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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 cellularly 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 decapeptides mimicking the C-terminal sequence of PC5 prosegment (pPC5) revealed that the native ^{107}QQVVKKRTKR^{116} 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 them to be very potent nanomolar inhibitors (IC_{50}) 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 Arg^{116} and Arg^{84} 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 (K/R)X_n(K/R)↓, 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*-(hydrophobic)Z↓, 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), prorenin (17), proneurotensin (18), pro-PTPμ 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).[2] 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 α_2-macroglobulin (35), α_1-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 α_2-macroglobulin can inhibit many other proteases in addition to the PCs. α1-PDX

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[1] The abbreviations used are: PC, proprotein convertase; BFA, brefeldin A; BTMD, before transmembrane domain; SKI-1, subtilisin kexin isozyme-1; pPC5, prosegment of PC5; ppPC5: preprosegment of PC5; MCA, amidomethylcoumarin; VEGF, vascular endothelial growth factor; ER, endoplasmic reticulum; CHO, Chinese hamster ovary; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; h-, human; m-, mouse; y-, yeast; WT, wild type; EGFP, enhanced green fluorescent protein; α1-PDX, α1-antitrypsin Portland.

[2] G. Siegfried, N. G. Seidah, and A.-M. Khatib, submitted for publication.

TABLE I
Sequence of oligonucleotides used for PC5 constructs

The indicated sense (S) and antisense (AS) oligonucleotides were used in pairs (S/AS) in PCR reactions, as indicated under "Experimental Procedures."

Primers	Sense (S)	Antisense (AS)
S1/AS1	CTCGAGCACCACCACCACCACCACTAATAAGATCCG	TGGCCCACTACGTGAACC
S2/AS2	ACATATGCGCGTCTACACCAACCACTGG	TGCGGCCGCATGGCTGAGGTCATAATC
S3/AS3	GACCATTAAAGCGTCTGTTCTCTCGAG	TGCGGCCGCATGGCTGAGGTCATAATCCGCCTTGGTTCT
S4/AS4	AAGCTTGGGACCATGGACTGGGACTGGGGGAACCGC	TGCGGCCGCATGGCTGAGGTCATAATCCCTCTTGGTTCTTTTGATCACCAC
S5/AS5	AGAACCAAG*GCG*GATTATGAC	TGCGGCCGCATGGCTGAGGTCATAATCCCTCTTGGTTCTTTTGATCACCAC
S6/AS6	CACTTCTACCAT*CG*TAGGACC	TGCGGCCGCATGGCTGAGGTCATAATCCCTCTTGGTTCTTTTGAGCACCAC
S7/AS7	CTACCATAGT*GC*GACCATTAAAAGG	TGCGGCCGCATGGCTGAGGTCATAATCCCTCTTGGTTCTTTTTGGCACCAC
S8/AS8	CATAGTAGGA*GG*ATTAAAAGGTCTG	TGCGGCCGCATGGCTGAGGTCATAATCCCTCTTGGTTCTTTTCACCACCAC
S9/AS9	TGCCGGACGGATTATGACCTCAGCCATG	CTCGAGAGAACAGACGCTTTAATGGTC
S10/AS10	GCGCGCCGCGTCTACACCAACCACTGG	GGGCCCTCATTAGTCATAATCCCTCTTGGTTCTTTT
AS11		GGGCCCTCATTAGTCATAATCCGCCTTGGTTCTTTT
AS12		CCGCGGAGATCTTCAGTCATAATCCCTCTTGGTTCTTTTGTGCACCAC
AS13		CCGCGGAGATCTTCAGTCATAATCCCTCTTGGTTCTTTTGATCACCAC
AS14		CCGCGGAGATCTTCAGTCATAATCCCTCTTGGTTCTTTTGAGCACCAC
AS15		CCGCGGAGATCTTCAGTCATAATCCCTCTTGGTTCTTTTTGGCACCAC
AS16		CCGCGGAGATCTTCAGTCATAATCCCTCTTGGTTCTTTTCACCACCAC
AS17		CCTTTTAATGGTC*GC*ACTATGGTAG
AS18		CCTTTTAATGGTC*GC*ACTATGGTAG
AS19		CAGACCTTTTAAT*CC*TCCTACTATG
AS20		GTCATAATCCGTCCGGCATACCGGGAG
AS21		CCGCGGTCAGTCATAATCCCTCTTGGTTCT

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 α_1-antitrypsin (AVRR^{352}) 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 (45, 46), whereas the prodomain of yeast kexin behaves as a mixed inhibitor with an IC_{50} of 160 nM (43). *In vitro* experiments demonstrated that the prosegment of Furin (pFurin) is 10-fold more potent toward PC5A (IC_{50} ~ 0.4 nM) than Furin (IC_{50} ~ 4 nM), whereas the prosegment of PC7 (pPC7) is a relatively selective inhibitor of its cognate enzyme (IC_{50} ~ 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).

As for other convertases, autocatalytic zymogen activation of pro-PC5 involves cleavage at the specific primary site KKRTKR$^{116}\downarrow$ found at the C terminus of the prosegment (Fig. 1*A*) (14). This primary cleavage occurs in the endoplasmic reticulum (ER) and is a prerequisite for the exit of PC5A from this compartment (14) as for all PCs (4, 7, 48) except PC2 (50, 51). Similar to subtilisin (52, 53), Furin (44, 54), and SKI-1 (7, 47), 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 and (ii) once the complex reaches an adequate lower pH and high calcium concentrations, presumably in the trans-Golgi network, the prodomain dissociates and is cleaved at an internal secondary site HSRTIKR$^{84}\downarrow$, found in a similar position in Furin (55).

Because 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 pro-VEGF-C. The data show that a PC5A isoform lacking the prosegment (PC5A-Δpro) and Ala mutants of the P1 Arg^{116} or Arg^{84} at the primary and secondary zymogen cleavage sites, respectively, are unable to process pro-VEGF-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* pro-VEGF-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-Δpro were obtained by PCR (7) using the pair of oligonucleotides S6/AS17, S7/AS18, S8/AS19, S3/AS9, S5/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 pIRES2-mPC5A digested with *Bgl*II. Each recombinant cDNA was transfected using LipofectAMINE 2000 (Invitrogen) into CHO-FD11 cells stably expressing VEGF-C. 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 antiserum anti-rabbit horseradish peroxidase-coupled IgGs (Invitrogen) (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 4 h with 250 μCi/ml [^{3}H]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-PerSeptive Biosystems, Framingham, MA) following the *O*-hexafluorophospho-[7-azabenzotriazol-1-yl]-*N*, *N*, *N*′, *N*′- tetramethyluronium (HATU)/di-isopropylethylamine (DIEA)-mediated Fmoc (*N*-(9-fluorenyl)-methoxycarbonyl) chemistry (56). The crude peptides were purified by reverse phase high performance liquid chromatography using an analytical Vydac C18 column with 300-Å pore diameter (5 μm, 4.6 × 250 mm). Peptides were eluted with a 1%/min linear gradient (10–27%) of 0.1% (v/v) aqueous trifluoroacetic acid/acetonitrile 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-

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 K_i values were calculated using a GrapFit4 program for competitive inhibition.

Inhibitor	Peptide sequence	K_i		
		mPC5A	hPC5A	hFurin
			nM	
WT	^{107}QQVVKKRTKR^{116}	16 ± 1	31 ± 4	190 ± 20
Q108A	QAVVKKRTKR	5.3 ± 0.1	6.6 ± 0.2	21 ± 4
K111H	QQVVHKRTKR	12 ± 2	22 ± 5	330 ± 40
K111L	QQVVLKRTKR	24 ± 4	22 ± 3	950 ± 120
K111R	QQVVRKRTKR	63 ± 12	60 ± 16	160 ± 14
K111P	QQVVPKRTKR	84 ± 17	88 ± 18	100 ± 10
K111Q	QQVVQKRTKR	130 ± 19	120 ± 16	2020 ± 250
K111A	QQVVAKRTKR	140 ± 16	110 ± 13	960 ± 120
K111V	QQVVVKRTKR	190 ± 20	240 ± 23	1100 ± 100
K111I	QQVVIKRTKR	380 ± 50	406 ± 40	1680 ± 90
K111S	QQVVSKRTKR	410 ± 62	440 ± 38	3030 ± 380
K115T	QQVVKKRTTR	970 ± 190	1000 ± 380	6900 ± 1800
K111W	QQVVWKRTKR	1080 ± 180	1000 ± 140	6710 ± 780
K111H/R113I	QQVVHKITKR	1200 ± 270	910 ± 200	3900 ± 800
K111E	QQVVEKRTKR	2300 ± 790	2300 ± 790	14000 ± 5900
R113I	QQVVKKITKR	7300 ± 3300	7500 ± 2000	8000 ± 2500

scribed (45, 47, 56), and the peptide concentrations were determined by quantitative amino acid 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 18 h post-infection and concentrated 80-fold using a Centriprep YM-30 concentrator (Millipore). The concentrated media were kept at −20 °C in 40% glycerol. The enzymatic activity of each protease was measured by its ability to cleave the fluorogenic substrate pERTKR-MCA (Peptide International). The substrate concentration added in the reaction was 5-fold the K_m value of each enzyme. In the rPC7-BTMD reaction, a final concentration of 350 μM 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 was 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 Ca^{2+}, 0.1 mM β-mercaptoethanol, and varying concentrations of pERTKR-MCA in a final volume of 100 μl. A 30-min preincubation of the enzyme in the solution mix was done before 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 (PerkinElmer Life Sciences) spectrofluorimeter.

Inhibition Assays (K_i Determination)—The K_i values (nM) of the various synthetic peptides were determined using Lineweaver-Burk plots. For each assay, six 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 K_i 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 Prosegments—The bacterial expression vector pET-24b(+) (Novagen) was cut at the 5′ and 3′ ends with *Xho*I and *Dra*III, respectively, to replace the stop codon by double stop codons after a His_6 insert using the pairs of oligonucleotides S1/AS1 (Table I). The cDNAs coding for the mPC5 prosegments (pPC5) were isolated by a three-step PCR using elongase (Invitrogen) 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′ *Nde*I/3′ *Not*I sites of the modified bacterial expression vector pET-24b(+), N-terminal to the hexahistidine tag. For protein production of these recombinants plasmids, they were transferred into host *Escherichia coli* strain called BL21(DE3) (Novagen). Protein expression was induced by the addition of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. The cells were then harvested by centrifugation at 5000 × *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 6 M guanidine·HCl, 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 14% 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 11675Da; 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 6–7 × 10^5 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 24 h of incubation 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 μg/ml; Epicentre). The cells were washed with phosphate-buffered saline) and lysed with radioimmune precipitation assay buffer as described (23). 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 (IC_{50})—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% bovine serum albumin (45). The fluorogenic substrate pERTKR-MCA was then added, and the released AMC was measured at different times over the course of 3 h. The reactions were performed at saturating conditions (5× K_m value) of the fluorogenic

substrate pERTKR-MCA. The K_m 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 IC_{50} 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-step 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 (23)) were produced using the primer pairs S4/AS12, S4/AS13, S4/AS14, S4/AS15, S4/AS16, and S10/AS21 (Table I), respectively. The cDNAs of the WT, R84A, and R116A ppPC5 were transferred into the *Eco*RI site of the pIRES2-EGFP vector. The other mutants (K111(H/I/L/P/V)) were cloned into 5′-*Sac*I/*Sac*II-3′ sites of the pIRES2-EGFP. HK293 cells (6.5×10^5 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 [^{35}S]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 α1-PDX. A total of 3 μg of DNA was transfected using LipofectAMINE 2000 (Invitrogen) for 0.75×10^6 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 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 fluorescence-activated cell sorter-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 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-Δpro, is not secreted from HK293 cells (Fig. 1*A*). 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 in the production of PC5-ΔC (65 kDa) (14), is not significantly affected in the R84A mutant since the PC5A/PC5A-ΔC ratio is relatively constant (Fig. 2). Because PC5A is capable of cleaving pro-VEGF-C,[2] the latter substrate was therefore used to estimate the cellular activity of PC5A and its prosegment mutants. The ability of the PC5A mutants (Fig. 1*A*), P4 T81R, P5 R80A, and P6 S79R, to process pro-VEGF-C at the ^{222}HSIIRR↓SL^{229} site (Fig. 1*B*) was tested in the Furin-deficient CHO-FD11 cells (58) (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 pro-VEGF-C, similar to gp160 (59). Extensive processing of pro-VEGF-C was observed when it was co-expressed with WT mPC5A (~86%) and its P6 S79R mutant (~76%; Fig. 3*B*). All other mutants either resulted in partial (R80A and T81R) or almost complete (R84A, R116A, and PC5-Δpro) loss of activity (Fig. 3). This indicates that P1 Arg of both primary and secondary prosegment cleavage sites is 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 pro-VEGF-C processing by PC5A.

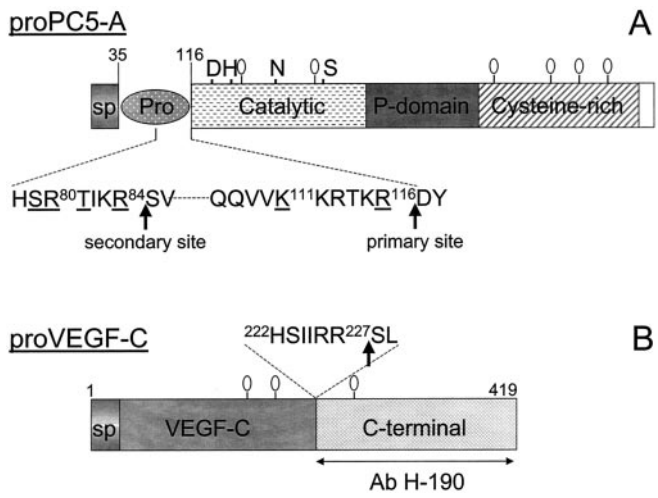

FIG. 1. **Schematic representation of full-length mouse PC5A and human pro-VEGF-C.** The structure of PC5A includes a signal peptide (*sp*) followed by a prosegment, a catalytic domain, and a P domain in addition to the C-terminal cysteine-rich domain. The selected *underlined* residues within the prosegment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

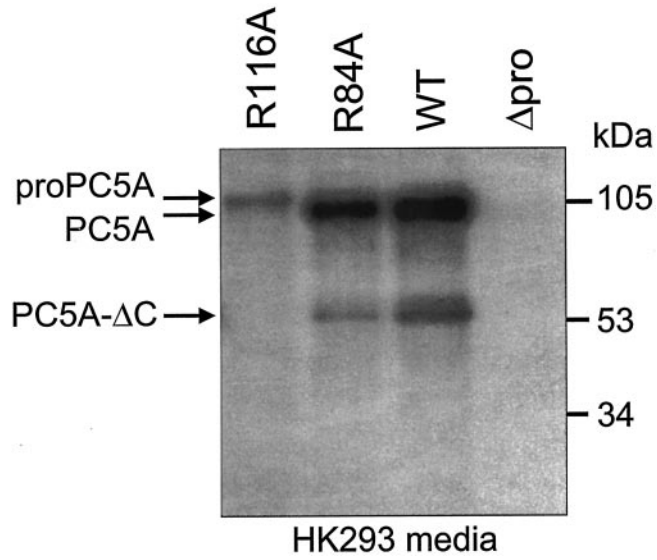

FIG. 2. **Biosynthetic analysis of PC5A and its primary and secondary site mutants.** HK293 cells were transfected either with a recombinant pIRES2 vector expressing PC5A-WT, -R84A, or -R116A or PC5A-Δpro. The cells were pulse-labeled for 4 h with [^{3}H]leucine. Cell lysates and media were immunoprecipitated with the anti-PC5-MAP antibody directed at the N terminus of the catalytic subunit (14), thus recognizing the ~115-kDa pro-PC5A, ~105-kDa mature PC5A, and the ~65-kDa PC5A-ΔC.

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 proregions of Furin and PC7 were potent inhibitors of these enzymes (45, 60). To determine whether similar peptides de-

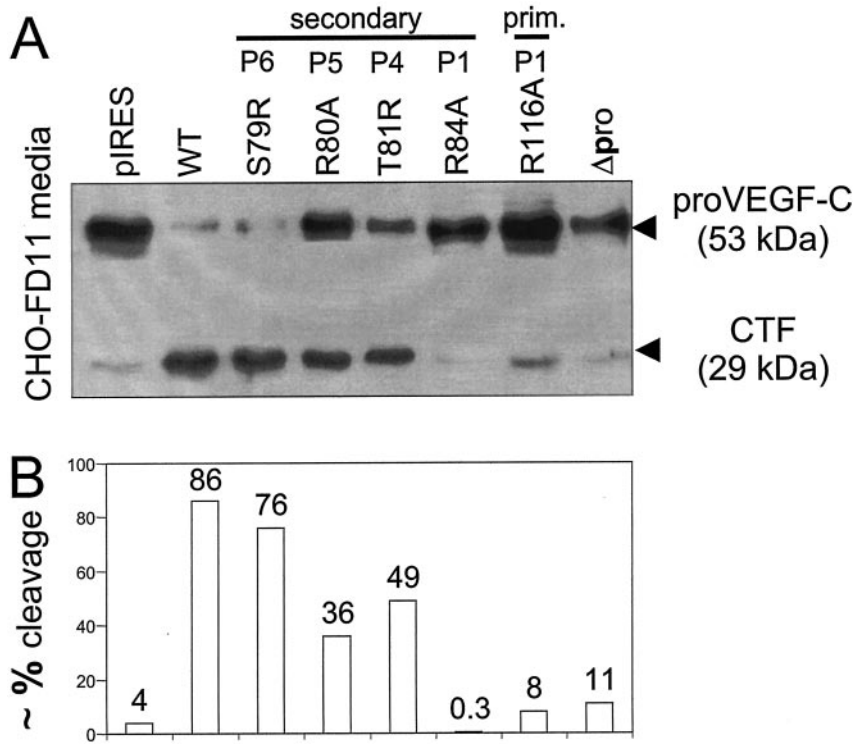

FIG. 3. **Processing of pro-VEGF-C by mPC5A and its mutants in CHO FD11 cells.** *A*, Western blot comparing the processing of stably expressed pro-VEGF-C by either the empty pIRES2-EGFP vector (*pIRES*) or its recombinants encoding mPC5A and mutants of its P1 (primary and secondary sites), secondary site P4, P5, and P6 (S79R, R80A, T81R, R84A, R116A), and Δpro. *B*, histogram representing the percent cleavage of pro-VEGF-C by mPC5A and its mutants as estimated by quantitative analysis (ImageQuant). The percentages were obtained from the ratio of VEGF-C/(pro-VEGF-C + VEGF-C).

rived 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_i) 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 ^{107}*QQ*VV*KK*R*TK*R^{116} is a very potent inhibitor of mPC5A and hPC5A, with K_i 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_i 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_i of ~7.5 μM. 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_i ~ 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

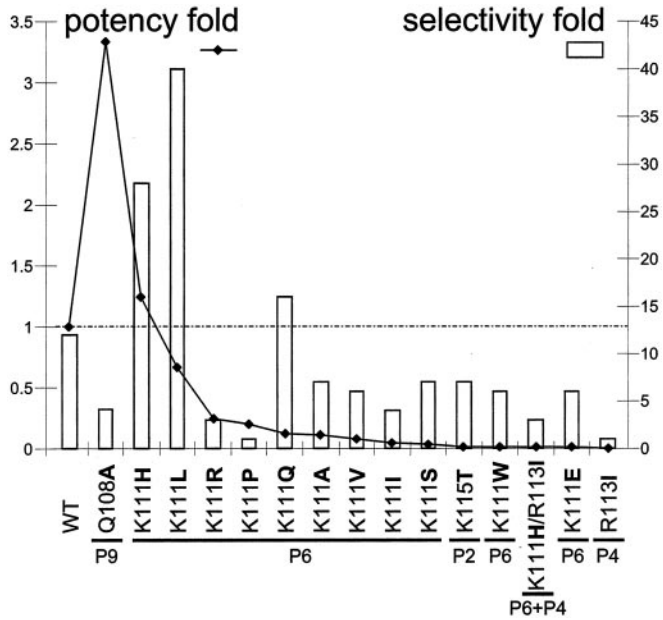

FIG. 4. **Graphical representation of the comparative inhibitory potency (on mPC5A; *line graph*) and selectivity (*versus* Furin; *histogram*) of each PC5-derived prosegment decapeptide (*see also* Table II).**

mouse and human PC5A, which exhibit an identical decapeptide 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 decapeptides, 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 K111V, 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,

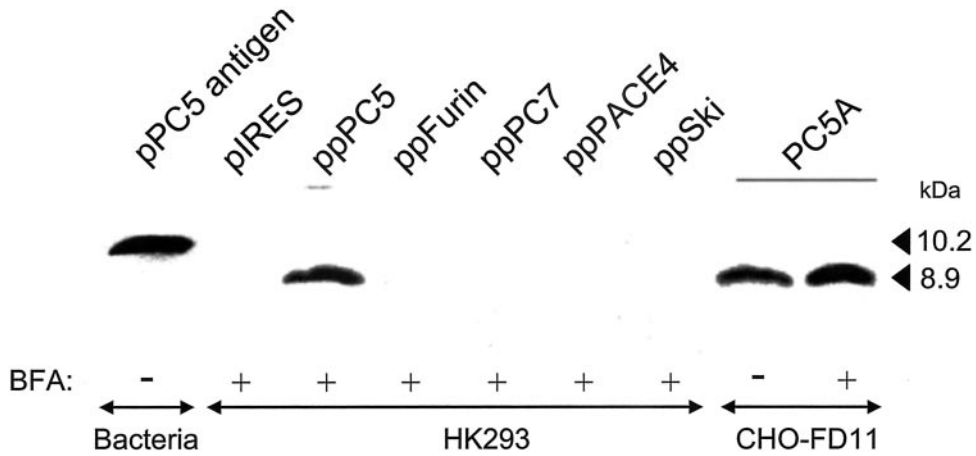

FIG. 5. **pPC5 polyclonal antibody specificity.** The polyclonal antibody generated was obtained against the purified, bacterially produced mouse pPC5. HK293 and CHO-FD11 cells were transiently transfected with different ppPCs or full-length PC5A, respectively. The next day the cells were incubated for 6 h 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) was compared with 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 hexahistidine tag (DYDLSHAAALEHHHHHH), explaining its slower migration (10.2 kDa) as compared with its cellularly derived form (8.9 kDa).

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 zymogen 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} ≤ 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} ~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} ~ 267 nM), whereas the R84A mutant is only 3-fold less potent (IC_{50} ~ 18 nM) than WT pPC5.

TABLE III
Inhibitory potency of pPC5s 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 was measured at different times over 3 h. The IC_{50} values were obtained using a GrapFit4 program. The values in parentheses represent the selectivity ratio with respect to mPC5A.

Prosegment	IC_{50}			
	mPC5A	hFurin	rPC7	yKexin
			nM	
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)

Biosynthetic 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. 6*A*, 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]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

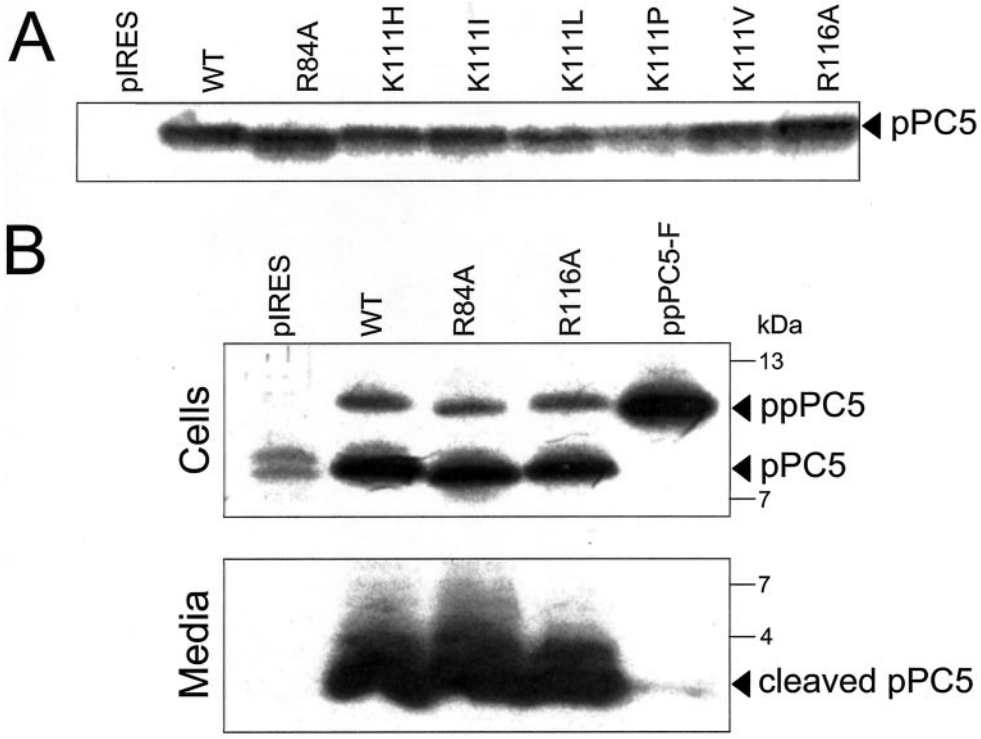

FIG. 6. **Biosynthetic analysis of ppPC5s.** *A*, HK293 cells were transfected with either the empty vector (*pIRES*) or the WT, R84A, K111H, K111I, K111L, K111P, K111V, or R116A ppPC5s. The cells were pulse-labeled for 6 h with [^{35}S]Met in the presence of BFA. Cell lysates were immunoprecipitated 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 [^{35}S]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.

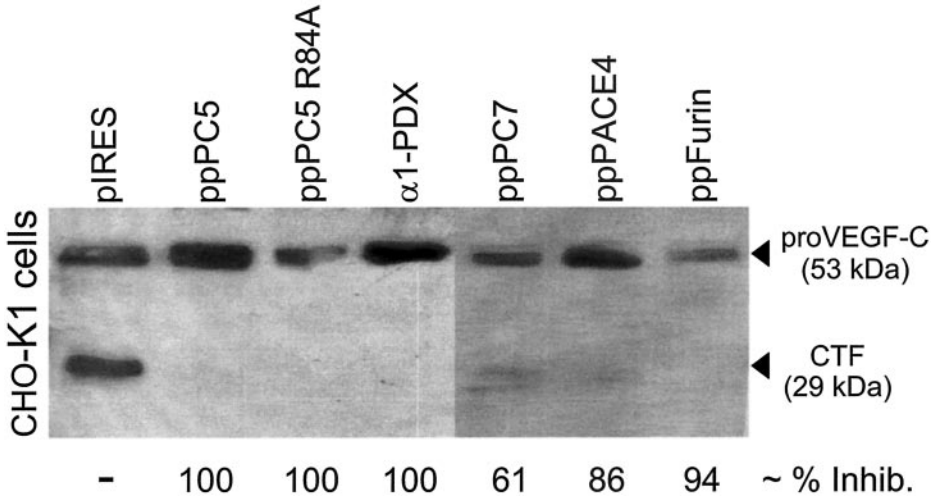

FIG. 7. **Inhibition of pro-VEGF-C processing in CHO-K1 cells.** Shown in a Western blot analysis of the parental CHO-K1 cells transiently co-expressing pro-VEGF-C and either empty vector (*pIRES*), WT ppPC5, R84A ppPC5, α1-PDX, ppPC7, ppPACE4, or ppFurin. The media were resolved by SDS-PAGE on a 12%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

revealed the secretion of a smaller fragment (~3 kDa) (Fig. 6*B*). 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 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 (23) 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 (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. 6*B*). 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 α1-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 with either mPC5A (FD11/VEGF-C/PC5A; Fig. 8*A*) or hFurin (FD11/VEGF-C/Furin; Fig. 8*B*). 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

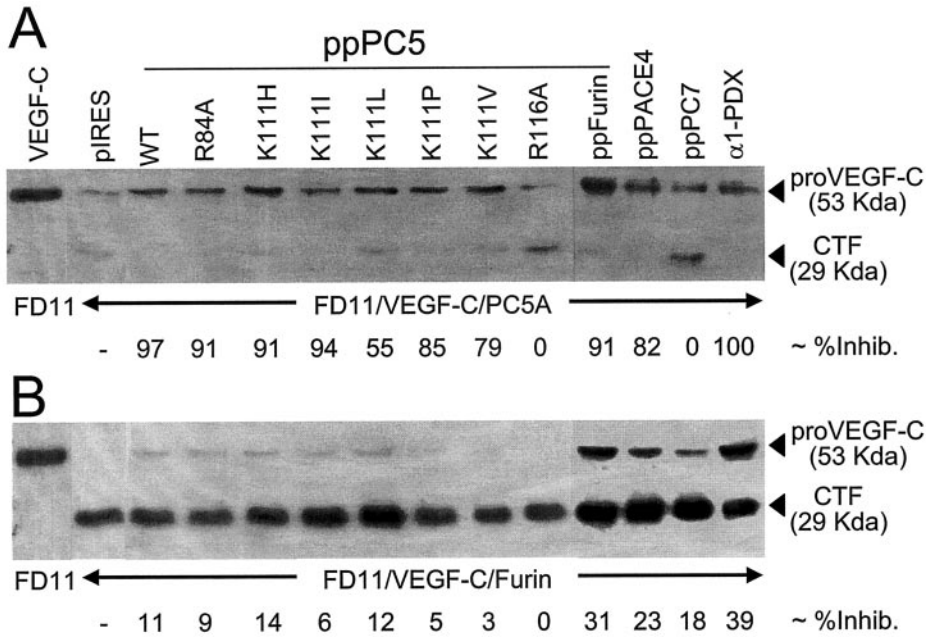

FIG. 8. **Inhibition of pro-VEGF-C processing in FD11 cells overexpressing PC5A or Furin.** Western blot analysis of the media of CHO-FD11 cells overexpressing VEFG-C and (*A*) FD11/VEGF-C/PC5A cells or (*B*) FD11/VEGF-C/Furin cells transiently transfected with either the empty vector (*pIRES*), WT, 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. *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 (Fig. 8*A*) or FD11/VEGF-C/Furin (Fig. 8*B*) cells. We used the CHO-FD11 cells since the data show that in these cells pro-VEGF-C is practically not processed. Stable co-expression of PC5A (Fig. 8*A*) or Furin (Fig. 8*B*) with pro-VEGF-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 α1-PDX and most prosegments tested were ≥80% inhibitory. Exceptions included ~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. 8*A*). In the FD11/VEGF-C/Furin cells, α1-PDX and ppFurin were ~31–39% inhibitory, whereas ppPACE4 (~23%), ppPC7 (~18%), and the ppPC5s (≤14%) were relatively weak inhibitors, and ppPC5-R116A was non-inhibitory (Fig. 8*B*). These data support the concept that most ppPC5s tested best inhibit PC5A as compared with 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 (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 Arg^{84} occupies the P1 position of the secondary processing site of pro-PC5A, within the sequence ^{79}SR-TIKR^{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 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 [^{3}H]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^{80} and P4 Thr^{81} 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^{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 (7), PC5A-Δpro 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 pPC5 is a potent *in vitro* inhibitor of PC5A and Furin, with K_i 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

[3] N. Nour and N. G. Seidah, manuscript in preparation.

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 because they are found in PC5A-specific substrates (16, 19, 21, 24), and a Q108A mutant was chosen because Gln 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, 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 IC_{50} 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 decapeptides (compare Tables II and III). The structure and/or additional interactions offered by the entire pPC5s as compared with 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 toward inhibition of cellular pro-VEGF-C processing by WT or mutant ppPC5s, ppPACE4, ppFurin, ppPC7, or α1-PDX. In native CHO-K1 cells, all tested ppPCs inhibit pro-VEGF-C processing (Fig. 7) except for ppPC5-R116A (data not shown). In the FD11/VEGF-C/PC5A cells, ppPC5s inhibit pro-VEGF-C processing by ≥80% except ppPC5-K111L (~55% inhibition) and ppPC7 (no inhibition; Fig. 8*A*). Interestingly, both ppPC5-K111L and ppPC7 (67) contain a P6 Leu, which may hinder their PC5-directed inhibitory properties. It was recently reported that *in vitro* pPC7 could inhibit PC5A with a K_i of 0.1 nM and was more selective toward PC5A than its cognate enzyme, PC7 (65). However, our previous (45) and present data do not agree with this conclusion. Indeed, although the PC5A-directed pro-VEGF-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. 8*A*). In FD11/VEGF-C/Furin cells, no significant inhibition of pro-VEGF-C processing by any of the prosegments was observed, and only ~30–40% inhibition was achieved by α1-PDX and ppFurin (Fig. 8*B*). 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, whereas the latter and ppPACE4 are similarly effective on lower endogenous Furin levels (compare Figs. 7 and 8*B*).

In conclusion, this work dealt with the zymogen processing of PC5A, and the data showed that although 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 although 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 (28, 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 convertase(s) (28).

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REFERENCES

1. Seidah, N. G., and Chretien, M. (1999) *Brain Res.* **848,** 45–62
2. Nakayama, K. (1997) *Biochem. J.* **327,** 625–635
3. Zhou, A., Webb, G., Zhu, X., and Steiner, D. F. (1999) *J. Biol. Chem.* **274,** 20745–20748
4. Seidah, N. G. (2002) in *Co- and Post-translational Proteolysis of Proteins* (Dalbey, R. E., and Sigman, D. S., eds) pp. 237–258, Academic Press, Inc, San Diego
5. Seidah, N. G., Mowla, S. J., Hamelin, J., Mamarbachi, A. M., Benjannet, S., Toure, B. B., Basak, A., Munzer, J. S., Marcinkiewicz, J., Zhong, M., Barale, J. C., Lazure, C., Murphy, R. A., Chretien, M., and Marcinkiewicz, M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 1321–1326
6. Sakai, J., Rawson, R. B., Espenshade, P. J., Cheng, D., Seegmiller, A. C., Goldstein, J. L., and Brown, M. S. (1998) *Mol. Cell* **2,** 505–514
7. Elagoz, A., Benjannet, S., Mammarbassi, A., Wickham, L., and Seidah, N. G. (2002) *J. Biol. Chem.* **277,** 11265–11275
8. Lusson, J., Vieau, D., Hamelin, J., Day, R., Chretien, M., and Seidah, N. G. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 6691–6695
9. Seidah, N. G., Chretien, M., and Day, R. (1994) *Biochimie* (*Paris*) **76,** 197–209
10. Beaubien, G., Schafer, M. K., Weihe, E., Dong, W., Chretien, M., Seidah, N. G., and Day, R. (1995) *Cell Tissue Res.* **279,** 539–549
11. Dong, W., Marcinkiewicz, M., Vieau, D., Chretien, M., Seidah, N. G., and Day, R. (1995) *J. Neurosci.* **15,** 1778–1796
12. Stawowy, P., Marcinkiewicz, J., Graf, K., Seidah, N., Chretien, M., Fleck, E., and Marcinkiewicz, M. (2001) *J. Histochem. Cytochem.* **49,** 323–332
13. Wong, B. S., Liu, S., Schultz, G. A., and Rancourt, D. E. (2002) *Mol. Reprod. Dev.* **61,** 453–459
14. De Bie, I., Marcinkiewicz, M., Malide, D., Lazure, C., Nakayama, K., Bendayan, M., and Seidah, N. G. (1996) *J. Cell Biol.* **135,** 1261–1275
15. Xiang, Y., Molloy, S. S., Thomas, L., and Thomas, G. (2000) *Mol. Biol. Cell* **11,** 1257–1273
16. Nachtigal, M. W., and Ingraham, H. A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 7711–7716
17. Mercure, C., Jutras, I., Day, R., Seidah, N. G., and Reudelhuber, T. L. (1996) *Hypertension* **28,** 840–846
18. Barbero, P., Rovere, C., De, B., I, Seidah, N., Beaudet, A., and Kitabgi, P. (1998) *J. Biol. Chem.* **273,** 25339–25346
19. Campan, M., Yoshizumi, M., Seidah, N. G., Lee, M. E., Bianchi, C., and Haber, E. (1996) *Biochemistry* **35,** 3797–3802
20. Cain, B. M., Vishnuvardhan, D., and Beinfeld, M. C. (2001) *Peptides* **22,** 1271–1277
21. Lissitzky, J. C., Luis, J., Munzer, J. S., Benjannet, S., Parat, F., Chretien, M., Marvaldi, J., and Seidah, N. G. (2000) *Biochem. J.* **346,** 133–138
22. Decroly, E., Wouters, S., Di Bello, C., Lazure, C., Ruysschaert, J. M., and Seidah, N. G. (1996) *J. Biol. Chem.* **271,** 30442–30450
23. Benjannet, S., Elagoz, A., Wickham, L., Mamarbachi, M., Munzer, J. S., Basak, A., Lazure, C., Cromlish, J. A., Sisodia, S., Checler, F., Chretien, M., and Seidah, N. G. (2001) *J. Biol. Chem.* **276,** 10879–10887
24. Ulloa, L., Creemers, J. W., Roy, S., Liu, S., Mason, J., and Tabibzadeh, S. (2001) *J. Biol. Chem.* **276,** 21387–21396
25. Krysan, D. J., Rockwell, N. C., and Fuller, R. S. (1999) *J. Biol. Chem.* **274,** 23229–23234
26. Tsuji, A., Hashimoto, E., Ikoma, T., Taniguchi, T., Mori, K., Nagahama, M., and Matsuda, Y. (1999) *J. Biochem.* (*Tokyo*) **126,** 591–603
27. Chretien, M., Mbikay, M., Gaspar, L., and Seidah, N. G. (1995) *Proc. Assoc. Am. Physicians* **107,** 47–66
28. Khatib, A. M., Siegfried, G., Chretien, M., Metrakos, P., and Seidah, N. G. (2002) *Am. J. Pathol.* **160,** 1921–1935
29. Garten, W., Hallenberger, S., Ortmann, D., Schafer, W., Vey, M., Angliker, H., Shaw, E., and Klenk, H. D. (1994) *Biochimie* (*Paris*) **76,** 217–225
30. Jean, F., Boudreault, A., Basak, A., Seidah, N. G., and Lazure, C. (1995) *J. Biol. Chem.* **270,** 19225–19231
31. Jean, F., Basak, A., DiMaio, J., Seidah, N. G., and Lazure, C. (1995) *Biochem. J.* **307,** 689–695
32. Angliker, H. (1995) *J. Med. Chem.* **38,** 4014–4018
33. Basak, A., Schmidt, C., Ismail, A. A., Seidah, N. G., Chretien, M., and Lazure,

C. (1995) *Int. J. Pept. Protein Res.* **46,** 228–237
34. Basak, A., Cooper, S., Roberge, A. G., Banik, U. K., Chretien, M., and Seidah, N. G. (1999) *Biochem. J.* **338,** 107–113
35. Van Rompaey, L., Ayoubi, T., Van, D., V, and Marynen, P. (1997) *Biochem. J.* **326,** 507–514
36. Anderson, E. D., Thomas, L., Hayflick, J. S., and Thomas, G. (1993) *J. Biol. Chem.* **268,** 24887–24891
37. Benjannet, S., Savaria, D., Laslop, A., Munzer, J. S., Chretien, M., Marcinkiewicz, M., and Seidah, N. G. (1997) *J. Biol. Chem.* **272,** 26210–26218
38. Jean, F., Stella, K., Thomas, L., Liu, G., Xiang, Y., Reason, A. J., and Thomas, G. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 7293–7298
39. Dahlen, J. R., Jean, F., Thomas, G., Foster, D. C., and Kisiel, W. (1998) *J. Biol. Chem.* **273,** 1851–1854
40. Lu, W., Zhang, W., Molloy, S. S., Thomas, G., Ryan, K., Chiang, Y., Anderson, S., and Laskowski, M., Jr. (1993) *J. Biol. Chem.* **268,** 14583–14585
41. Tsuji, A., Ikoma, T., Hashimoto, E., and Matsuda, Y. (2002) *Protein Eng.* **15,** 123–130
42. Fu, X., Inouye, M., and Shinde, U. (2000) *J. Biol. Chem.* **275,** 16871–16878
43. Lesage, G., Tremblay, M., Guimond, J., and Boileau, G. (2001) *FEBS Lett.* **508,** 332–336
44. Anderson, E. D., VanSlyke, J. K., Thulin, C. D., Jean, F., and Thomas, G. (1997) *EMBO J.* **16,** 1508–1518
45. Zhong, M., Munzer, J. S., Basak, A., Benjannet, S., Mowla, S. J., Decroly, E., Chretien, M., and Seidah, N. G. (1999) *J. Biol. Chem.* **274,** 33913–33920
46. Boudreault, A., Gauthier, D., and Lazure, C. (1998) *J. Biol. Chem.* **273,** 31574–31580
47. Toure, B. B., Munzer, J. S., Basak, A., Benjannet, S., Rochemont, J., Lazure, C., Chretien, M., and Seidah, N. G. (2000) *J. Biol. Chem.* **275,** 2349–2358
48. Nagahama, M., Taniguchi, T., Hashimoto, E., Imamaki, A., Mori, K., Tsuji, A., and Matsuda, Y. (1998) *FEBS Lett.* **434,** 155–159
49. Cameron, A., Appel, J., Houghten, R. A., and Lindberg, I. (2000) *J. Biol. Chem.* **275,** 36741–36749
50. Benjannet, S., Rondeau, N., Paquet, L., Boudreault, A., Lazure, C., Chretien, M., and Seidah, N. G. (1993) *Biochem. J.* **294,** 735–743
51. Muller, L., and Lindberg, I. (1999) *Prog. Nucleic Acid Res. Mol. Biol.* **63,** 69–108
52. Shinde, U., Li, Y., Chatterjee, S., and Inouye, M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 6924–6928
53. Yabuta, Y., Takagi, H., Inouye, M., and Shinde, U. (2001) *J. Biol. Chem.* **276,** 44427–44434
54. Anderson, E. D., Molloy, S. S., Jean, F., Fei, H., Shimamura, S., and Thomas, G. (2002) *J. Biol. Chem.* **277,** 12879–12890
55. Seidah, N. G., Day, R., Marcinkiewicz, M., and Chretien, M. (1998) *Ann. N. Y. Acad. Sci.* **839,** 9–24
56. Basak, A., Toure, B. B., Lazure, C., Mbikay, M., Chretien, M., and Seidah, N. G. (1999) *Biochem. J.* **343,** 29–37
57. Munzer, J. S., Basak, A., Zhong, M., Mamarbachi, A., Hamelin, J., Savaria, D., Lazure, C., Hendy, G. N., Benjannet, S., Chretien, M., and Seidah, N. G. (1997) *J. Biol. Chem.* **272,** 19672–19681
58. Gordon, V. M., Klimpel, K. R., Arora, N., Henderson, M. A., and Leppla, S. H. (1995) *Infect. Immun.* **63,** 82–87
59. Gu, M., Rappaport, J., and Leppla, S. H. (1995) *FEBS Lett.* **365,** 95–97
60. Bhattacharjya, S., Xu, P., Zhong, M., Chretien, M., Seidah, N. G., and Ni, F. (2000) *Biochemistry* **39,** 2868–2877
61. Dufour, E. K., Denault, J. B., Bissonnette, L., Hopkins, P. C., Lavigne, P., and Leduc, R. (2001) *J. Biol. Chem.* **276,** 38971–38979
62. Miranda, L., Wolf, J., Pichuantes, S., Duke, R., and Franzusoff, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 7695–7700
63. Lippincott-Schwartz, J., Yuan, L., Tipper, C., Amherdt, M., Orci, L., and Klausner, R. D. (1991) *Cell* **67,** 601–616
64. Taylor, N. A., Shennan, K. I., Cutler, D. F., and Docherty, K. (1997) *Biochem. J.* **321,** 367–373
65. Fugere, M., Limperis, P. C., Beaulieu-Audy, V., Gagnon, F., Lavigne, P., Klarskov, K., Leduc, R., and Day, R. (2002) *J. Biol. Chem.* **277,** 7648–7656
66. Tangrea, M. A., Bryan, P. N., Sari, N., and Orban, J. (2002) *J. Mol. Biol.* **320,** 801–812
67. Seidah, N. G., Hamelin, J., Mamarbachi, M., Dong, W., Tardos, H., Mbikay, M., Chretien, M., and Day, R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 3388–3393
68. Khatib, A. M., Siegfried, G., Prat, A., Luis, J., Chretien, M., Metrakos, P., and Seidah, N. G. (2001) *J. Biol. Chem.* **276,** 30686–30693
69. Bassi, D. E., Lopez, D. C., Mahloogi, H., Zucker, S., Thomas, G., and Klein-Szanto, A. J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 10326–10331

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