The Prosegments of Furin and PC7 as Potent Inhibitors of Proprotein Convertases

IN VITRO AND EX VIVO ASSESSMENT OF THEIR EFFICACY AND SELECTIVITY*

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All proprotein convertases (PCs) of the subtilisin/kexin family contain an N-terminal prosegment that is presumed to act both as an intramolecular chaperone and an inhibitor of its parent enzyme. In this work, we examined inhibition by purified, recombinant bacterial prosegments of furin and PC7 on the in vitro processing of either the fluorogenic peptide pERTKR-MCA or the human immunodeficiency virus envelope glycoprotein gp160. These propeptides are potent inhibitors that display measurable selectivity toward specific proprotein convertases. Small, synthetic decapeptides derived from the C termini of the prosegments are also potent inhibitors, albeit less so than the full-length proteins, and the C-terminal P1 arginine is essential for inhibition. The bacterial, recombinant prosegments were also used to generate specific antisera, allowing us to study the intracellular metabolic fate of the prosegments of furin and PC7 expressed via vaccinia virus constructs. These vaccinia virus recombinants, along with transient transfectants of the preprosegments of furin and PC7, efficiently inhibited the ex vivo processing of the neurotrophins nerve growth factor and brain-derived neurotrophic factor. Thus, we have demonstrated for the first time that PC prosegments, expressed ex vivo as independent domains, can act in trans to inhibit precursor maturation by intracellular PCs.

Limited proteolysis of proproteins is an archetypal mechanism responsible for the generation of diverse bioactive peptides and proteins from inactive precursors (1–3). Within the secretory pathway, these cleavages involve the processing of precursors at either single or paired basic residues (1, 2) or at specific hydrophobic and small residues (3). The recently characterized enzymes responsible for many of these intracellular conversions are calcium-dependent subtilisin-like serine proteases related either to the yeast kexin (1–5) or to the pyrolysin (6) subfamilies of subtilases (7). The mammalian kexin-like proteases, known as proprotein convertases (PCs), form a family comprising seven members: furin (PACE), PC1 (PC3), PC2, PC4, PACE4, PC5 (PC6), and PC7 (LPC, PC8) (1–5). These enzymes cleave precursor polypeptides at specific sites within the general motif (R/K)n – (X)n – (R/K)m, where n = 0, 2, 4, or 6, and X is any amino acid (aa) except Cys (1–5). The only mammalian pyrolysin-like enzyme known to date is SKI-1/SIP, which appears to recognize the motif RXXL(X,T) (6, 8).

The synthesis of most proteases as inactivezymogens provides cells with the means to regulate spatially and temporally their proteolytic activities (9), thereby minimizing the occurrence of premature enzymatic activity which could lead to inappropriate protein degradation. The inhibitory mechanism often involves the presence of an inactivating (pro)segment at the N terminus of the zymogen. In the case of bacterial subtilases,zymogen activation involves the autocatalytic excision of their prosegments, which are thought to act both as intramolecular chaperones (10) as well as specific inhibitors of the parent protease (10–13). The prosegments of mammalian PCs, which exhibit 30–67% sequence identity to each other (1, 3) and an absolute conservation of 8 aa (1), are autocatalytically processed in the endoplasmic reticulum (ER). With the exception of PC2 (1–4), this event is a prerequisite for the efficient egress of PCs from this compartment (14–17). The initial cleavage of the prodomain of PCs does not result in the immediate activation of the enzyme; rather the prosegment appears to remain tightly associated with the convertase until it reaches its final cellular destination. At this point, the increase in H+ and/or Ca2+ concentrations in the TGN or secretory granules triggers a secondary cleavage(s), resulting in the dissociation of the prosegment (12–14). Whereas PC1, PC2, furin, PACE4, and PC5 contain a secondary cleavage site KR or

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1 The abbreviations used are: PC, proprotein convertase; ER, endoplasmic reticulum; TGN, trans-Golgi network; pFurin, furin prosegment; ppFurin, furin preprosegment; ppPC7; PC7 prosegment; ppPC7, PC7 prosegment; BTMD, before transmembrane domain; VV, vaccinia virus; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; aa, amino acid(s); bp, base pair; gp, glycoprotein; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxyethyl)glycine]; pfu, plaque-forming units; HIV, human immunodeficiency virus; Fmoc, N-[9-fluorenyl]methoxycarbonyl; hfurin, human furin; POMC, pro-opiomelanocortin; AMC, 7-aminomethylcoumarin; MCA, methylcoumarinamide.
RR within their prosegments, PC4 and PC7 have only an RR site. In the latter cases, it is not yet known if cleavage at these sites occurs or is required for the effective activation of these enzymes (1, 3). Moreover, it remains to be determined whether the subsequent trimming of the C-terminal basic residues known to be mediated by specific carboxypeptidases (18) is required for full activation of these enzymes.

Furin and PC7, the major convertases of the constitutive secretory pathway (1–5, 19–22), process precursors either within the trans-Golgi network (TGN) or at the cell surface. As such, they mediate a wide range of processing events, which, in pathological situations, may exacerbate a disease state (23). Examples include the participation of furin and possibly PC7 in the processing of the viral surface glycoproteins gp160 of HIV (24–26) and glycoprotein of Ebola virus (27). Furthermore, at least furin has been implicated in the processing of toxins such as those of Aeromonas hydrophila (pore-forming proaerolysin) (28), anthrax (29), Pseudomonas, and diphtheria (30). Experiments in which the activation of these proteins has been prevented through the inhibition of furin and possibly other PCs indicate that there is considerable promise in these novel approaches to treating such pathologies.

Some of the previous attempts to inhibit the substrate processing activity of PCs ex vivo have included the use of irreversibly chloromethylketone inhibitors (31, 32) and reversible peptide inhibitors (33–35). Major limitations of these agents include either their cytotoxicity (through interfering with the biosynthesis of many important cellular proteins) and/or their relatively poor cellular permeability and targeting (31, 32). Alternatively, recombinant protein-based inhibitors have been developed (36–41). These strategies are based on the expression of proteins that contain a furin-like recognition sequence (RXXR) within the inhibitor binding region of either human α1-antitrypsin (36–38), α2-macroglobulin (39), proteinase-3 (40), or the turkey ovomucoid third domain (41). Although often reasonably effective, the inability of these recombinant proteins to inhibit selectively furin and not other PCs remains problematic (37, 38).

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

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Bacterial Recombinant hFurin and PC7 Prosegments**

The bacterial expression vector pET24b (+) (Novagen) was cut with 5′ NdeI and 3′ BamHI to remove the N-terminal T7 tag. It was then ligated with a linker composed of pre-anneled sense 5′ TACACATATG 3′ and antisense 5′ GATCCCATATG 3′ oligonucleotides (the underlined codon represents the initiator Met, which is followed in the recombinant vector by an Aas-Pro sequence). The cDNA for the N-terminal prosegments of human furin (aa 27–107 of hfurin (42), referred to as pFurin) and rat PC7 (aa 37–140 of rPC7 (20), referred to as pPC7), were isolated by a three-step PCR using Elongase (Life Technologies, Inc.) for 25 cycles, i.e., 94 °C for 25 s, 50 °C for 50 s, and 68 °C for 50 s. The hFurin and rPC7 cDNAs were used as templates (5′ GGATCCGAGGCTACTACGCTTGGC 3′ and antisense, which contains a hexa-His anti-coding sequence (5′ TCTGAGTCTGAGGTCGACGATG TGTTGAGGTCGACGATG TTGGTGGTTGGTGGTGGTGCCGTTAGTCCG 3′ and 5′ TCTGAGTCTGAGGCTACTACGCTTGGC 3′), respectively. These 277- and 346-bp cDNAs were cloned into the PCR 2.1 TA cloning vector for sequencing and then were transferred into the BamHI/XhoI sites of the above modified bacterial expression vector pET 24b. These recombinants were transformed into the Escherichia coli strain BL21 (DE3). Protein expression was induced with 1 mM isopropyl-β-D-thiogalactoside, after which the cultures were grown for 4 h at 37 °C. The cells were harvested by centrifugation at 4,000 × g. Since the prosegments were localized to the inclusion bodies, the cell pellets were sonicated on ice in binding buffer (20 mM Tris-HCl, 0.5 mM NaCl, 5 mM imidazole, 6 μM guanidine HCl, pH 7.9). The supernatant was applied to a 1 ml column containing a Ni2+-immobilized resin (Novagen), pre-equilibrated at room temperature with binding buffer. The column was washed with binding buffer containing 20 mM imidazole in order to eliminate weakly bound species. Elution was then carried out with the same buffer now containing 1 M imidazole. The eluant was dialyzed against 50 mM sodium acetate, pH 5.5, at 4 °C overnight. The prosegments were further purified with a Varian 5050/8010 instrument by reverse-phase high performance liquid chromatography using a 5-μm C4 column (0.94 × 25 cm; Vydac). After binding in an aqueous phase containing 0.1% trifluoroacetic acid, proteins were eluted at 2 ml/min with a 1% linear gradient (10–70% of 0.1% aqueous trifluoroacetic acid/CH3CN) (monitored at 210 nm). The purity, concentrations, and masses of the prosegments were determined by Coomassie staining of 15% Tricine/SDS-PAGE gels (Fig. 1), quantitative amino acid analysis, and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry.

**Synthesis of Prosegment-derived Peptide Derivatives**

All Fmoc protected amino acid derivatives (1-c-configuration), the coupling reagents, and the solvents were purchased from PE Biosystems Inc. (Framingham, MA). Calbiochem, and Richelieu Biotechnologies (Montréal, Québec, Canada). The furin-derived synthetic peptides are as follows: Fur-M1, 425QYHFWRFHVTTRKS(43); Fur-C24, 425HSLRFQVPQWLRQVKRRTK(43); Fur-C10, 425QQVKRRTKR(43); and Fur-C10A, 425QQVAKRRTKR107. The rPC7-derived synthetic peptides are as follows: PC7-C10, 425KHEAVRWHSEQRLLKRAKR140; PC7-C10, 425KHEAVRWHSEQRLLKRAKR140; PC7-C10, 425KHEAVRWHSEQRLLKRAKR140; and PC7-C18A, 425HEAVRWHSEQRLLKRAKR140, respectively. The PC7 N20, M18, and C18 peptides were synthesized with the C terminus in the amide form (CONH2), on a solid phase automated peptide synthesizer instrument (Pioneer), PE Biosystems, following the O-hexafluorophos- pho-[7-azabenzotriazol-1-yl-N,N,N′,N′-tetramethyluronium)/diisopro- pyl ethyl amine-mediated Fmoc chemistry. All syntheses were accomplished using an unloaded polyamino linker-polyethylene glycol resin. The following side chain-protecting groups were used: 2,2,4,6,7-pentamethyldihydrobenzofuran 5-sulfanyl for Arg; t-buty1 for Ser, Thr, Asp, and Tyr, and trityl for His, Asn, and Gln, respectively. The crude peptides were cleaved from the resin and fully deprotected by 3 h treatment with reagent B (trifluoroacetic acid/pheno1/water/triisopro- pyl silane = 88:5:5:2). The peptides were purified by reverse-phase high performance liquid chromatography using a semi-preparative C8-C18 C18 column (0.94 × 25 cm, Chromatography Specialty Co.), followed by further purification on a semipreparative C18 analytical column (0.46 × 25 cm, Beckman) following the conditions as described above.

**MALDI-TOF Mass Spectrometry**

High performance liquid chromatography fractions were mixed with one of two matrix solutions as follows: 3:5-dimethoxy-4-hydroxyni- namic acid (Aldrich) for prosegments, and α-cyano-4-hydroxyni- namic acid (Aldrich) for synthetic peptides. Spectra were obtained on a Voyager DE-Pro MALDI-TOF instrument (PE PerSeptive Biosystems).

**In Vitro Inhibition of gp160 Processing by Furin**

HIV-1 [125I]Met gp160 was affinity purified from CV-1 cells overexpressing VV.gp160 as described (33). For the inhibition assay, furin was first preincubated with increasing concentrations of pFurin or pPC7 for 10 min at 25 °C according to Decroy et al. (33), followed by the addition of 10,000 cpm of [125I]Met gp160 and an overnight incubation at 25 °C. The products were analyzed by SDS-PAGE as described (24, 33).

2 J. S. Munzer and N. G. Seidah, unpublished results.
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Enzymatic Activity Determination

Media of BSC40 cells infected with vaccinia virus recombinants of soluble PC7 (VV:pPC7-BTMD (21)), furin (VV:hfurin-BTMD (24)) (a generous gift from G. Thomas, Vollum Institute, Portland, OR), PC5 (VV:PC5A), PACE4 (VV:PACE4A), and the shed form of yeast kexin (VV:ykexin) (21, 24) were concentrated 50-fold and kept in 40% glycerol at −20 °C. Enzymatic activity was determined by the cleavage of the fluorogenic substrate PERTKR-MCA (Peptides International) (24). For each assay, 5–10 μl of enzyme that will cleave an equal amount of substrate was added to a solution containing 50 mM Tris-HCl, pH 7.0, 2 mM Ca²⁺, and 100 μM PERTKR-MCA in a final volume of 100 μl. Fluorescence was measured at 0, 30, 60, and 90 min using a model LS 50B (Perkin-Elmer) spectrofluorimeter (21).

Inhibition Studies

Stop-time Assay—Enzymes were preincubated for 15 min at room temperature with various concentrations of the prosegments (mixed with 0.1% BSA to avoid nonspecific binding of the dilute prosegments to the microtiter plate) or synthetic peptides. The fluorogenic pERTKR-MCA substrate was then added, and the released AMC was measured as above. Kᵢ values were derived from Dixon (43) and Cornish-Bowden (44) plots using substrate concentrations of 75, 100, and 200 μM for PC7, and 5, 25, and 50 μM for furin.

On-line Assay—Here we sequentially added to the microtiter plate the prosegment (at various concentrations), 100 μM pERTKR-MCA, and finally the buffered enzyme mixture (see above). Fluorescence was continually recorded over a 5- to 10-min period to follow the progress of inhibition (13).

Cellular Expression of Preprofurin and Prepro-PC7

The preprosegments of rFurin (ppPC7, 450-bp coding for aa 1–142) and furin (ppFurin, 351-bp coding for aa 1–109) were amplified for 25 cycles by PCR (94 °C for 25 s, 50 °C for 50 s, and 68 °C for 50 s using Elongase). The sense and antisense pairs of oligonucleotides for PC7 and furin that contain 5′ HinIII and 3′ BamHI sites were as follows: 5′-AAGCTTGAAGCCATGGAGCT-3′ and 5′-GGATCCTCTAGATGCTCGCTTGGCCCTCTT-3′, and 5′-AAGCTTGAAGCCATGGAGCT-3′ and 5′-GGATCCTCTAGATGCTCGCTTGGCCCTCTT-3′, and 5′-AAGCTTGAAGCCATGGAGCT-3′ and 5′-GGATCCTCTAGATGCTCGCTTGGCCCTCTT-3′. Note that in both sense oligonucleotides the initiator methionine codon is underlined and that in both antisense oligonucleotides we have introduced two tandem stop codons (underlined). The PCR products were cloned into the PCR 2.1 TA cloning vector for sequencing and then introduced two tandem stop codons (underlined). The PCR products were cloned into the PCR 2.1 TA cloning vector for sequencing and then ligated into the pcDNA3 vector (Invitrogen) for transient transfection and the PMJ602 vaccinia virus transfer vector (45) which led to the isolation of the recombinant VV:ppFurin and VV:ppPC7 virus stocks.

Prosegment Antibodies and Vaccinia Virus Expression

The purified, bacterially produced prosegments pFurin and pPC7 were treated with carboxypeptidase B to remove the C-terminal dibasic and hexa-his tag and then used to raise specific antisera in rabbits. For the cellular expression of the prosegments, 5 × 10⁶ BSC40 or AtT20 cells were infected for 2 h with 2 plaque-forming units (pfu)/cell of either VV:ppFurin or VV:ppPC7. Following overnight incubation in minimal essential medium without serum, the cells were extracted in 5M acetic acid, sonicated, and then applied to a SepPak (Waters) C18 cartridge.

Silencing of Proprotein Convertases by their Prosegments

Expression, purification, and characterization of the prosegments of human furin (pFurin) and rat PC7 (pPC7), we first needed to obtain large quantities of purified proproteins. Bacterial expression systems are a logical venue, especially since, based on their amino acid sequences, neither prosegment is expected to undergo post-translational modifications (e.g. N-glycosylation or Tyr-sulfation) of its backbone (20, 42). We therefore expressed these prosegments in BL21 cells, starting from their signal peptide cleavage sites and finishing with their primary autoactivation sites, along with an additional C-terminal hexa-His tag. Therefore the constructs are of the type: MDP... R(T,A)KR-(H)₆, where the additional N-terminal MDP and C-terminal hexa-His sequences are common to both proteins. Following a 4-h induction with isopropyl-1-thio-β-D-galactopyranoside, both prosegments were purified from cell pellet extracts using a Ni²⁺-affinity column (Fig. 1A). The typical yield observed for each prosegment is 7–8 mg/liter of bacterial culture. Note that the 90-aa pFurin construct runs faster on SDS-PAGE than the 113-aa pPC7 product. Further confirmation of the identity of the two polypeptides was obtained from analyses of their MALDI-TOF mass spectrometry spectra. pFurin and pPC7 have molecular masses of 10,735 Da (versus the calculated value of 10,737) and 12,348 Da (versus the calculated value of 12,356). These values are well within the expected experimental error of ±20 Da for this mass range.

The inhibitory characteristics of the furin and PC7 prosegments toward furin-mediated processing in vitro of HIV gp160 into gp120/gp41 (24, 25, 33) is shown in Fig. 2. At concentrations exceeding 12 nM pFurin, processing of gp160 by the quantity of furin used in this assay is almost completely inhibited (Fig. 2A). The low levels of gp77/gp53 (⁎) that are detected are due to further cleavage of gp120 by furin, as previously reported (49). The small amount of gp120 observed in control incubations (lane C, Fig. 2), which is equivalent to that seen with pFurin concentrations ≥25 nM, is due to its co-purification with gp160 on the lentil lectin affinity column (24, 33). The prosegment pPC7 (Fig. 2B) partially inhibits the furin-mediated cleavage of gp160 but only at concentrations exceeding 200–300 nM, indicating that it is a much less effective inhibitor of furin than pFurin. We did not test this reaction with PC7, since this enzyme does not cleave gp160 very efficiently in vitro (25).
Inhibition of Proprotein Convertases by Their Prosegments

**Specificity of pFurin and pPC7 and Their Derived Peptides: Importance of the C-terminal P1 Arg**—In order to assess the extent of prosegment inhibitory selectivities toward their cognate enzymes, we tested the inhibition of pFurin and pPC7 on the processing of the small fluorogenic substrate pERTKR-MCA. Five different soluble convertases were used as follows: human BTMD-furin, human PACE4-A, rat BTMD-PC7, murine PC5-A, and the shed form of yeast kexin (21, 24). Starting with equal amounts of pERTKR-MCA cleavage activity, the data (Fig. 3A) reveal that pFurin is a potent inhibitor of these convertases at the nanomolar level. The concentration of pFurin needed to inhibit 50% of each processing reaction (IC\textsubscript{50}) is presented in Table I, which depicts the average of five separate experiments. Unexpectedly, the observed order of inhibition for pFurin was PC5 > furin > PC7 > PACE4 > kexin. pFurin inhibits PC5-A (IC\textsubscript{50} = 0.4 nM) at 10-fold lower concentrations than furin (IC\textsubscript{50} = 4 nM). A similar experiment carried out with pPC7 (Fig. 3B, Table I) revealed a different rank order of preference as follows: PC7 > PC5 > PACE4 > furin > kexin. PC7 was inhibited at 50-fold lower concentrations of pPC7 than furin and PC5. Neither prosegment was very effective against PACE4 or kexin, which required between 300- and 2500-fold more peptide than the parent enzyme in order to achieve 50% inhibition. Therefore, pPC7 thus seems to be much more selective toward its parent enzyme than pFurin. Finally, from progress curves of on-line assays, we deduced that both proteins exhibit slow binding inhibition kinetics of their parent enzymes (not shown), in a manner similar to that reported for the prosegment of PC1 (13).

To define the regions of the prosegments that are essential for inhibition, a series of peptides was synthesized as indicated in Table II. Thus, the Fur-M15, PC7-M18 peptides which are derived from the middle portion of the prosegments and the N-terminal PC7-N20 peptide are very poor inhibitors (IC\textsubscript{50} > 50,000 nM). It is interesting to note that although Fur-M15 encompasses the secondary cleavage site of furin (11), it is a very poor inhibitor (Table II) and is not a substrate (not shown), possibly because of the absence of extended P' residues. In contrast, the PC7 C-terminally derived C-24, C-18, and C-10 synthetic peptides (K\textsubscript{i} values ~5–7 nM and IC\textsubscript{50} values ~20–28 nM) and the furin-derived C-24 and C-10 peptides (K\textsubscript{i} values ~35–40 nM and IC\textsubscript{50} values ~150 nM) arc relatively good inhibitors of their parent enzymes (Table II). In both cases, peptides as small as 10 aa (C-10) can still potently inhibit their cognate enzymes, albeit at ~50-fold higher concentrations than their respective full-length prosegments. Kinetic analyses using Dixon plots (43, 50) revealed that all of the C-terminal synthetic peptides tested act as purely competitive inhibitors of their parent enzyme (not shown). Finally, mutation of the C-terminal Arg to Ala in Fur-C10A or PC7-C18A almost completely abolished the inhibitory activity (Table II). This demonstrates the critical importance of the C-terminal P1 Arg for the inhibitory function. In agreement, carboxypeptidase B digestion of the full-length prosegments, which removed the C-terminal LysArg-(H)\textsubscript{6} sequence as demonstrated by MALDI-TOF spectrometry, also caused a more than 2,500-fold decrease in the inhibitory activity of these proteins (not shown).

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

**Cellular Overexpression of the Prosegments of Furin and PC7 and Their ex Vivo Inhibitory Properties**—BSC40 and AtT20
cells were infected with recombinant vaccinia viruses expressing the prosegments of furin (VV:ppFurin) or PC7 (VV:ppPC7) which are extended by two amino acids past the primary cleavage sites. The cell lysates were analyzed by Western blotting using the specific rabbit polyclonal antisera raised against bacterially produced pFurin and pPC7 (see "Experimental Procedures"). As shown in Fig. 5, Western blot analyses of these prosegments revealed that each antiserum is quite specific for its original antigen such that no cross-reactivity is observed. Although both prosegments are well expressed in both cell lines, ppFurin exhibits two major immunoreactive forms migrating with apparent molecular masses of 12.5 and 10 kDa (Fig. 5A), with the latter migrating slightly faster than the bacterially produced product that contains an additional 9 aa (MDP and (H)6) (not shown). This −2.5-kDa difference in apparent molecular masses agrees well with the expected theoretical mass difference between ppFurin and pFurin (2,778 Da), suggesting that the 10-kDa protein is pFurin. Thus, we deduced that the upper band could be ppFurin that still contains the N-terminal signal peptide. Accordingly, and in contrast to the full-length enzyme, overexpression of the ppFurin domain by itself results in a delayed excision of its signal peptide, especially in BSC40 cells. A similarly delayed signal peptide removal has already been demonstrated using baculovirus overexpression of prepro-PC1 in Sf9 cells (51). Although not shown, when BSC40 cells were pulse-labeled with [3H]Leu for 10 min and then chased in absence of radiolabel, our data revealed that it took about 1 h to completely transform ppFurin into pFurin.3 In the case of PC7, only a very low level of an 15.5-kDa protein can be detected in BSC40 cells, and mostly an 11-kDa product is observed in both cell types (Fig. 5B).

An inhibition of proprotein convertases by their prosegments

The IC50 values were obtained as described under "Experimental Procedures" from nonlinear curve fitting (SigmaPlot software) using a general equation for competitive enzyme inhibition. The K values are representative of triplicate Dixon plots.

### Table II

| Inhibitor | Peptide sequence | Enzyme | Furin IC50 (nM) | PC7 IC50 (nM) | PC7 K (nM) |
|-----------|-----------------|--------|----------------|---------------|------------|
| hFur-FL*  | hFur-M15        |       | 4              | -50,000       | -          |
| hFur-C10  | hFur-C24        |       | -              | 40            | 500        |
| hFur-C10 (R107A) |          |       | -              | 35            | 90         |
| rPC7-FL*  | rPC7-N20        |       | -              | 100,000       | -          |
| rPC7-M18  | rPC7-C10        |       | 28             | 81            | 6          |
| rPC7-C18  | rPC7-C24        |       | 23             | 65            | 7          |
| rPC7-C10 (R107A) |          |       | -              | 100,000       | -          |

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

- indicates that this value was not determined.

3 S. Benjannet and N. G. Seidah, manuscript in preparation.
The cells were then pulse-labeled with [35S]Met for 4 h and infected with either VV:POMC (antigen control) or co-infected with ppPC7. The cells were then pulse-labeled with [35S]Met for 4 h and the medium immunoprecipitated with an NGF antiserum. The migration positions of the 35-kDa pro-NGF and the 13.5-kDa NGF are shown.

**DISCUSSION**

The ability of prosegments to inhibit their parent enzymes is a well established phenomenon (9, 10). Among the best studied prosegments of serine proteinases are those of the bacterial subtilases, in particular those of subtilisin E (10, 53) and a-lytic protease (54). In addition to being essential for the proper folding of these enzymes during synthesis, the prosegments are powerful, specific inhibitors with Ki values typically in the nanomolar range (10, 55). These observations appear to hold true also for members of the eukaryotic kexin family of subtilases. Studies of the prosegment of yeast kexin-like krp1 from *Schizosaccharomyces pombe* (14) confirm that it plays a critical role in the folding of the nascentzymogen during synthesis.

![Image](image1.png)

**FIG. 6. Inhibition of pro-NGF processing.** Rat Schwann cells were infected with either VV:POMC (antigen control) or co-infected with VV:NGF and the pro-NGF into the 13.5-kDa NGF is apparent when rat Schwann cells are co-infected with VV:NGF and the control vector VV:POMC. This predominantly furin-mediated processing (47) is inhibited by co-expression of the serpin a1-PDX, as previously reported (36). It is also apparent that overexpression of ppFurin results in a similar inhibition of pro-NGF to NGF processing and that ppPC7 only partially blocks this reaction. Since PC7 does not effectively process pro-NGF, the latter result suggests that overexpressed ppPC7 may partially inhibit furin activity.

The differential ability of overexpressed ppFurin and ppPC7 to inhibit the processing of pro-BDNF was next investigated in COS-1 cells, wherein this neurotrophin precursor is best cleaved by PC1 and furin (52). In this experiment we further compared the inhibitory potency of ppFurin and ppPC7 by transient transfection. The vector pcDNA3 containing sense (S)-oriented cDNAs, as well as negative controls carrying these cDNAs in an antisense (AS) orientation, were tested. Thus, Western blot analyses of the proteins in conditioned media using one of the commercial BDNF antibodies (Santa Cruz Biotechnology) revealed that only the expression of the sense ppFurin construct results in an effective inhibition of the processing of the 32-kDa pro-BDNF into the 14-kDa BDNF (Fig. 7). Again, ppPC7 does not inhibit this processing, suggesting that its overexpression does not affect endogenous furin. The antisense controls do not exhibit significant inhibition of pro-BDNF processing.

Subsequent to an autocatalytic intramolecular cleavage at the prosegment-catalytic domain junction, the prosegment remains noncovalently attached to the enzyme, serving as a potent autoinhibitor of its activity (9, 10, 14). In eukaryotes, as this complex progresses along the secretory pathway, additional events mediate the degradation and dissociation of the prosegment, leading to a fully activated enzyme (11). For kexin, both prosegment cleavage and enzyme activation occur within the ER (56). Except in the case of PC2, prosegment cleavage of all other members of the mammalian PC family occurs within the ER. Enzyme activation, however, occurs later in the secretory pathway, typically in the TGN or in immature secretory granules (1–5, 12).

Other studies (9) have characterized the nature of prosegment inhibition in *vitro*. With respect to the mammalian PCs, there have been several studies involving prosegment inhibition in *vitro*. Anderson et al. (12) showed that a bacterially expressed furin prosegment potently (Ki, ~14 nM) inhibited this enzyme in *trans* at an equimolar ratio. A more detailed kinetic study (13) confirmed that, similar to subtilisins, an extended prosegment of PC1 purified from SF9 insect cells acted as a slow, tight-binding inhibitor of both PC1 and furin but not of PC2. In a preliminary study, Basak et al. (57), using synthetic peptides based on the prosegment of PC1, observed differential inhibition of PC1 and furin. Moreover, the authors established that the region most effective for inhibition resides within the C-terminal 34 amino acids of the PC1-prosegment, with the C-terminal dibasic Lys-Arg residues being critical.

The data presented here on the inhibition of PCs by prosegments in *vitro* confirm and extend previous observations. Using full-length prosegments of furin (pFurin) and PC7 (pPC7) purified from bacterial lysates, we demonstrate differential inhibition of the activity (hydrolysis of the synthetic fluorogenic peptide pERTKR-MCA) of five distinct soluble PC preparations (Fig. 3). As expected, pPC7 is most inhibitory toward its parent enzyme (Table 1). These findings suggest that the structural conformation of pPC7 imparts considerable specificity to this polypeptide as a PC inhibitor. As seen in Fig. 3, this selectivity is powerful enough to inhibit ~90% of the activity of PC7 but only 10% of the activity of furin, laying the foundation for the possibility of developing even more specific inhibitors.

Identical experiments using pFurin reveal that it is less effective against PC7, PACE4, and kexin than against its parent enzyme. This selectivity also holds true for a large, biological substrate. Thus, the processing in *vitro* of HIV gp160 by furin (Fig. 2) is only partially inhibited by 300 nM pFurin, whereas 25 nM pFurin causes full inhibition (a difference of at least 10-fold). Surprisingly, pFurin inhibits PC5 approximately 10-fold better (IC50 value) than furin (Table 1). Previous cellular expression studies have demonstrated that both furin and PC5 can effect similar processing of the precursors of Mullerian

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*S. Benjannet and N. G. Seidah, unpublished results.*
inhibiting substance in testicular Sertoli cells (58), the receptor PTP$_{\mu}$ in endothelial cells (59), and gp160 in LoVo cells (49).

Also, in vitro data regarding the processing of gp160 (24), the $\alpha_5$, $\alpha_e$, and $\alpha_i$ integrin chains$^6$ inhibition by the serpin $\alpha$-PDX (38), and $K_v$ values for the fluorogenic peptide substrate pERTKR-MCA$^7$ all suggest similar and probably overlapping substrate cleavage abilities of furin and PC5. Moreover, homology alignments of the prosegment of furin with that of other PCs show that it is 58% identical to that of PC5 (3). Taken together, these findings argue considerable functional and probably structural similarity (within the catalytic subsites) between furin and PC5. In contrast with active enzyme in these experiments. We also point out, however, that the rank order of inhibition using synthetic peptides derived from pPC7 (Fig. 4) and pFurin (not shown) is different from that of the full-length prosegments (Table I), suggesting that the latter contain additional structural determinants modulating PC inhibition.

The peptide truncation series (Table II) shows clearly that most of the inhibitory potency of pFurin and pPC7 resides in the 10 aa preceding the prosegment primary cleavage site. According to NMR analyses of pPC7 peptides$^8$ and secondary structure predictions of pFurin (60), these residues, with the exception of the last pair of basic residues, are part of an amphiphilic $\alpha$-helix. This combination of a common structure and conserved aa within this C-terminal region of the prosegments probably explains their similar nanomolar inhibitory potencies. Although these observations may explain minor discrepancies in the selectivity of inhibition, major ones (e.g. pPC7 inhibition of kexin) presumably must take into account differences in enzyme structure. Finally, P1 Arg to Ala mutants demonstrated that this C-terminal residue is crucial for the inhibitory potency of these peptides (Table II). A similar finding was also confirmed using carboxypeptidase B-digested peptides (not shown). These effects of carboxypeptidases are reminiscent of the overall decrease in endopeptidase activity in fat/fat mice lacking carboxypeptidase E (61), suggesting that the removal of C-terminal basic residues is essential for maximal convertase activity.

Although peptides derived from other regions of pFurin or pPC7 were ineffective as inhibitors, they nonetheless appear to play some role in the inhibitory potency of the full-length prosegment. For example, comparing the IC$_{50}$ values of the full-length prosegments with those of the C-24 peptides reveals a difference of 40–70-fold. Also, removal of the N-terminal 17 aa of pPC7 resulted in a substantial decrease in the inhibitory potency of this polypeptide (not shown). These effects of carboxypeptidases are reminiscent of the overall decrease in endopeptidase activity in fat/fat mice lacking carboxypeptidase E (61), suggesting that the removal of C-terminal basic residues is essential for maximal convertase activity.

We next turned our attention to the question of whether the prosegments, expressed as independent domains, can act in trans to inhibit intracellular furin and PC7. Successful inhibition would require not only that these polypeptides enter the secretory pathway but also that they remain there long enough to interact with the mature target PC (i.e. most likely within the TGN). Current evidence suggests that a polypeptide must have a minimum size of at least 50 aa in order to be recognized by the signal recognition particle and threaded through the membrane of the ER (62, 63). In agreement with this hypothesis, a 64-aa long prepropeptide derived from frog skin is the smallest known to date (64). Including their signal peptides, the propresequences of furin (ppFurin) and PC7 (ppPC7) contain 107 and 140 aa, respectively (1). To test whether these propresequences can independently enter the secretory pathway, we produced antisera specific for each of the propresequences (Fig. 5). Overexpression of either ppFurin or ppPC7 using vaccinia virus infection of BSC40 or AT202 cells revealed that the former loses its signal peptide very slowly (Fig. 5) and that only ppPC7 is secreted into the medium (not shown). We interpreted these data to mean that the independently expressed propresequences were reasonably stable within the secretory pathway and, at least in the case of PC7, were able to pass through it intact. In order to test the ex vivo inhibitory function of these polypeptides, we examined the processing of pro-NGF to NGF in Schwann cells infected with wild-type or prosegments vaccinia virus constructs. As seen in Fig. 6, ppFurin significantly inhibits the maturation of this neurotrophin, which is known to occur in the TGN and be best carried out by furin (47). In fact, it is nearly as effective as the serpin $\alpha$-PDX, which was included as a positive control, having been shown previously to be a potent inhibitor of the pro-NGF to NGF conversion (36). In contrast, ppPC7 has only a slight inhibitory effect (Fig. 6). Although PC7 is expressed in Schwann cells (46), it is a poor effector of pro-NGF maturation.$^5$ Thus, the mild inhibition seen in this experiment is most likely due to cross-reactivity between pPC7 and furin (see Table I).

The processing of pro-BDNF to BDNF represents another well characterized neurotrophin maturation event on which to test the inhibitory potency of our propresequence constructs (50). In these experiments, cellular co-expression of ppFurin and pro-BDNF via transient transfection in COS-1 cells completely inhibits the production of the 14-kDa BDNF (Fig. 7). Transfection with the ppPC7 polypeptide was noticeably less effective, again demonstrating the selective nature of these propresequences as ex vivo inhibitors. Even though PC7 is expressed in COS-1 cells (20), it is a poor effector of pro-BDNF maturation in this system.$^5$ Thus, we presume that the inhibition seen in this experiment is also due to cross-reactivity between pPC7 and furin. Interestingly, the antisense propresegment constructs employed as controls in this experiment were not significantly inhibitory. This is of interest in view of the reported use of antisense full-length furin for the down-regulation of its expression (65). We conclude from these data that the PC propresequences tested here selectively inhibit the maturation of pro-BDNF by furin-like enzymes. These events likely occur in the TGN, where the processing of neurotrophins in constitutively secreting cells has been localized (47, 52).

In conclusion, this work on the inhibitory properties of PC propresequences provides the first evidence that these polypeptides can be used to inhibit the cellular processing of precursors ex vivo. This technology represents a novel enzyme silencing strategy that will enhance our understanding of the basic cellular functions of these proteases. Future work will involve improving the performance of these propresequences using site-directed mutagenesis, leading to the design of more selective and powerful convertase inhibitors that may provide novel

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$^6$ J.-C. Lissitzky, J. Luis, J. S. Munzer, S. Benjannet, F. Parat, M. Chrétien, J. Marraldi, and N. G. Seidah, submitted for publication.

$^7$ S. Munzer and N. G. Seidah, unpublished results.

$^8$ S. Bhattacharjya, P. Xu, M. Zhong, M. Chrétien, N. G. Seidah, and F. Ni, in preparation.
approaches to the treatment of a variety of pathologies including proliferative, microbial, and viral diseases (23).

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