as glycosylated zymogen (101KDa) and processed into mature PC7 (89KDa) in endoplasmic reticulum (16). SKI-1 is active in the *cis/medial*-Golgi. Furin, PACE4, PC5 and PC7 are widely expressed and function in the secretory pathway of many different mammalian cell types (4, 7, 9, and 10). These four widely expressed members of the SPC family are the focus of this project.

Importance of SPCs

SPC's have important roles in development and homeostasis. They cleave and activate peptide hormones, neuropeptides, growth factors, receptors, adhesion molecules, plasma proteases, matrix degrading metalloproteinases, and other enzymes. Their importance is illustrated by knockouts in mouse models, which are either embryonic lethal or result in severe developmental abnormalities (17).

SPCs also activate proteins involved in infectious disease. These include bacterial toxins, such as *Pseudomonas* exotoxin A, diphtheria toxin, anthrax toxin, aerolysin, botulinum neurotoxin and tetanus neurotoxin. SPC's activate viral glycoproteins, notably gp160 of the Human Immunodeficiency Virus (HIV), the fusion protein of measles virus, and the envelope glycoprotein of Ebola virus. SPC's are also capable of activating respiratory viruses such as human influenza virus and Sendai virus (17).

SPCs also play a role in non-infectious pathological conditions. For example, they activate the Alzheimer's disease β-secretase, which is one of the enzymes involved in cleaving the amyloid precursor protein (18-20). Aberrant cleavage by SPC's causes three rare autosomal dominant neurodegenerative diseases, namely familial amyloidosis of Finnish type, familial British dementia, and familial Danish dementia (17). Furin activates the insulin proreceptor and pro-clotting factor IX; lack of furin activity versus these proproteins can result in insulin-resistant diabetes and hemophilia B (21-23).

SPCs are also over-expressed in some cancers, and expression level appears to correlate with malignancy (24-25). SPC activity may contribute to tumor development through activation of paracrine factors and receptors involved in controlling cell differentiation and proliferation (26). SPCs may facilitate metastasis through activation of matrix-degrading metalloproteinases (24). Furin

and PACE4 process stromelysin 3, an MMP involved in tumor invasion. Similarly, MT-MMPs (Membrane-type Matrix degrading Metalloproteinase) and adhesion molecules, such as E-cadherin, may be activated by furin and PACE4 (24). PC7 is expressed abundantly in activatable immune cells such as CD4+ T-lymphocytes, suggesting PC7 may play an important role in processing of tumor necrosis factor and integrins (16). Activation of transforming growth factor-beta by furin and other SPCs may contribute to the immunosuppression seen in many types of cancer (27).

Despite the high degree of homology in the PC family, only PACE4 and furin localize to the same chromosome: mouse chromosome 7 and human chromosome 15. Furin knockouts are embryonic lethal in mouse models (28), and cell strains harboring loss-of-function mutation in *fur* alleles are deficient in the activation of variety of proproteins (26, 28, 30-32). PACE4 plays a critical role in axis formation during mouse development (27). Thus, widely expressed SPCs have enormous biomedical importance, with key roles in development, homeostasis and pathology.

Substrate Binding and Specificity

SPCs are similar at the level of primary structure and have many common biochemical characteristics. However, these enzymes differ in their substrate specificity. Examination of amino acid sequences around the cleavage site of many mammalian precursor polypeptides shows a common motif, typically N-

Arg-X-X-Arg-C. The substrate binding region of all SPC's contains subsites (designated as S1-S4) with negatively charged residues, which may interact with the positively charged residues of substrates. The affinity of the substrates to the endoproteases may be determined by the number of interactions between the negative charges of the subsites and the positive charges of the substrates.

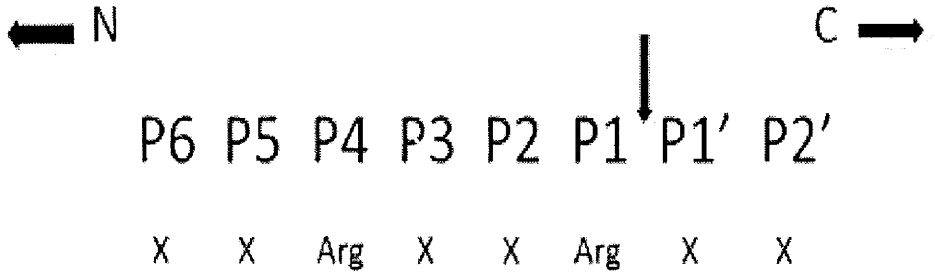

Figure 2: The canonical SPC cleavage site and nomenclature of amino acids in the cleavage site. P1 is the carboxy most Arginine, P2 is the residue amino terminal to P1, P1'and P2' are the residues carboxy terminal to P1. Arg indicates Arginine and ⬇ is the peptide bond that is hydrolyzed.

It was demonstrated that negatively charged residues in or near the S1-S4 subsites of the substrate binding region are critical in furin (12). The Sl binding pocket is predicted to be a large, elongated pocket involved in electrostatic interaction with the Pl basic residue (29). However, according to the number and distribution of these negative charges the PCs differ slightly from each other. Furin, with 16 acidic residues (including Asp153, Asp228, Glu299, Glu301, Glu331 and Asp522) in the immediate vicinity of the active-site cleft, possesses the highest number of negative charges. PACE4, PC5 and PC4, with 15 acidic residues each, exhibit a similar negative charge density, while PC7, PC1 and PC2

contain 13 acidic residues (12). Therefore, the overall requirement for positively charged residues N-terminal of the cleavage site of bound substrates might be expected to decrease in the same order (33).

The requirement of Arg at position P1 is a common feature of the SPCs. The published sequence rules (29-34) that govern the constitutive precursor cleavage are:

1. A basic residue at the P4 or P6 position upstream of the cleavage site is usually required.
2. At position P2, a basic residue may be required, with Lys more favorable than Arg.
3. At position P4, Arg is more favorable than Lys.
4. At position P1', a hydrophobic aliphatic residue is not suitable.

Substrate specificities of furin and PACE4 have been most thoroughly investigated, with less known about PC5 and PC7. PACE4 is unable to process several proproteins acted upon by furin. The restricted substrate range of PACE4 may reflect a unique physiological role for PACE4. Some proteins activated by furin but not by PACE4 are involved in disease development, including *Pseudomonas* exotoxin A (35), avian influenza hemagglutinin (36), and Newcastle disease virus F protein (37). gp 160 of the human immunodeficiency virus type I can be processed by furin, but is less efficiently processed by PACE4 (38-40). Furin can effectively recognize the cleavage sequence N-Arg-X-X-Arg-C

(41-42). The PACE4 cleavage sequence appears to be more restrictive. PACE4 may require a basic residue--Lysine or Arginine--in the P2 position and optimal cleavage sequence for PACE4 has been described as N-Arg-X-Lys/Arg-Arg-C (11, 43). Interestingly, PACE4 is unable to process several proteins containing the N-Arginine-X-Lysine/Arginine-Arginine-C cleavage sequence, suggesting others factors influence substrate selectivity of PACE4. Other experimentation on the sequence motif necessary for cleavage by PACE4 is ambiguous. Some reports suggest that PACE4 can recognize the Arg-X-X-Arg sequence and sequences that lack a P4 arginine residue. Thus, the precise role of the P2 residue in the substrate specificity of PACE4 is not clear. While less is known about substrate specificity of PC5 and PC7, each member of SPCs appears to have different substrate specificity. This could impart a specific physiological role to each enzyme, as well as influence their ability to activate proteins involved in the development of disease conditions (44-46).

Enzyme Substrate Interaction

Molecular modeling studies have suggested that all residues which are closely associated with the substrate binding cleft of SPCs interact with amino acids in cleavage site of substrate. Amino acids in the S1 subsites are conserved among SPC's (Table 1). Amino acids in the S4 subsites are also conserved expect for PC1/PC3. However, the amino acids at the 90 and 94 positions (residue number corresponding to PACE4) in the S2 subsite are different for furin and PACE4. These residues may therefore play an important role in substrate preference of these SPC's by mediating interaction with the P2 residue in

substrate proproteins (13). Specifically, the presence of an acidic residue at position 90 in PACE4 and the longer side chain of Glu 94 (PACE4) compared to Asp (furin) could be important in mediating interactions with basic P2 residues. These differences might account for the cleavage site selectivity of furin and PACE4.

	S4		**S3**			**S2**			**S1**
	E	D	D	D	D	E	N	D	D
	140	168	161	63	90	94	95	210	162
Furin	E	D	E	D	T	D	N	D	D
PACE4	E	D	D	D	D	E	N	D	D
PC5/6	E	D	D	D	D	E	N	D	D
PC4	E	D	E	D	T	E	N	D	D
PC1/3	E	E	N	D	D	E	N	D	D
PC7	E	D	D	D	D	G	N	D	D
PC2	E	D	T	D	T	F	N	D	D
Kexin	E	Q	A	D		D	D	D	D

Table 1: Amino acids in SPC subsites that may interact with substrateresidues. Residue numbering is for PACE4. Single letter amino acid abbreviations are used. The S2 subsite is highlighted and potentially important differences at positions 90 and 94 are indicated.

Our Study

We examined if the sequence variation at P2 residues determines the ability of SPCs to recognize, bind and act upon a proprotein. Amino acids within the catalytic domain of the SPCs that may play a role in substrate preferences, particularly Asp90 and Glu94 which may interact with P2 side chains, are also examined for PACE4. According to molecular modeling studies, comparison of negatively charged and presumably critical amino acid residues in the S2 subsites of human furin and human PACE4 revealed differences (Table 1). These could constitute an important factor for observed difference in cleavage site selectivity between furin and PACE4 (12). To understand where the actual interaction of amino acids takes place between PACE4 and substrates, we created three mutants of PACE4, called E94D, D90T and double mutant (a combination of both E94D and D90T). These changes might make the S2 site of PACE4 mimic that of furin, with a corresponding change in the substrate specificity. Finally, to examine the substrate preferences of furin in TGN and secreted furin, substrate specificity of furin was examined at pH 6 and pH 7.

Substrate specificity was analyzed both qualitatively and quantitatively. For qualitative analyses we used von Willebrand's factor (vWF) as a model substrate and examined the ability of widely expressed SPCs to act upon wild type or mutant versions differing in the P2 residue. von Willebrand's factor is synthesized as a 2,813 residue proprotein and is a good substrate of SPCs. Maturation of pro-von Willebrand factor to its active form requires proteolytic

processing. Correct processing of vWF is essential for its ability to bind and stabilize factor VIII during clotting. Quantitative analysis was done using synthetic peptides as substrates, which are linked to a fluorescent dye. We used 7 peptides, with wild type substrate having Arginine at the P2 position and the others having substitution of polar, non polar, or acidic amino acids.

Furin, PACE4, PC5 and PC7 are the major group of enzymes involved in processing many proproteins that traverse the secretory pathway and are involved in number of infectious and non-infectious diseases. Designing an inhibitor for one or more SPC's could be very beneficial in treatment of several pathologies. Therefore, knowledge about substrate specificity of each enzyme is critical. Hence, the result of our research could be used as preliminary data for drug designing.

Materials and Methods

Cell Culture

Cells used in this study were RPE.40 cells, which are a strain of SPC-deficient Chinese hamster ovary cells. Cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 (Gibco-BRL; Gaithersburg, MD) containing 5% fetal bovine serum (Gibco-BRL) at 37° C in a 4.7% CO_2 atmosphere.

Construction of Mutant PACE4 Expression Vector for Stable Expression

Three mutants of human PACE4 cDNA were previously created, namely D90T, E94D and Double Mutant (DM), which is a combination of both D90T and E94D. All three mutants were cloned in the pSVL expression vector (Pharmacia/GE Healthcare; Piscataway, NJ), which does not contain a selectable marker. To generate cells stably expressing these mutants, the PACE4 cDNAs were excised from pSVL using a BamHI/XhoI double digest. The mutant PACE4 cDNAs were then purified from agarose by using the Wizard PCR preps DNA purification system (Promega; Madison, WI), in accordance with the manufacturer's instructions. The cDNAs were then directionally ligated into the expression vector pcDNA 3.1 (-) (Invitrogen; Carlsbad, CA), which harbors a gene conferring resistance to geneticin (G418), by using the same restriction sites (BamHI/Xho1). Ligation products were transformed into *Escherichia coli* XL1-Blue cells (Stratagene; La Jolla, CA) and transformants harboring the constructs were selected by growth on Ampicillin-containing (200mg/ml) Luria-Bertani

medium. The presence of the constructs was verified by standard plasmid prep (QIAGEN; Valencia, CA) and restriction digestion analysis. Large scale isolation of the recombinant constructs, designated pcDNA 3.1-E94D, pcDNA3.1-D90T and pcDNA3.1-DM, was done by using a plasmid isolation kit obtained from Marligen (Ijamsville, MD) according to manufacturer's instructions.

Stable Transfection

The generation of cells stably expressing human PACE4 mutants (E94D, D90T and DM) was done by transfecting pcDNA3.1-mutant PACE4 constructs into RPE.40 cells. For all transfections, 10^5 cells were seeded in 3 ml of growth medium in 60mm×15mm (P60) tissue culture dishes and incubated for 12h before transfection was initiated. Transfections were done with TransIt transfection reagent from Mirus (Madison, WI) in accordance with manufacturer's instructions. After 24h cells were supplemented with new serum-free medium (HyQ-CCM, from HyClone Laboratories; Logan, UT). After 48h G418 was added (850μg/mL). After 60 hours cells were washed with 1X Phosphate Buffered Saline (PBS) and supplemented with new medium containing G418 (850μg/ml). Two days later cells were transferred to four 60mm×15mm tissue culture dishes and supplemented with growth medium containing G418 (850μg/mL). After 60 hours, 3 colonies were selected for each mutant and transferred into three, 35mm×10mm tissue culture dishes (P35s). After 60h the cells were transferred to 25 centimeter square flask (T25). Freezer stocks of these cells were made for future use.

Synthesis and Ultra-purification of SPCs

RPE.40 cells harboring constructs for furin, wild type PACE4, PC5 and PC7 were previously generated. RPE.40 cells harboring these constructs, as well as the E94D, D90T, and DM plasmids, were grown in 150 cm^2 flasks (T150) using growth medium supplemented with 850μg/mL G418. After 12 h cells were washed two times with Phosphate-Buffered Saline (PBS) containing magnesium and calcium ions. Then cells were supplemented with serum-free OPTI-MEM + GlutaMAX (Gibco-BRL) with 850μg/mL G418.

After 72 h medium was harvested and centrifuged for 5 min at 2900 RCF to separate the cells from the medium. The enzymes in the conditioned medium were obtained by passing the medium through Centriplus YM-50 ultrafiltration tubes (Millipore; Bedford, MA) at 2900 RCF for 45 min. The enzymes were then stored at -20°C for future analysis.

Production of Pro-von Willebrand's Factor (Pro-vWF)

Escherichia coli XL1- Blue cells harboring constructs for wild type and mutant pro-vWF were previously created. Wild type pro-vWF has Lys at position 762 (K762, which is the P2 position), and mutant pro-vWF has different amino acid substitutions to the P2 residue, including K762S, K762G, K762D, K762A, K762V, and K762L. These cells were grown in Ampicillin-containing (200 μg/mL) Luria-Bertani medium. Purification of recombinant constructs was done by using a plasmid isolation kit from QIAGEN (Valencia, CA). These constructs

were transiently introduced into RPE.40 cell using Lipofectamine reagent (Gibco-BRL) in accordance with the manufacturer's protocol; for these transient transfections, 5×10^5 RPE.40 cells were seeded in 3mL of growth medium in P60 tissue culture dishes and incubated for 12 h before transfections were initiated. After transfections, cells were incubated in growth medium for 5 hours and then switched to serum-free medium (HyQ-CCM). After 60 h, medium containing secreted vWF was harvested and centrifuged for 5 min at 13,000×g. The conditioned medium containing vWF was stored at -20°C for future analysis.

Generation and Production of Pro-von Willebrand's Factor Mutant K762N

The construct encoding the precursor to von Willebrand's factor (pro-vWF) was provided by Dr. Jan A. van Mourik and Dr. Jan Voorberg of the central laboratory of the Netherland Red Cross Blood Transfection Service (Amsterdam, The Netherlands). Site-directed mutagenesis was done to create the K762N mutant of pro-vWF using the QuikChange XL kit (Stratagene) according to manufacturer's instruction. The primers used to create K762N were

Top: 5'-CCCCTGTCTCATCGCAGCAACAGGAGCCTATCCTGTCGG-3'

Bottom: 5'-CCGACAGGATAGGCTCCTGTTGCTGCGATGAGACAGGGG-3'

The product of site-directed mutagenesis was sequenced by University of Michigan Sequencing Core Facility (Ann Arbor, MI) to confirm the mutation. The plasmid containing K762N pro-vWF was transformed into *Escherichia coli*

XL1-Blue cells and transformants harboring the construct were selected by growth on Ampicillin-containing Luria-Bertani medium (200μg/ml). Freezer stocks were made for future use.

The plasmid harboring K762N was transiently introduced into RPE.40 cells as described previously for other mutant forms of vWF. After transfections, cells were incubated in growth medium for 5 hours and then switched to serum-free medium OPTI-MEM + GlutaMAX (Gibco-BRL). After 60 h, medium containing secreted vWF was harvested and centrifuged for 5 min at 13,000×g. The conditioned medium containing vWF was stored at -20 for future analysis.

Quantitative Analysis: Fluorimetric Reactions

The peptides for fluorimetric analysis contain four amino acids linked to a fluorescent dye called 7-Amino-4-Methylcoumarin. They were obtained as granules from Peptides International (Louisville, KY). Each was re-suspended in 100% DMSO at a concentration of 10 mM, according to the manufacturer's instructions. For reactions, 5 mM working solutions of the peptides were used, which were diluted from the stock solution using 1X IVP (*In Vitro* Processing Buffer; 0.1 M HEPES and 3 mM $CaCl_2$). For Km determination, increasing amounts of substrates were added to the reaction mixture. The setup for Fluorimetric reactions to analyze substrate specificity was done as described in the table 2.

Tube#	Enzyme (μL)	Substrate (μL) 5 mM	10X IVP Buffer (μL)	Water (μL)	Fluorescent Intensity Unit (FIU)	FIU
1	0	1	20	179	Background fluorescence	Actual Fluorescence
2	5	1	20	174		
3	0	2	20	178		
4	5	2	20	173		
5	0	4	20	176		
6	5	4	20	171		
7	0	8	20	172		
8	5	8	20	167		
9	0	10	20	170		
10	5	10	20	165		
11	0	20	20	160		
12	5	20	20	155		
13	0	40	20	150		
14	5	40	20	145		
15	0	50	20	140		
16	5	50	20	135		

Table 2: Template for fluorimetric reactions. Indicated volumes of each reagent were added to each reaction; note that even numbered reactions were controls lacking any processing enzyme. Reactions were incubated for 37°C for 30 min.

The reaction tubes were incubated at 37°C for 30 min in a PCR machine and then analyzed for fluorescence.

The flourimeter (Quantech Model Number FM109515, Barnstead International; Dubuque, IA) was standardized using 7-amino-4-methylcoumarin (AMC), with 0.1 mM set to generate 1000 Fluorescent Intensity Units (FIU) according to manufacturer's instruction. The background fluorescence was subtracted from the reaction fluorescence to obtain FIU corresponding to fluorescent dye released during the reaction.

Michaelis-Menten Constant (Km) Calculation

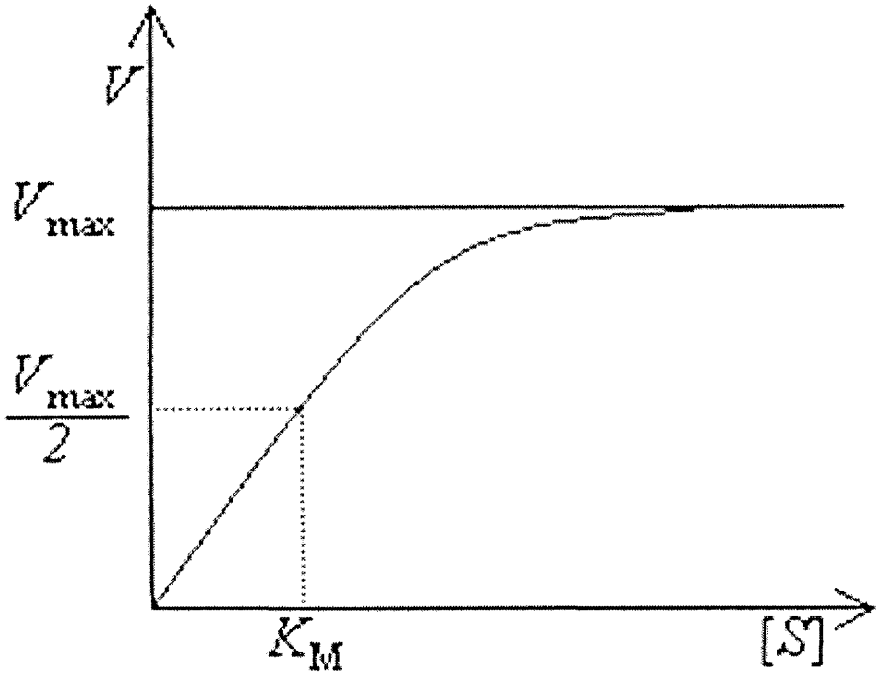

Figure 3: Calculation of Km from a graph plotting substrate concentration versus reaction rate (49).

Km is a constant that describes the relative affinity of enzymes for particular substrates. Km is equal to the substrate concentration at which an enzyme reaction proceeds at half the maximum velocity (Figure 1). Therefore, the lower the Km the higher the affinity of enzyme for substrate.

The Km for all reactions was calculated in μM, using a software program called GraphPad Prism (GraphPad Software, Inc; La Jolla, CA). Both graphs (like shown in Figure 1) and values for Km were generated.

Quantitative Method: Western Blotting

Activity of SPCs was tested using wild type and mutant pro-vWF as substrate.

The SPC's used in these reactions are not purified enzymes, so a volume of each enzyme processing approximately 90% of wild type pro-vWF to its mature form was standardized (Table 3).

SPC's	Volume (μL)
Furin	3
PACE4	2
E94D	1
D90T	16
DM	8.5
PC5	16
PC7	16

Table 3: Volumes used for each SPC during quantitative processing reactions.

The amount of vWF used in every reaction was standardized by using wild type vWF as control, and is shown in table 4.

vWF	Volume (µL)
Wild type	20
K762S	15
K762G	15
K762D	15
K762A	10
K762L	20
K762V	20

Table 4: Volumes used for wild type and mutant forms of pro-vWF during quantitative processing reactions.

For every pro-vWF processing reaction, 4µL of 10X IVP buffer at pH 7.0 was used except for the reactions to check the substrate specificity of furin at pH6, where 4µL of 10X IVP buffer at pH 6.0 was used. Total volume for every reaction was maintained at 40µL by using sterile water. Reaction tubes were incubated at 37°C for 16 h. Western blotting was done as described by Sucic et.al, (35).

Results

Quantitative Results

	Furin	Furin	PACE4	E94D	D90T	DM	PC5	PC7
R-T-K-R	21.87 ± 2.80	17.02 ± 2.17	8.86 ± 1.10	39.44 ± 6.75	161.33 ± 12.96	34.91 ± 14.04	12.43 ± 2.77	74.20 ± 13.87
R-T-S-R	39.75 ± 8.32	39.50 ± 4.22	96.60 ± 12.90	141.40 ± 24.68	27.46 ± 9.20	82.91 ± 11.10	84.57 ± 6.14	185.10 ± 28.55
R-T-N-R	29.01 ± 4.85	34.23 ± 4.04	82.51 ± 14.53	75.94 ± 15.10	76.87 ± 11.59	80.78 ± 15.24	55.13 ± 6.41	136.10 ± 23.42
R-T-G-R	94.14 ± 6.65	Not Determined	157.80 ± 22.75	173.53 ± 23.35	Not Determined	137.86 ± 44.16	97.10 ± 23.77	206.18 ± 42.3
R-T-D-R	181.78 ± 29.55	87.68 ± 6	205.20 ± 30.08	340.63 ± 48.71	40.73 ± 7.79	Not Determined	119.67 ± 27.62	156.00 ± 2.94
R-T-A-R	120.00 ± 16.24	55.85 ± 7.16	215.53 ± 28.26	217.17 ± 28.30	68.59 ± 15.1	252.13 ± 39.18	142.80 ± 4.69	224.33 ± 28.44

Table 5: Quantitative in vitro processing results. Each SPC was incubated with fluorogenic substrates for 30 minutes, following the reaction protocol shown in Table 2. Fluorescence liberated by SPC cleavage was then quantified by fluorimetry and was used to generate Km values, which are shown along with standard error. Km values are in μM. In a few cases, values were not determined because of limited substrate availability.

For quantitative analysis of substrate specificity, each wild type or mutant enzyme was used with a synthetic peptide conjugated to a fluorescent dye. The peptides differed in the amino acid at the P2 position. Recognition and cleavage of the peptide by the enzyme resulted in liberation of the fluorescent dye, which was quantified and used to generate a Km. Results are shown in Table 2; each Km represents the mean of at least three separate experiments.

Results show that each enzyme has unique substrate preferences. Furin (at pH 7.0) showed the highest affinity for the wild type substrate (R-T-K-R; Km of 21.87) with a basic P2 residue. Furin also showed relatively high affinity for substrates with polar residues in the P2 position (R-T-N-R, with a Km of 29.01, and R-T-S-R, with a Km of 39.75). Substrates with non-polar P2 residues were processed much less efficiently (R-T-G-R, with a Km of 94.14, and R-T-A-R, with a Km of 120.00). Furin showed the least affinity for R-T-D-R (Km of 181.78), which has an acidic P2 residue. At pH 6.0, furin exhibited the same preference of substrates, although the Kms for each substrate were generally lower that at pH 7.0 (see Table 1). This suggests that furin may work more efficiently within the Golgi apparatus than in other environments.

PACE4 (at pH 7.0) showed the highest affinity for the wild type substrate (R-T-K-R; Km of 8.86) with a basic P2 residue. PACE4 also showed relatively high affinity for substrates with polar residues in the P2 position (R-T-N-R, with a

Km of 82.51, and R-T-S-R, with a Km of 96.60). Substrates with non-polar P2 residues were processed much less efficiently (R-T-G-R, with a Km of 157.80, and R-T-A-R, with a Km of 215.53). PACE4 showed low affinity for R-T-D-R (Km of 181.78), which has an acidic P2 residue. Overall, PACE4 had generally higher Kms compared to furin, suggesting that PACE4 acts with less efficiency.

E94D (at pH 7.0) showed the highest affinity for the wild type substrate (R-T-K-R; Km of 39.44). E94D also showed relatively high affinity for substrates with polar residues in the P2 position (R-T-N-R, with a Km of 75.94, and R-T-S-R, with a Km of 141.40). Substrates with non-polar P2 residues were processed less efficiently (R-T-G-R, with a Km of 173.53, and R-T-A-R, with a Km of 217.17). E94D showed the low affinity for R-T-D-R (Km of 181.78), which has an acidic P2 residue.

Overall substrate preferences of D90T were markedly different, suggesting a key role for Asp 90 in mediating interaction with P2 residue. D90T showed highest affinity for a substrate with a polar residue in the P2 position (R-T-S-R, with a Km of 27.46). D90T showed relatively high affinity for the R-T-D-R (Km of 40.73) with an acidic P2 residue. A substrate with a non-polar P2 residue was processed less efficiently (R-T-A-R, with a Km of 68.59). The affinity of D90T for R-T-N-R (Km of 76.87) was low. Interestingly, D90T showed the least affinity for wild type substrate (R-T-K-R, with a Km of 161.33), which has a basic P2 residue.

DM showed the highest affinity for the wild type substrate (R-T-K-R; Km of 34.91). DM also showed relatively high affinity for substrates with polar residues in the P2 position (R-T-N-R, with a Km of 80.78, and R-T-S-R, with a Km of 82.91). Substrates with non-polar P2 residues were processed much less efficiently (R-T-G-R, with a Km of 137.86, and R-T-A-R, with a Km of 181.78. DM substrate preferences largely did reflect those of furin, but with higher Km.

PC5 showed the highest affinity for the wild type substrate (R-T-K-R; Km of 12.43) with a basic P2 residue. PC5 also showed relatively high affinity for substrates with polar residues in the P2 position (R-T-N-R, with a Km of 55.13, and R-T-S-R, with a Km of 84.57). Substrates with non-polar P2 residues were processed much less efficiently (R-T-G-R, with a Km of 97.10, and R-T-A-R, with a Km of 142.80). PC5 showed the least affinity for R-T-D-R (Km of 181.78).

PC7 showed the highest affinity for the wild type substrate (R-T-K-R; Km of 74.20). PC7 also showed relatively high affinity for substrates with polar residues in the P2 position (R-T-N-R, with a Km of 136.10, and R-T-S-R, with a Km of 185.10). PC7 showed the low affinity for R-T-D-R (Km of 156.00). Substrates with non-polar P2 residues were processed much less efficiently (R-T-G-R, with a Km of 206.18, and R-T-A-R, with a Km of 224.33). Overall, PC7 appeared to be the least efficient enzyme, with relatively high Kms for each substrate.

Qualitative Results

For qualitative analysis of substrate specificity, each wild type or mutant enzyme was used with wild type and mutant von Willebrand's factor. The mutant von Willebrand's factor differed in the amino acid at the P2 position (K762 in wild type von willebrand's factor).

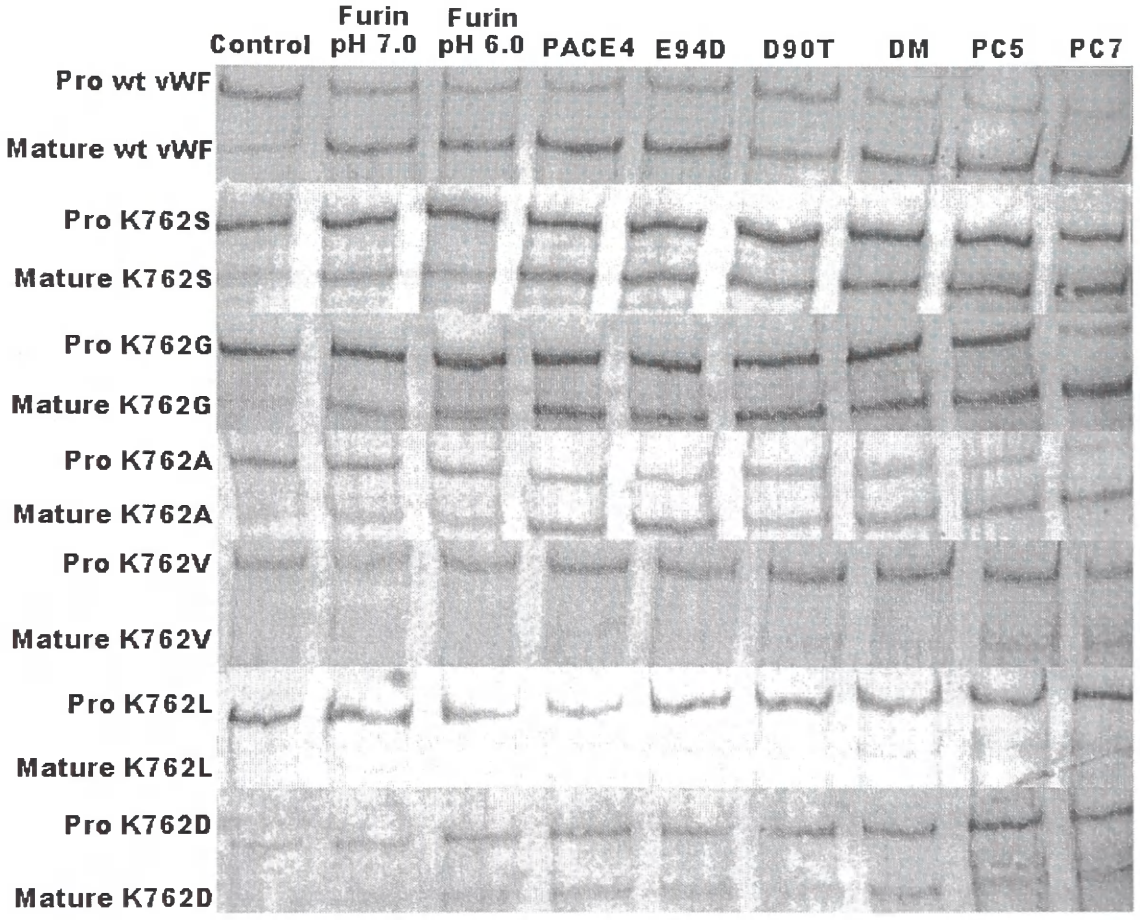

Figure 4: Processing ability of SPCs against wild type and mutant vWF. Each enzyme was incubated with each protein at 37°C for 16hrs. Proteins in the reaction were resolved using SDS-PAGE, and von Willebrand's factor was detected using western blotting. Processing of pro-von Willebrand's factor causes a significant molecular weight decrease, as indicated.

Furin (at pH 7.0) processed wild type vWF (with a basic P2 residue) approximately 90%. Furin also processed K762G and K762A efficiently (with non-polar residues in the P2 position), but processing of K762V and K762L (also non-polar P2 residues) was minimal (approximately 2%). K762S, with a polar P2 residue, was processed at the same efficiency as K762A (approximately 20%). Furin did not process K762D, which has an acidic P2 residue. At pH 6.0, furin exhibited the same preference of substrates.

PACE4 processed wild type vWF efficiently. PACE4 also processed K762G and K762A (approximately 40% and 60%), but very little processing was seen for K762V and K762L (approximately 10% and 1%); all of these have non-polar residues. K762S, with a polar P2 residue, was processed at the same efficiency as K762G (approximately 40%). Processing of K762D, which has an acidic P2 residue, was minimal.

E94D processed wild type vWF very efficiently. Among substrates with non-polar residues, E94D processed K762G and K762A (approximately 75% and 30%), but very little processing was seen for K762V and K762L (approximately 10% and 1%). K762S, with a polar P2 residue, was processed somewhat more efficiently than K762G (approximately 40%), but less efficiently than K762A. Processing of K762D, which has an acidic P2 residue, was minimal.

D90T processed approximately 50% of wild type vWF. D90T also processed K762G and K762A (non-polar residues in the P2 position) at the same efficiency as wild type vWF, but very little processing was seen for K762V and K762L (approximately 10% and 1%), which also have a non-polar amino acid at P2 position. K762S was processed less efficiently than substrates with non-polar amino acid at P2 position (approximately 35%). Processing of K762D was minimal (approximately 5%). Thus, D90T also exhibits markedly different preferences for mutants of pro-vWF compared to the other enzymes.

DM efficiently processed wild type vWF. DM also processed K762G and K762A, but less efficiently than the wild type (approximately 60% and 50%); but very little processing was seen for K762V and K762L (approximately 20% and 1%). K762S (polar P2 residue) was processed less efficiently than K762G and K762A (approximately 40%). Processing of K762D, which has an acidic P2 residue, was minimal.

PC5 processed wild type efficiently. PC5 also processed K762G and K762A, with non-polar residues in the P2 position, but less efficiently than wild type (approximately 60%); very little processing was seen for K762V and K762L (approximately 20% and 25%), which also have non-polar amino acid at P2 position. K762S (polar P2 residue) was processed less efficiently than substrates with non-polar amino acids at P2 position (approximately 50%). Processing of

K762D, which has an acidic P2 residue, was minimal, but did occur more efficiently than for either furin or PACE4.

PC7 processed wild type vWF very efficiently. PC7 also processed K762G and K762A (approximately 80%), but less processing was seen for K762V and K762L (approximately 30% and 25%). K762S was processed less efficiently than substrates with non-polar amino acids at the P2 position (approximately 65%). Processing of K762D was less efficient, but more pronounced than for furin or PACE4.

Discussion

Widely expressed SPCs play critical roles in development, homeostasis, and pathology; however, specific roles for each enzyme remain elusive, in part because of some redundancy in substrate preferences. While some redundancy does exist, many reports also suggest that each enzyme has unique substrate preferences, which could underlie unique physiological and pathological roles. In particular, furin and PACE4 have been reported to vary in their requirements for a P2 residue, with PACE4 having more stringent requirements; indeed, some reports have suggested that PACE4 requires a basic P2 residue, although other experimentation does not support this (12 and 43). We set out to systematically examine the P2 requirements of all four widely expressed SPCs, using a combination of quantitative and qualitative methods.

Overall quantitative results suggest that P2 substrate specificities of the widely expressed SPCs are similar, with basic and polar amino acids being generally preferred over non-polar and acidic residues at the P2 position. However, each enzyme does appear to have unique preferences. Furin, particularly at pH 6.0, appears to be the broadest and most efficient enzyme. Others have shown that furin can have different substrate specificities at pH 6.0 versus 7.0. The most well-documented example occurs during furin maturation, where the inhibitory propeptide is auto-cleaved in the Golgi apparatus at the sequence N-Arg-Ala-Val-Thr-Lys-Arg-C (47 and 48). Furin has a much lower Km for this sequence at pH 6.0 than pH 7.0, ensuring that activation does not

occur until transit to the Golgi. Since furin primarily localizes to the Golgi, the increased efficiency of furin at pH 6.0 suggest that furin is extremely proficient at processing proteins that traverse through the Golgi.

PACE4 efficiency versus peptides is not in complete agreement with some published results done with protein substrates (12 and 43). For example; our results show PACE4 has a higher affinity for peptide substrates with an acidic P2 residue compared to a hydrophobic residue; published results done on protein substrates showed the opposite preference. Overall, quantitative results show that PACE4 has stringent P2 requirements. The stringent specificity may be because of aspartic acid at position 90 (D90) and glutamate at position 94 (E94) in the S2 subsite. In contrast, furin has threonine at position 90 and aspartic acid at position 94, which might give furin more tolerance for substrates that lack a basic (positively charged) P2 residue. PC7 appears to be the least efficient, perhaps consistent with its proposed role as a completely redundant member of the family; its relatively high Kms toward substrates with basic, polar, and non-polar P2 residues could be the result of a glycine substitution for E94, which removes a negative charge from the S2 subsite and replaces it with a non-polar aminoacid.

Overall qualitative results were not always in agreement with results obtained with the peptides, but generally support the conclusions that P2 preferences are generally similar among the widely-expressed SPCs, although each enzyme may have some unique properties. Furin and PACE4 results are

somewhat inconclusive and in conflict with published reports (need future experimentation). PC7 appears broader against the protein substrates. It processed mutant proteins with aspartic acid at the P2 position more efficiently than furin and PACE4. This may be because of effects of the amino acid substitutions on overall conformation of the von Willebrand's factor. Such structural changes may result in impaired interaction with enzymes that are not directly due to interactions between the P2 residue and the S2 subsite.

We also attempted to investigate the molecular basis for the restricted range of PACE4 regarding P2 preferences. Toward this end, we used site-directed mutagenesis to change two amino acids that may be important at the S2 subsite (D90 and E94) to the residues seen at those positions in furin (threonine and aspartic acid, respectively)(Figure 2). We hypothesized that these changes, individually or together, might result in an enzyme with the P2 preferences of furin. Results do suggest an important role for D90. The D90T mutant had markedly different substrate preferences than all other mutants. D90T was much more tolerant of acidic and hydrophobic P2 residues and also it showed low affinity towards a basic P2 residue. This suggests that in PACE4, D90 (negatively charged) may interact with positively charged P2 residues. When D90 is changed to a threonine (lacking the negative charge of D90) the S2 subsite may be much more accommodating of side chains with negative charges or hyprohobic properties. Except for K762D, these results were largely also seen with a protein substrate.

Results suggest that E94, alone, plays a limited role, if any, in mediating P2 preferences in PACE4. In the double mutant, substrate preferences did mimic those of furin, but with overall higher Kms. Substitution of two different amino acids at 90 and 94 the position may cause structural changes in the S2 subsite of DM. The structural changes may be the reason for higher Kms of DM, though the range of substrate preferences was the same for both furin and DM. We are looking only at the amino acids (S2 subsite) which may be interacting with P2 residue at substrate, but change in the overall conformation of substrate binding cleft may cause a different pattern of interaction with other amino acids (i.e., P1 or P1') in the substrate.

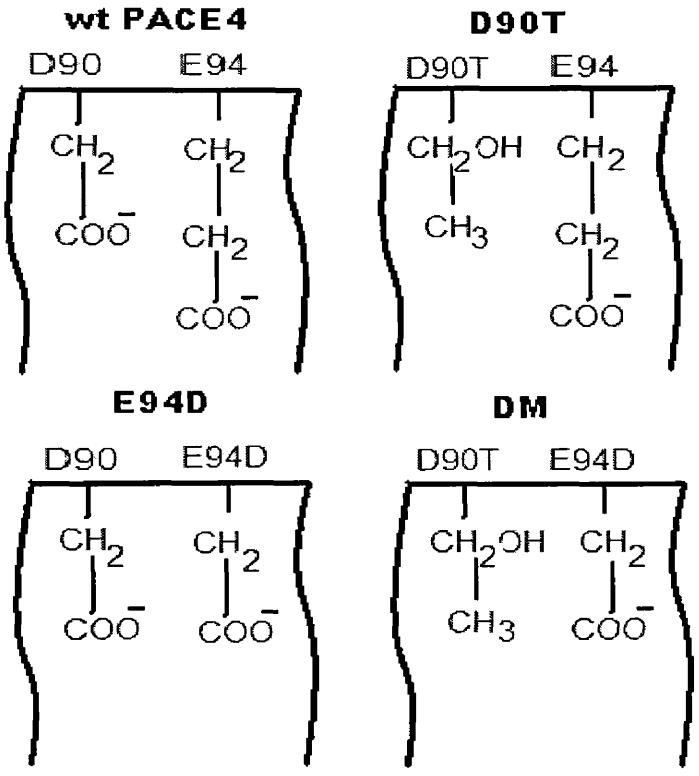

Figure 5: The side chain of amino acids at 90 and 94th position in the S2 subsite of wild type (wt) PACE4 and mutants (E94D, D90T and DM). Note that DM mimics furin residues at these positions.

It is known that widely expressed SPCs are involved in many physiological and pathological functions. They are involved in processing many proteins like receptors, growth factors, hormones and neuropeptides, to their mature forms. In addition to processing cellular precursor proteins, SPCs are also involved in processing viral proteins and bacterial toxins. Thus, designing inhibitors is potentially very important. To design an inhibitor precise knowledge of cleavage preferences of any individual enzyme is important. The cleavage preferences of SPCs at the P2 residue have not been described in a level of detail sufficient for designing specific inhibitors for individual SPCs. These results can provide preliminary data for designing specific inhibitors for individual SPCs.

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