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

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To investigate if some residues within the prosegment of PC5A are important for its optimal proteolytic function, various PC5A mutants were cellulary expressed, and their processing activities were compared using pro-vascular endothelial growth factor C (pro-VEGF-C) as a substrate. Although wild type PC5A almost completely processes pro-VEGF-C, a prosegment deletion 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 decaptapetides mimicking the C-terminal sequence of PC5 prosegment (pPC5) revealed that the native 107QQVVKKRTKR116 peptide is a nanomolar inhibitor, whereas its P6 mutant K111H is more selective toward PC5A than Furin. In vitro activity assays using the bacterially expressed pPC5 and its mutants revealed that they 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 Chinese hamster ovary FD11 cells overexpressing pro-VEGF-C with either PC5A or Furin showed them to be as good inhibitors of PC5A as the serpin α1-antitrypsin Portland (α1-PDX), ppFurin, or ppPACE4 but less potent toward overexpressed Furin. In conclusion, cleavages of the prosegment of PC5A at both Arg115 and Arg116 are required for PC5A cellular activity, and ppPC5 is a very potent but modestly selective cellular inhibitor of PC5A.

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)1 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 (KRX(R/K)R)1 where n = 0, 2, 4, or 6, and X is any amino acid (1–3). The PC family counts eight known members; that is, 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, which cleaves at the consensus motif (R/K)X-hydrophobicZ1, where Z is variable (5–7).

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. Although 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 (8–11), it is one of the least understood enzymes of the convertase family. Its levels are up-regulated in proliferating vascular smooth muscle cells (12) as well as during embryo implantation (13). PC5 exists in two different isoforms, a soluble PC5A sorted to regulated secretory granules (8, 14) and a membrane-bound PC5B cycling between the trans-Golgi network and the cell surface (14, 15). Active PC5A can cleave a variety of secretory precursors; that is, pro-mullerian-inhibiting substance (16), proenin (17), prorenotensin (18), pro-PTP receptor (19), pro-cholecystokinin (20), integrin pro-α subunits (21), human immunodeficiency virus gp160 (22), Alzheimer disease β-secretase BACE1 (23), transforming growth factor-TGF-β-like Lefty (24), and vascular endothelial growth factor C (VEGF-C). Recent development on its cleavage specificity showed that, in contrast to Furin (2, 25), purified active mouse PC5A (mPC5A) cleaves in vitro tri- and tetrapeptides 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), 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 chloromethyl ketone 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 (35), α1-antitrypsin, Portland (α1-PDX) (36–38), proteinase inhibitor 8 (39), and the turkey ovomucoid third domain (40). However, these effective inhibitors lack selectivity toward members of the PC family. Furthermore, both proteinase inhibitor 8 and α2-macroglobulin can inhibit many other proteases in addition to the PCs. α1-PDX

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was shown to inhibit all the PCs in the constitutive secretory pathway (37). Recently, Tsuji et al. (41) showed that a reactive site loop variant of α1-antitrypsin (AVRR352) is ~100-fold more selective in vitro toward 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 (46), whereas 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 toward PC5A (IC50 (pFurin) is 10-fold more potent toward PC5A (IC50

Table I

Sequence of oligonucleotides used for PC5 constructs

| Primers | Sense ($) | Antisense ($) |
|---------|-----------|---------------|
| S1/AS1  | CTCGAGCACACACGACCCACACTAATAGTCCG | TGGCCGACTAGTGGAACCC |
| S2/AS2  | AACTATGGGGCGTGCTACACACCACCACTTGGGCGTACAGGCTGCTGAGGTCATAATCCCTCTTGGTTCTTTTTGGCACCAC |
| S3/AS3  | GACATTAAAGCGGCTGTCCCTTGCGGAGATCTTGGCACCAC |
| S4/AS4  | AAGCTTGGGACATAGGCTGGGACTGGGGAGCCC |
| S5/AS5  | AGGACGGCGGCGGCTGATGATTAGAC |
| S6/AS6  | CAGCTTCACTAGCTGAGGAC |
| S7/AS7  | CTACATATGCGGACATTTAAAGGAAAG |
| S8/AS8  | CATAGTGATGATATTAAAGGAGCT |
| S9/AS9  | TGGCGGCGGATGATAGGCTGGGACTGGGGAGCCC |
| S10/AS10| GGGCGGGCGGGGTATACACCAACCCACACAGGCTGCTGAGGTCATAATCCCTCTTGGTTCTTTTTGGCACCAC |
| AS11    | GGGCCGACTAGTGGAACCC |
| AS12    | GGGCGACGGCTGTCCCTTGCGGAGATCTTGGCACCAC |
| AS13    | GGGCGGCGGCGGCTGATGATTAGAC |
| AS14    | GGGCGGCGGCGGCTGATGATTAGAC |
| AS15    | GGGCGGCGGCGGCTGATGATTAGAC |
| AS16    | GGGCGGCGGCGGCTGATGATTAGAC |
| AS17    | CTTTTAATAGGCTGATATGAG |
| AS18    | CTTTTTAACTGGCTGATAGGTAAG |
| AS19    | CAGCAGTCTTACATACGTGAAC |
| AS20    | GTACATATGCGGACATTTAAAGGAAAG |
| AS21    | GGGCGGACGGCTGTCCCTTGCGGAGATCTTGGCACCAC |

### EXPERIMENTAL PROCEDURES

#### Cellular Activity and Biosynthetic Analysis of PC5A and Its Mutants

The various mutants 7S9, R80A, 81R, R84A, R116A, and PC5A-Δpro were obtained by PCR (7) using the pair of oligonucleotides S6/AS17, S7/AS18, S8/AS19, S9/AS3, S9/AS3, and S9/AS20, respectively (see 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 pIRE2-mPC5A digested with BglII. Each recombinant cDNA was transfected using LipofectAMINE 2000 (Invitrogen) into CHO-FD11 cells stably expressing VEG-F-G. Media were analyzed by Western blot on a 12% SDS-polyacrylamide electrophoresis gel using as the primary antibody a polyclonal anti-VEGF-C antibody, H-190, directed against the C-terminal end of VEGF-C (Santa Cruz Biotechnology; dilution 1:500) and as secondary antisera against rabbit horseradish peroxidase-coupled IgGs (Invitrogen) (dilution 1:10,000). Biosynthetic analysis was performed in HK293 cells expressing either pRRES2-EFGP (control), the full-length WT PC5A, or its mutants R84A and R116A. Forty-eight hours post-transfection, the cells were pulse-labeled for 4 h with 250 μCi/ml [3H]leucine (Amersham Biosciences), and cell lysates and media were immunoprecipitated using a polyclonal anti-PC5 antibody directed against the N terminus of the active enzyme, i.e., amino acids 117–132 (dilution, 1:200 (14)). Immunoprecipitates were then resolved by SDS-PAGE (8% Tricine gel) and autoradiographed (14, 37).

#### 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-PerSepive Biosystems, Framingham, MA) following the O-hexafluoroisopropyl-[7-azabenzotriazol-1-yl]-N, N', N'-tetramethyluronium (HATU)/diisopropylcarbodiimide (DIEA)-mediated Fmoc (N-$\text{F}$-fluorenyl)-methoxycarbonyl (Fmoc) chemistry (56). The crude peptides were purified by reverse phase high performance liquid chromatography using an anodisc (500 μm) C18 column with a linear gradient of 0.0005% TFA in H2O (0% A) to 0.0005% TFA in CH3CN (100% A) at a flow rate of 2 mL/min. The purified peptides were characterized by matrix-assisted laser desorption ionization-time of flight mass spectrometry as de-
Inhibitory potency of PC5 C-terminal decapetides

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

| Inhibitor Peptide sequence | \( K_i \) (nM) |
|---------------------------|----------------|
| mPC5A                     | hPC5A          | hFurin  |
| WT                        | 16 ± 1         | 31 ± 4  | 190 ± 20 |
| Q108A                     | 5.3 ± 0.1      | 6.6 ± 0.2 | 21 ± 4  |
| K111H                     | 12 ± 2         | 22 ± 5  | 330 ± 40 |
| K111L                     | 24 ± 4         | 22 ± 3  | 950 ± 120 |
| K111R                     | 63 ± 12        | 60 ± 16 | 160 ± 14 |
| K111P                     | 85 ± 10        | 85 ± 16 | 250 ± 50 |
| K111Q                     | 130 ± 19       | 120 ± 16 | 2020 ± 250 |
| K111A                     | 140 ± 16       | 110 ± 13 | 960 ± 120 |
| K111V                     | 190 ± 20       | 240 ± 23 | 1100 ± 100 |
| K111I                     | 380 ± 50       | 406 ± 40 | 1680 ± 90 |
| K111S                     | 410 ± 62       | 440 ± 38 | 3030 ± 380 |
| K115T                     | 970 ± 190      | 1000 ± 380 | 6900 ± 1800 |
| K111W                     | 1080 ± 180     | 1000 ± 140 | 6710 ± 780 |
| K111R/H113I               | 1200 ± 270     | 910 ± 200 | 3900 ± 800 |
| K111E                     | 2300 ± 790     | 2300 ± 790 | 14000 ± 5900 |
| K113I                     | 7300 ± 3300    | 7500 ± 2000 | 8000 ± 2500 |

**Expression and Purification of Bacterial Mouse PC5 Prosegment—**The expression vector pET24b(+) (Novagen) was transformed into the E. coli strain called BL21(DE3) (Novagen). Protein expression was induced by the addition of 0.4 mm isopropyl-1-thio-\( \beta \)-galactoside for 3 h at 37 °C. The cells were then harvested by centrifugation at 5000 \( \times g \) 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 0.15 M sodium chloride, and purification of these pPC5s was done under these denaturing conditions on a Ni\(^{2+} \) 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 Coomassie staining of SDS-PAGE (1% Tricine gels. The average yield of each purified prosegment varied between 10 and 20 mg/liter 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 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 11,684 Da, obtained 11675 Da; R84A pPC5 and R116A pPC5 expected 11,599 Da, obtained 11,593 and 11,599 Da respectively; K111H pPC5 expected 11,694 Da, obtained 11,714 Da; K111L pPC5 expected 11,670 Da, obtained 11,695 Da; K111P pPC5 expected 11,654 Da, obtained 11,684 Da; K111V pPC5 expected 11,656 Da, obtained 11,671 Da; K111I pPC5 expected 11,670 Da, obtained 11,695 Da).

**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 \( \mu \)g/liter 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 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 11,684 Da, obtained 11675 Da; R84A pPC5 and R116A pPC5 expected 11,599 Da, obtained 11,593 and 11,599 Da respectively; K111H pPC5 expected 11,694 Da, obtained 11,714 Da; K111L pPC5 expected 11,670 Da, obtained 11,695 Da; K111P pPC5 expected 11,654 Da, obtained 11,684 Da; K111V pPC5 expected 11,656 Da, obtained 11,671 Da; K111I pPC5 expected 11,670 Da, obtained 11,695 Da).

**Stop-time Inhibition Assays (IC\textsubscript{50})—**The different enzymes (VV: pPC5-BTMD, mppPC5, rppPC7, mppPC7, mppPC5, hppFurin, hppFAC4-A, hppSK2-K1) were incubated with 1.2 \( \mu \)g/ml of each of the prosegments for 3 h at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal calf serum (Invitrogen), the cells were rinsed with serum-free DMEM and incubated for another 6 h with serum-free DMEM containing brefeldin A (BFA, 2.5 \( \mu \)g/ml; Epicentre). The cells were washed with phosphate-buffered saline) and lysed with radiolabeled immunoprecipitation assay buffer (described). The radiolabeled proteins 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 1:2500 dilution.

**Inhibitor Peptide sequence**

- **WT**: QQVKKR
- **Q108A**: QQVKKRTKR
- **K111H**: QQVVKVRTR
- **K111L**: QQVVKVRTR
- **K111R**: QQVVKVRTR
- **K111P**: QQVVKVRTR
- **K111Q**: QQVVKVRTR
- **K111A**: QQVVKVRTR
- **K111V**: QQVVKVRTR
- **K111I**: QQVVKVRTR
- **K111S**: QQVVKVRTR
- **K115T**: QQVVKVRTR
- **K111W**: QQVVKVRTR
- **K111R/H113I**: QQVVKVRTR
- **K111E**: QQVVKVRTR
- **K113I**: QQVVKVRTR

**Table II**

| Inhibitor Peptide sequence | \( K_i \) (nM) |
|---------------------------|----------------|
| mPC5A                     | hPC5A          | hFurin  |
| WT                        | 16 ± 1         | 31 ± 4  | 190 ± 20 |
| Q108A                     | 5.3 ± 0.1      | 6.6 ± 0.2 | 21 ± 4  |
| K111H                     | 12 ± 2         | 22 ± 5  | 330 ± 40 |
| K111L                     | 24 ± 4         | 22 ± 3  | 950 ± 120 |
| K111R                     | 63 ± 12        | 60 ± 16 | 160 ± 14 |
| K111P                     | 85 ± 10        | 85 ± 16 | 250 ± 50 |
| K111Q                     | 130 ± 19       | 120 ± 16 | 2020 ± 250 |
| K111A                     | 140 ± 16       | 110 ± 13 | 960 ± 120 |
| K111V                     | 190 ± 20       | 240 ± 23 | 1100 ± 100 |
| K111I                     | 380 ± 50       | 406 ± 40 | 1680 ± 90 |
| K111S                     | 410 ± 62       | 440 ± 38 | 3030 ± 380 |
| K115T                     | 970 ± 190      | 1000 ± 380 | 6900 ± 1800 |
| K111W                     | 1080 ± 180     | 1000 ± 140 | 6710 ± 780 |
| K111R/H113I               | 1200 ± 270     | 910 ± 200 | 3900 ± 800 |
| K111E                     | 2300 ± 790     | 2300 ± 790 | 14000 ± 5900 |
| K113I                     | 7300 ± 3300    | 7500 ± 2000 | 8000 ± 2500 |
substrate pERTKR-MCA. The Kcat values of the pERTKR-MCA processing are 70 μM for rPCT7-BTMID, 45 μM for yxekin, and 7 μM for hFurin-BTMID and mPC5A (30, 57). The IC50 values were obtained by plotting the results with the GraFit4 program.

**Transfections and Biosynthetic Analysis of ppPC5a**—The preprosegments of mPC5 (coding for residues 1–116) were amplified for 20 cycles by a three-step PCR reaction. The primer pairs used to amplify the ppPC5 WT and R116A were S4/A/S10 and S5/A/S11 (Table I), respectively. All Fp ppPC5 mutants were amplified with a sense primer corresponding to a region upstream of the multiple cloning sites of pRES2-EGFP. The ppPC5 K111H, K111L, K111P, K111V, and ppPC5-FLAG (signal peptide of β-secretase BACE1-FLAG epitope (23)) were produced using the primer pairs S4/A/S12, S4/A/S13, S4/A/S14, S4/A/S15, S4/A/S16, and S10/A/S21 (Table I), respectively. The cDNAs of the WT, R84A, and R116A ppPC5 were transferred into the EcoRI site of the pRES2-EGFP vector. The other mutants (K111(H/I/L/P)V) were cloned into 5’-SacI/SacI 13’- sites of the pRES2-EGFP. HK293 cells (6.5 x 10⁶ 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 6 h with 250 μCi/ml [³⁵S]Met (Amersham Biosciences) and BFA. Cells were lysed in radioimmune precipitation assay buffer containing a mixture 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 (23).

**Inhibition of VEGF-C Processing ex Vivo**—CHO-K1 cells were transiently transfected with hVEGF-C and either various ppPCs or control. A total of 3 μg of DNA was transfected using LipofectAMINE 2000 (Invitrogen) for 0.75 x 10⁶ cells. The CHO-FD11 cells (kindly supplied by Dr. Stephen H. Leppla; National Institutes of Health), which are derived from CHO-K1 cells that are Furin-deficient, were stably cloned into 5’-SacI/SacI 13’- sites of the pRES2-EGFP vector. The various mutants (K111(H/I/L/P)V) were cloned into 5’-SacI/SacI 13’- sites of the pRES2-EGFP. HK293 cells (6.5 x 10⁶ 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 6 h with 250 μCi/ml [³⁵S]Met (Amersham Biosciences) and BFA. Cells were lysed in radioimmune precipitation assay buffer containing a mixture 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 (23).

**RESULTS**

**Biosynthesis of PC5A and Its Mutants and Their Proteolytic Activities**—Biosynthetic analysis demonstrated that PC5A lacking its prosegment, PC5A·Δpro, is not secreted from HK293 cells (Fig. 1A). In addition, wild type PC5 and its P1 mutant of the secondary-processing site (R84A) were secreted as processed PC5A, whereas only trace amounts of pro-PC5A were 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 endo H-sensitive (14). Finally, the reported late C-terminal processing of PC5A, resulting mainly in the secretion of the precursor form with small amounts of pro-PC5A, does not seem to play a major role, as its substitution by Arg did not significantly affect pro-VEGF-C processing by PC5A.

**Inhibitory Potency and Specificity of C-terminal pPC5-derived Decapeptides**—Previously, we showed that peptides as small as 10 amino acids corresponding to the C-terminal end of the prorregions of Furin and PC7 were potent inhibitors of these enzymes (45, 60). To determine whether similar peptides de-
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 (21) 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 (K<sub>i</sub>) on the in vitro processing of the fluorogenic pERTKR-MCA substrate by mPC5A, hPC5A, or soluble hFurin-BTMD (22). As compared with Furin, the selectivity and potency of the various peptides revealed that 1) the native decapeptide<sup>107</sup>Q<sup>108</sup>VV<sup>109</sup>K<sup>110</sup>K<sup>111</sup>R<sup>112</sup>T<sup>113</sup>K<sup>114</sup>R<sup>115</sup> is a very potent inhibitor of mPC5A and hPC5A, with K<sub>i</sub> values of 16 and 31 nM, respectively. This peptide exhibits 6–12-fold selectivity toward PC5A as compared with Furin. 2) The most potent inhibitor of PC5A is the P9 Q108A peptide with a K<sub>i</sub> of 5–6 nM, except that it is only 4-fold more selective toward Furin (Table II and Fig. 4). The worst inhibitor of PC5A is the P4 mutant R113I, with a K<sub>i</sub> of 7.5 M. 3) None of the 11 P6 mutants tested were more potent than Q108A, but 2 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 pro-VEGF-C, resulted in a low potency inhibitor K111H/R113I (K<sub>i</sub> ~ 1.2 μM). 5) Although 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 (1) resulted in a lower inhibitory potency, as compared with 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 α1-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 decapetide at the C terminus of their prosegment (8, 62).

**Expression of Various pPC5s and Antibody Production**

Based on the inhibition constants obtained from the above decapetides, we next verified if the full-length 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 K111L, were bacterially produced and purified (see “Experimental Procedures”). The purified WT pPC5 was used to raise a polyclonal antibody in rabbit. To test the antiserum selectivity, we expressed the preprosegments of PC5, Furin,
PC7, PACE4, and SKI-1 in HK293 cells treated with 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 because of the 17-amino acid C-terminal extension, which includes a hexahistidine tag, of the bacterial pPC5 antigen (see the legend to Fig. 5 and “Experimental Procedures”). Furthermore, this antiserum recognizes the intracellular 8.9-kDa pPC5 that is generated by the yzomyn processing of the full-length enzyme in CHO-FD11 cells in the absence or presence of BFA (Fig. 5).

Comparative in Vitro Inhibition Efficacies of pPC5s—The IC$_{50}$ 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 IC$_{50}$ 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 nanomolar 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 inhibitors than their corresponding decapeptides and showed IC$_{50}$ 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 (IC$_{50} \leq 10$ nM), whereas 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 (IC$_{50} \sim 71$ 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 significantly affect the inhibitory and selectivity properties of the prosegment. The various prosegments were also very potent inhibitors of hFurin and rPC7, with the IC$_{50}$ in the nanomolar range. The best inhibitor of mPC5A, hFurin, and rPC7 is also the K111H mutant with an IC$_{50}$ of ~4, 21, and 23 nM, respectively. All pPC5s tested failed to potently inhibit ykexin (IC$_{50}$ in the nanomolar range; Table III). Finally, the P1 mutant R116A exhibits an ~40-fold lower potency (IC$_{50} \sim 267$ nM), whereas the R84A mutant is only 3-fold less potent (IC$_{50} \sim 18$ nM) than WT pPC5.

**Bioisynthetic Fate of the Cellularly Expressed ppPC5 and Its Mutants**—We first tested the integrity and total level of the various prosegment constructs after their expression in the easily transfected HK293 cells that were pulse-labeled for 6 h with $^{[35]S}$Met in the presence of BFA, ensuring their retention in the ER. The 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 $^{[3]}$H$^{[3]}$Val 8.9-kDa form revealed Val at positions 2 and 9, confirming that the sequence starts at Arg$^{35}$ of mPC5A (data not shown), as for the whole enzyme (14). Analysis of the media

**TABLE III**

| Prosegment | mPC5A | hFurin | rPC7 | yKexin |
|------------|-------|--------|------|--------|
| WT         | 6.5 ± 0.1 | 41 ± 2 | 47 ± 2 | 1550 ± 60 |
| (1)        | (6)     | (7)    | (240)  |        |
| K111H      | 4.4 ± 0.4 | 21 ± 3 | 23 ± 1 | 1730 ± 65 |
| (1)        | (5)     | (5)    | (390)  |        |
| K111V      | 8.5 ± 0.1 | 30 ± 3 | 37 ± 2 | 1530 ± 160 |
| (1)        | (3.5)   | (4)    | (180)  |        |
| K111I      | 9.3 ± 0.8 | 42 ± 2 | 44 ± 1 | 1550 ± 211 |
| (1)        | (5)     | (5)    | (167)  |        |
| R84A       | 17 ± 1  | 43 ± 1 | 44 ± 2 | 6580 ± 3200 |
| (1)        | (2.5)   | (2.5)  | (370)  |        |
| K111P      | 18 ± 1  | 126 ± 13 | 134 ± 7 | 1370 ± 89 |
| (1)        | (7)     | (8)    | (75)   |        |
| K111L      | 71 ± 6  | 148 ± 4 | 131 ± 2 | 3330 ± 196 |
| (1)        | (1.6)   | (1.6)  | (50)   |        |
| R116A      | 258 ± 18 | 419 ± 21 | 541 ± 14 | 2350 ± 903 |
| (1)        | (1.6)   | (2)    | (10)   |        |
revealed the secretion of a smaller fragment (~3 kDa) (Fig. 6B). The fact that the R84A mutant is similarly processed to the other forms suggests processing of pPC5 into the secreted ~3-kDa form does not seem to require Arg84 and may, thus, be performed by another enzyme. In contrast, the removal of the signal peptide was practically abolished in a construct connecting a -secretase signal peptide-FLAG (23) to the N terminus of the wild type pPC5 sequence (pFpPC5). This signal peptide 2 FLAG motif was previously shown to be efficiently cleaved in the ER when connected to -secretase, resulting in an N-terminally flagged-BACE1 (23). However, when connected to pPC5, it either did not enter the ER or resisted 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 pro-VEGF-C Processing—We next compared the ability of the various ppPC5s, ppFurin, ppPACE4, ppPC7, and a1-PDX, to inhibit the cellular processing of pro-VEGF-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 pro-VEGF-C and either mPC5A (FD11/PC5A; Fig. 8A) or hFurin (FD11/VEGF-C/Furin; Fig. 8B). As a control, we analyzed the fluorescence level of EGFP of each transfected cell pool by fluorescence-activated cell sorter, which indicated equivalent transfection efficiencies (data not shown). In the parental CHO-K1 cells, we note that WT and R84A

Fig. 6. Biosynthetic analysis of ppPC5s. A, HK293 cells were transfected with either the empty vector (pIRES) or the WT, R84A, K111H, K111I, K111L, K111V, or R116A ppPC5s. The cells were pulse-labeled for 6 h with [35S]Met in the presence of BFA. Cell lysates were immunoprecipitated with the anti-pPC5 antibody, and the immunoprecipitates were 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 6 h with [35S]Met. Cell lysates and media were immunoprecipitated with the Ab:pPC5, and the proteins were 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 pro-VEGF-C processing in CHO-K1 cells. Shown in a Western blot analysis of the parental CHO-K1 cells transiently co-expressing pro-VEGF-C and either empty vector (pIRES), WT ppPC5, R84A ppPC5, a1-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 percent inhibitions are shown at the bottom of the gel. CTF, C-terminal fragment.
ppPC5 are equivalent to α1-PDX and ppFurin as inhibitors of pro-VEGF-C processing, whereas ppPACE4 and ppPC7 were less effective (Fig. 7). Because 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 pro-VEGF-C in these cells and to what extent each convertase is blocked by the above inhibitors. 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 or (B) FD11/VEGF-C/Furin cells transiently transfected with either the empty vector (pRES), 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. CTF, C-terminal fragment.

**FIG. 8.** Inhibition of pro-VEGF-C processing in FD11 cells overexpressing PC5A or Furin. Western blot analysis of the media of CHO-FD11 cells overexpressing VEGF-C and (A) FD11/VEGF-C/PC5A cells or (B) FD11/VEGF-C/Furin cells transiently transfected with either the empty vector (pRES), 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. CTF, C-terminal fragment.

**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 (55), an event thought to be favored within the acidic environment of the trans-Golgi network (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 pro-PC5A zymogen, mostly blocked in the ER, barely secreted even in an overexpression system (Fig. 2), and unable to cleave pro-VEGF-C (Fig. 4). These data extend the notion that primary cleavage of the prosegment is a prerequisite for PC5 to exit from the ER (14) 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 (55) suggested that Arg84 occupies the P1 position of the secondary processing site of pro-PC5A, within the sequence 79SR-TIKR84 ↓. 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 pro-VEGF-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 although the primary site processing occurred, the secondary one may not have occurred, 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 (data not shown).

The fact that the inactive PC5A-R84A (Fig. 3) is C-terminally processed to its 65-kDa form, similar to the WT enzyme (Fig. 2), suggests this cleavage is not autocatalytic as was originally suspected (14) but, rather, implicates another enzyme that is yet to be defined.3 With respect to the other secondary site mutants, the data revealed that P6 Ser is not critical but that P5 Arg and P4 Thr seem to play prominent roles, since their replacement by Ala significantly reduced pro-VEGF-C processing (Fig. 3). In that context, it is interesting to note that in the Furin mutation of the corresponding secondary processing site P4 Val 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 (7), PC5AΔpro remained in the ER (data not shown), was not secreted (Fig. 2), and was inactive (Fig. 3).

We demonstrated that a 10-amino acid peptide corresponding to the C terminus of ppPC5 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 dedocapeptides.

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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, decapetides containing P6 His or Arg and P4 Ile were tested because they are found in PC5A-specific substrates (16, 19, 21, 24), and a Q108A mutant was chosen because Gin at P9 is conserved in all PC prosegments (55). Compilation of our results revealed that although selectivity toward PC5A as compared with Furin can be improved, especially for the P6 K111H and K111L mutants, although selectivity toward PC5A as compared with 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, although 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 toward 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 decapetides (compare Tables II and III). The structure and/or additional interactions offered by the entire pPC5s as compared with decapetides 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 mimicking the C terminus of the wild type prosegment of each of the seven known PCs (65). In an effort to improve the selectivity 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 p1-PDX (38, 68, 69). It is hoped that pharmacological use of PC inhibitors including those presented above and novel ones such as polyarginines (7) will be more exploited in the future as novel tools in pathologies clearly implicating one or more convertases (28).

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