Inhibitory Potency and Specificity of Subtilase-like Pro-protein Convertase (SPC) Prodomains*

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The SPCs (subtilisin-like pro-protein convertases) are a family of enzymes responsible for the proteolytic processing of numerous precursor proteins of the constitutive and regulated secretory pathways. SPCs are themselves synthesized as inactive zymogens. Activation of SPCs occurs via the intramolecular autocatalytic removal of the prodomain. SPC prodomains have been proposed as templates in the development of potent and specific SPC inhibitors. In this study, we investigated the specificity and potency of complete prodomains and short C-terminal prodomain peptides of each SPC on highly purified, soluble enzyme preparations of human SPC1, SPC6, and SPC7. Progress curve kinetic analysis of prodomain peptides and complete prodomains showed competitive inhibitory profiles in the low nanomolar range. Complete prodomains were 5–100 times more potent than C-terminal prodomain peptides, suggesting that N-terminal determinants are involved in the recognition process. However, complete prodomains and prodomain peptides exhibit only a partial specificity toward their cognate enzyme. Ala-scan structure activity studies indicated the importance of basic residues in the P4, P5, and P6 positions for inhibition of SPC1. In contrast, hydrophobic residues in P8 and P7, as well as basic residues in P4 and P5, were critical for inhibition of SPC7. Our data demonstrated that the use of prodomains as specific inhibitors acting in trans would be of limited usefulness, unless modified into more specific compounds.

Proteolytic processing is a post-translational modification by which a cell diversifies and controls the protein products of its genes. In mammalian species, endoproteolytic activation of many secretory protein precursors is carried out by the SPCs1 (for a review see Ref. 1). The catalytic domains of the SPCs have a high structural similarity to the bacterial subtilisins and yeast kexin (2). The SPC family of enzymes consists of seven distinct members named, using the unified nomenclature of Chan et al. (3), SPC1 (furin/PACE), SPC2 (PC2), SPC3 (PC1/PC3), SPC4 (PACE4), SPC5 (PC4), SPC6 (PC5/PC6), and SPC7 (LPC/PC7/PC8). Each SPC contains at least five well conserved domains: 1) an N-terminal signal peptide, responsible for directing proteins into the secretory pathway; 2) a prodomain acting as a putative intramolecular chaperone for facilitated transportation, folding, and regulation of enzymatic activity (4–7); 3) a catalytic domain responsible for substrate-specific interactions and cleavage; 4) a P-domain with a conserved RGD motif essential for enzyme structural cohesion (8) and activity and that also regulates stability, calcium, and pH dependence (9, 10); and finally 5) a C-terminal-specific domain that contains membrane attachment sequences, Cys-rich regions, and intracellular sorting signals (1, 11).

The substrate cleavage specificity of SPCs is recognized to be C-terminal to either single or paired basic residues, with the Lys-Arg motif being the most common. The substrate specificity of SPC1 has been thoroughly studied, with a minimal recognition sequence for catalysis being RXRX. In general, it is evident that SPCs favor the presence of basic residues at subsites P1, P2, and, in many cases, P4 for efficient catalysis. The presence of an Arg residue at P4 is not mandatory for the activity of all SPCs but various studies indicate that the presence of an Arg residue in P2, P4, or even P6 enhances cleavage. The apparent close similarity of cleavage specificity between SPCs leads to the notion of possible redundant processing functions. Indeed, different SPCs have been shown to process various precursors at the same cleavage sites. Extensive mapping studies have demonstrated the frequent occurrence of overlapping cellular SPC expression patterns (12–16). Knock-out studies in mice have also provided support for some level of redundant functions, depending on the precursor studied (17).

SPCs are involved in many important biological processes, including zymogen activation (6, 7), peptide hormone processing (18–21), complement activation (22), clot formation and lysis (23), angiogenesis (24), and tissue remodeling (25, 26). These proteases have also been implicated in a number of pathophysiologicals, thus raising the possibility that SPC inhibitors may become useful therapeutic agents (1, 27–29). However, the use of SPC inhibitors as pharmacological agents is highly dependent on a clear understanding of their redundant/distinct functions. Furthermore, the development of highly potent and specific SPC inhibitors requires a better knowledge of the molecular determinants of catalytic activity that distinguish each member of the SPC family.

Endogenous inhibitors are often a good starting point in the development of pharmacological compounds. With regards to...
the SPCs, 7B2 CT (7B2 C-terminal) peptide and proSAAS are the only two endogenous inhibitors identified (30, 31) as they specifically inhibit SPC2 and SPC3, respectively. However, regulation of enzymatic activity can also be carried out by intramolecular mechanisms. Indeed, SPCs, like many other proteinases, are synthesized aszymogens (32). The inhibitory mechanism often involves the presence of a prodomain, whose function is to prevent premature enzymatic activity (6). Each SPC contains a distinct prodomain that acts in cis to regulate that enzyme’s activity. Based on the fact that SPC prodomains exhibit low levels of overall homology to each other, it has been suggested that they could be used in trans as potent and specific inhibitors of their cognate enzymes (33). Some evidence in support of the high inhibitory potency of prodomains has been provided. However, their degree of specificity remains somewhat unclear. The present study is an extensive comparative analysis of the inhibitory characteristics of SPC prodomains to address the issue of specificity. Our studies reveal that prodomains are potent inhibitors of SPCs in the low nanomolar range, that inhibition is highly dependent on the C-terminal structure of each prodomain, but also that some N-terminal elements within the prodomains are required for maximal inhibition. However, our results show that prodomains are not highly specific inhibitors of their cognate SPCs and that further structural modifications will be required to achieve higher levels of specificity.

MATERIALS AND METHODS

Materials—The fluorogenic substrate pyro-Glu-Arg-Thr-Lys-Arg-methylcoumaryl-7-amide (pERTKR-MCA) and the enzyme titrate deacetyl-Arg-Val-Lys-Arg-CH$_2$ (dec-RVKR-CH$_2$) were obtained from Bachem Biosciences. Production of specific polyclonal antibodies to hSPC1 and hSPC6 against peptides corresponding to sequences located N-terminally (for hSPC7: SVHFNDKPYQQWHLNRRRS; hSPC6: DVYDFSRAQSTYFNDPKW) and C-terminally (hSPC7: PGLKIEPEDIYTTFNLKT) were prepared by the Sheldon Biotechnology Center (McGill, Montreal, Quebec, Canada). The hSPC1 antibody raised against a peptide encompassing amino acids 187–198 of hSPC1 has been previously described (34). Short and long propeptides corresponded to the C-terminal region of prodomains as well as Ala-substituted peptides were also synthesized by the Sheldon Biotechnology Center (McGill).

Plasmids—Human SPC1 cDNA was truncated at position 714 (hSPC1Δ), so that the 86-kDa recombinant protein is lacking the C-terminal and transmembrane region of native human SPC1 (34). The 2.6-kb hSPC7 fragment corresponding to sequences located N-terminally (for hSPC7: PGLKIEPEDIYTTFNLKT) was prepared by the Sheldon Biotechnology Center (McGill). The expression vector dec-RVKR-CH$_2$ (35) was used for transfection in transient expression assays. The expression vector pC5.1V5-HisA (Invitrogen) was previously described (34). The constructs were all verified by DNA sequencing.

Expression of Recombinant hSPC1Δ, hSPC6A, and hSPC7Δ—Drosophila Schneider 2 cells (S2 cells, Invitrogen) were grown in complete DES expression medium supplemented with 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin G, 50 µg/ml streptomycin, and 2 mM L-glutamine to a density of 6–20 x 10$^6$ cells/ml at 22–24°C. Stable cell lines were established by co-transfecting the enzyme cDNA/pC5.1V5-HisA plasmid with pC5HYGRO plasmid (Invitrogen) at a weight ratio of 19:1 µg, respectively, by calcium-phosphate precipitation and selecting stable transformants with 300 µg/ml hygromycin B over a period of 3 weeks. The established resistant cell lines were then adapted to serum-free medium IPL-41-containing lipid supplement, 4 g/L ultratitrated yeastolate, 2 mM L-glutamine, 50 units/ml penicillin G, and 50 µg/ml streptomycin but no hygromycin B by successive passages of medium with 5, 2.5, 1, and 0% fetal bovine serum. The stable cell cultures were then scaled up to a volume of 600 ml in shake flasks spinning at 120 rpm supplying them with pluronic acid F68 to a final concentration of 0.1%. Typically, conditioned medium was collected every 6–7 days and submitted to purification procedures.

Purification of Active Recombinant hSPC1Δ, hSPC6A, and hSPC7Δ—All purification steps were accomplished at 4°C. Typically 1.5–2 liters of conditioned medium was filtered (0.22 µm) for the removal of insoluble material and submitted to high performance tangential flow ultrafiltration (Pellecino II, Millipore) against a 30-kDa cut-off Biomax membrane combined to a buffer exchange. The volume was generally lowered to 100–150 ml, filtered, and immediately injected on an equilibrated anion exchange column (MonoQ HR 10/10, Pharmacia Biotech). The injection procedure was done in a flow rate of 1 ml/min for 10 min with 500 mM NaCl, pH 7.5, 1 mM CaCl$_2$, and 0.1% (NH$_4$)$_2$SO$_4$, filtered, and submitted to hydrophobic interaction chromatography (HiTrap HIC 6F high sub, Pharmacia Biotech). The injection was performed at 1 ml/min with buffer C (100 mM HEPES, pH 7.5, 1 mM CaCl$_2$, 1 mM (NH$_4$)$_2$SO$_4$, and 0.1% (NH$_4$)$_2$SO$_4$ gradient over 200 ml with buffer A. Finally, enzymatically active and immuno-reactive fractions of 4 ml were pooled, concentrated to a minimal volume of 500–800 µl with a Centricron Plus-80 concentrator (30-kDa cut-off, Millipore) and the concentrate was applied at 1 ml/min to a gel filtration column (Superdex 200 26/60, Pharmacia Biotech) using buffer A. Enzymatically active and immuno-reactive fractions were then concentrated as described above. Gel filtration columns were packed with hSPC6A, and hSPC7A preparations were kept as 100–200 µl aliquots containing 15% glycerol (except for hSPC7A, which was kept without glycerol) at −25°C. The preparations of pure recombinant active enzymes were analyzed by Western blotting, silver staining, and Bradford protein assay.

Enzymatic Titration—Enzyme titration was performed for all enzyme preparations using the irreversible inhibitor dec-RVKR-CH$_2$. Assuming that all of the enzyme molecules were active, 1 nM of hSPC1Δ, hSPC6A, and hSPC7A preparations, as estimated by Bradford protein assay, was incubated with increasing concentrations of Dec-RVKR-CH$_2$ (1–51 µM) at room temperature for 15 min in a microtiter plate. Saturating concentrations of pERTKR-MCA (100 µM for hSPC1Δ and hSPC6A, 250 µM for hSPC7A) were then added to evaluate the residual activity after 1 h of incubation at 37°C, which was recorded on a SpectraMax Gemini XS spectrofluorometer (Molecular Devices) (37).

Enzymatic and Protein Assays—Enzyme activity was routinely evaluated during the expression and purification procedures as well as for inhibition assays. The enzymatic activities of hSPC1Δ and hSPC7Δ were carried out in 20 µl Bis-Tris, 1 mM CaCl$_2$, pH 6.5 (2 µg/µl bovine serum albumin was added for hSPC6A). The hSPC1Δ enzymatic assay was done in 100 mM HEPES, pH 7.5, 1 mM CaCl$_2$, 1 mM β-mercaptoethanol, 0.5 µg/ml bovine serum albumin. Unless otherwise stated, all initial rate enzymatic assays were performed at saturating conditions of fluorogenic substrate pERTKR-MCA (250 µM for hSPC1Δ and hSPC6A, 500 µM for hSPC7A) in the presence or absence of inhibitor at 37°C for 1 h or less in a total volume of 100 µl in 96-well microtiter plates. The resulting fluorometric signal due to released MCA was determined online as progress curves on a SpectraMax Gemini XS spectrofluorometer with SoftMaxPro 3.3.1 software (Molecular Devices) at EX/EM wavelengths of 370/460 nm with a 435-nm cut-off filter, and the amount of MCA released was calculated by reference to corresponding MCA standard curves. Protein level was also routinely determined by colorimetric assay using Bradford reagent with bovine serum albumin as the standard protein.

Determination of hSPC1Δ, hSPC6A, and hSPC7Δ Steady State Kinetic Constants—For hSPC7, these experiments were repeated for two different preparations. The enzyme’s pre-steady and steady states were first evaluated by performing time course experiments in saturating substrate conditions from 5 to 120 min in enzymatic assay conditions. The hydrolysis rate of pERTKR-MCA, measured by the amount of fluorescence versus time was plotted. Based on this, the Michaelis-Menten constant was determined by adding varying amounts of fluorogenic substrate (5 nM–1 µM) to 1–26 units of enzyme in activity buffer. The reactions were incubated and monitored online for 30 min at 37°C. The data, plotted as the rate of hydrolysis activity versus pERTKR-MCA concentration, was then fitted to a standard pseudo-first order rate equation using ENZFITTER (BioSoft Corp.) to determine the Michaelis-Menten constant (K$_m$), the limiting rate (V$_{max}$), the turnover number (K$_{cat}$/V$_{max}$), and the K$_{cat}$/K$_m$ ratio for the fluorogenic peptide pERTKR-MCA.

Production and Purification of Prodomains—Six prodomains were prepared using PCR amplification of full-length cDNAs of hSPC1, hSPC2, hSPC6, hSPC6A, and hSPC7. The following sense and antisense primers were used: for hSPC1: cagagagagctacctacaag and...
Comparative Inhibition of SPC Prodomains

RESULTS

Characterization of Recombinant Prodomains—Six of seven SPC prodomains were prepared for this study using a bacterial expression system and characterized using MALDI-mass spectrometry. A representative MALDI spectrum of SPC3 is shown (Fig. 1) while all the results are summarized in Table I.

Preparation of Recombinant hSPC1, hSPC6A, and hSPC7Δ Using Schneider 2 Cells—To investigate the structure/activity relationship of the SPCs, enzymatically pure preparations of selected enzymes are an important condition for accurate analysis. Previously, numerous expression systems, including vaccinia virus and baculovirus, were used to produce enzymatically active recombinant SPCs. In the present study, we used a non-viral and highly efficient expression system employing

Schneider 2 insect cells to produce soluble forms of hSPC1, hSPC6, and hSPC7. The recombinant enzymes were expressed continuously in serum-free media and then submitted to extensive purification procedures. First, we established three cell lines by transfecting S2 cells with plasmids containing the cDNA-encoding hSPC1Δ, hSPC6 (A isoform), and hSPC7Δ. Western blot analysis of the conditioned media of these selected stable lines, using hSPC1-, hSPC6-, and hSPC7-specific antibodies, showed strong immunoreactive signals of 83 and 80 kDa for hSPC1Δ (34), 90 and 75 kDa for hSPC6A (not shown), and 89 and 86 kDa for hSPC7Δ (Fig. 2). As a control, wild-type S2 cells did not show any hSPC1Δ, hSPC6A, and hSPC7Δ immunoreactivity. The same purification procedures were employed to obtain pure active recombinant hSPC1Δ, hSPC6A, and hSPC7Δ. Table II summarizes the results for hSPC7Δ’s purification. Only one major proteolytically active and immunoreactive peak was detected after the MonoQ HR 10/10 anion filtration, a single enzyme-specific peak was still observed, resulting in a 320-fold purification. Analysis of active peaks at all purification steps by Western blotting demonstrated that proteolytic activity coeluted with specific immunoreactivity throughout the entire procedure (Fig. 2, A and B). This provided strong evidence that the immunoreactive signal consisting in a doublet of 86 and 89 kDa is responsible for the enzymatic activity observed and corresponds to both isoforms of hSPC7Δ. These two isoforms may be the result of differential N'-glycosylation of the four putative asparagine-linked glycosylation sites or of a minor truncation at the C-terminal of hSPC7Δ. The final yield of protein was 1.84 mg (from 1.6 liters of conditioned media) with a 20% recovery. Therefore, extrapolation of this result suggests that the expression system established can produce up to 10 mg of active enzyme in the crude preparation and hence, more than 5 mg per liter of media.
When aliquots of each purification step were subjected to SDS-PAGE and overexposed silver staining, multiple protein bands were apparent including an 86–89-kDa band corresponding to the immunoreactive protein. Throughout the purification, this band was enriched compared with the non-immunoreactive proteins to a purity of greater than 80%, based on silver stain gel (Fig. 2C) and densitometry analysis.

In the case of hSPC1Δ, a single active peak with a strong immunoreactive signal at 81–83 kDa was also detected through the entire purification, which was purified 21.7-fold over the starting media. Again, a doublet of 83 and 81 kDa was observed by Western blotting analysis (34). A final yield of 2.81 mg was achieved. For hSPC6, two immunoreactive proteins of 90 kDa and 75 kDa coeluted in a single proteolytic activity peak. A purification of 16-fold and a final yield of 400 μg was achieved.

The high purity preparations of hSPC1Δ, hSPC6A, and hSPC7Δ and the precise knowledge of their active content allowed us to accurately establish the steady state kinetic parameters using pERTKR-MCA as the fluorogenic substrate. Very distinct values were obtained for parameters using pERTKR-MCA as the fluorogenic substrate. These comparative behaviors indicate their enzymatic activity measured in relative fluorescent units (RFU). B, Western blot of the enzymatically active fractions from gel filtration chromatography using the N-terminally directed hSPC7 antibody described in “Materials and Methods.” C, overexposed silver staining of the protein content from each purification step. Lane 1, crude extract (10 μg). Lane 2, post-ultrafiltration (10 μg). Lane 3, post-MonoQ (10 μg). Lane 4, post-HIC (3.2 μg). Lane 5, post-Superdex (10 μg).

We first compared the progress curves of three short propeptide variants and three complete prodomains on their respective enzymes (Fig. 4). Each peptide was a potent inhibitor in the nanomolar range, although complete prodomains were more potent. More importantly, these progress curves showed that short propeptide variants have a competitive behavior and that addition of the complete N-terminal region did not result in any significant changes in the kinetic profiles. The competitive inhibition behavior of the short propeptides and of the longer prodomains has been confirmed in saturation kinetic experiments that showed only a change in the affinity (i.e. higher K_{eff}) of the enzymes for the substrate, typical of competitive inhibitors that only bind to the catalytic site of the free enzyme (data not shown).

The next series of experiments investigated the specificity of each short propeptide on the three enzyme preparations (Fig. 5). We tested the seven different short propeptides on hSPC1Δ (Fig. 5A) and noted that four of the seven peptides tested had similar inhibitory potency, equivalent to the K of the hSPC1 propeptide which was 184 nM. This result suggests that each short propeptide is not a highly specific inhibitor. Similar results were obtained for hSPC6A, where five of the seven propeptides tested were determined to be potent inhibitors. In the case of hSPC7Δ, only the hSPC7 and the mSPC5 propeptides displayed significant inhibition (K values of 69 nm and 228 nm, respectively). Interestingly, the hSPC2 and hSPC3 short propeptides had very little inhibitory potency on any of the three enzyme preparations (Fig. 5). Furthermore, hSPC6A was the most sensitive to inhibition by the propeptides in general, while hSPC7Δ had the most specific response.

The lack of specificity of each short propeptide may in fact not be so surprising considering that the C-terminal regions of each prodomain has a high degree of homology (Fig. 3). We therefore investigated if longer peptides that included residues in the P' region could improve the specificity of each propeptide (Table IV). A comparative analysis revealed that extending the short propeptides at the C-terminal had essentially no effect on potency and specificity.

**Ala-scan Structural Analysis of hSPC1 and hSPC7 Short Propeptides—**While our results indicated that short propeptides are generally nonspecific, some distinctions were observed between the hSPC1 and hSPC7 short propeptides in their capacity to inhibit hSPC1Δ and hSPC7Δ. Indeed, the hSPC1 short propeptide is a 6-fold better inhibitor for hSPC1Δ as compared with hSPC7Δ (i.e. K, 184 nm for hSPC1Δ and 850 nm for hSPC7Δ, see Table IV). Also, the hSPC7 short propeptide is at least a 15-times more potent inhibitor of hSPC7Δ than of hSPC1Δ (i.e. K, 69 nm for hSPC7Δ and >1 μm for hSPC1Δ, Table IV). To understand why hSPC1 and hSPC7 short propeptides had such distinct inhibition properties on both enzymes, we designed hSPC1 and hSPC7 Ala-substituted short propeptides (Fig. 6). We did not substitute the P' and P'' C-terminal basic residues since it has already been shown that such substitutions completely abolishes inhibitory potency (33). The results showed that inhibition of hSPC1Δ decreased when basic residues in positions P'(Ala-9), P''(Ala-8), and D''(Ala-7) were substituted in the hSPC1 short propeptide (Fig. 6A). This is in good agreement with previous studies indicating that multiple positions...
basic residues are an essential requirement of inhibition for hSPC1 (41). In contrast, the Ala-substituted short propeptides designed for hSPC7Δ revealed that the most important positions for inhibition were the basic residues in the P6 (Ala-9) and P8 (Ala-8) positions, but also the hydrophobic residues (Leu) in the P4 (Ala-7) and P7 (Ala-6) positions (Fig. 6B). When Ala-modified hSPC7 short propeptides were tested on the hSPC1Δ preparation, or when Ala-modified hSPC1 short propeptides were tested on the hSPC7Δ preparation, no significant improvement in $K_i$ was observed (Table V).

**Complete Prodomains Inhibitory Potency and Specificity—**

The short C-terminal region of prodomains ending with the required KR is sufficient for potent inhibition of the SPCs (Fig. 5, Table IV). However, their lack of specificity toward their cognate enzyme triggered us to investigate whether the N-terminal extension of the complete prodomains would provide a greater degree of specificity and possibly higher inhibitory potency. These complete prodomains were tested for their potency and specificity of inhibition of hSPC1Δ, hSPC6AΔ, and hSPC7Δ (Table VI). The results showed that each of the prodomains, except for the SPC2 prodomain, were highly potent inhibitors of the three enzymes tested (0.1–150 nM). These data also demonstrated that addition of N-terminal region of each prodomain did not increase the specificity of inhibition. However, a comparison of the inhibitory potency of the complete prodomains with their related short propeptides did reveal an increase in potency (Fig. 7). In general complete prodomains were 5–100 times more potent than the corresponding short propeptides.

**Prodomain Structural Analysis—**

Recently, the global fold of mSPC3 prodomain was determined by NMR and CD spectroscopy and shown to closely correspond to the structure of the prodomain of subtilisin BPN’ (42). The secondary structure of the mSPC3 prodomain was found to be a mixture of a-helix (22%), $\beta$-strands (30%), and random coils and turns. To evaluate the secondary structure of the prodomains that we produced, we measured their far-UV CD spectra (Fig. 8). The CD spectra of prodomains hSPC1, mSPC3, mSPC5, and hSPC7 are quite similar in shape, and closely related to the CD spectrum of mSPC3 reported (42). This suggests that these prodomains have similar conformations of secondary structure. As for the hSPC2 prodomain, the CD spectrum is indicative of an unfolded protein. On the other hand, the structure of hSPC6 prodomain seems to contain more a-helical structure, as depicted by the double minimum at 223 and 210 nm and the maximum at 192 nm. These analyses strongly suggest that the prodomains that we have expressed and purified are folded, with the exception of hSPC2.

### DISCUSSION

Previous studies have established the ability of prodomains to act as intramolecular chaperones that are essential for the correct folding of their parent enzyme (43). For a number of peptidyl hydrolases including serine proteases, the prosegment has also been shown to be a potent inhibitor for its associated protease (4–7). Some well studied prosegments include those of the bacterial subtilases, such as subtilisin E (43, 44). These studies have demonstrated the high inhibitory potency of the prosegment. Thus, prodomains can also serve as a key component of the proteolytic machinery to regulate the intracellular processing of precursors. These observations have led to the proposal that prosegments could potentially be used in a strategy to regulate the enzymatic activity. As a strategy to develop potent inhibitors used in trans, prodomain sequences could be useful to develop lead compounds or molecular tools that can help in the study of SPC function.

Alignment of the SPC prodomain regions shows low levels of overall homology, but the most conserved region is found in their C-terminals (Fig. 3). Initial studies using the prosegment of SPC1, have demonstrated a potent inhibition for SPC1 ($K_i$ = 14 nm) when tested in vitro (6). Others have also shown the potent inhibitory effect of the prosegment of SPC3 on SPC3 as well as on SPC1 (7). These observations thus raised the question as to the specificity of each prosegment when used as an inhibitor in trans. In a preliminary attempt to address this issue, the prosegments of SPC1 and SPC7 as well as peptide fragments thereof were tested on concentrated media from cells infected with recombinant SPC vaccinia virus (33). This study concluded that SPC prosegments were indeed potent, but also specific inhibitors when used in trans. The sum of these data imply that each prodomain would in fact be a highly specific inhibitor of its cognate enzyme. In light of the implication of these observations, we carried out a detailed comparative analysis of at least six of the seven prodomains on three distinct highly purified enzyme preparations.

Since the C-terminal of the prodomain is essential for enzymatic inhibition (6–7, 38), we prepared seven short C-terminal propeptides (i.e. 12 aa) for initial experiments. These peptides proved to retain a high inhibitory potency when tested against their cognate enzyme (Table IV). For example, hSPC1-S inhibited hSPC1Δ with a $K_i$ of 184 nm, hSPC6-S inhibited hSPC6A with a $K_i$ of 23 nm and hSPC7-S inhibited hSPC7Δ with a $K_i$ of 69 nm. However, these propeptides were also potent inhibitors of the other SPC enzymes as well. For example, the hSPC1-S propeptide was a more potent inhibitor of hSPC6A ($K_i$ = 27 nm) than of hSPC1Δ ($K_i$ = 184 nm) itself. Furthermore, hSPC1-S, hSPC4-S, mSPC5-S, and hSPC6-S were also equipotent inhibitors of hSPC1Δ ($K_i$ values ranging from 123–184 nm) as well as of hSPC6A ($K_i$ values ranging from 12–27 nm). In contrast hSPC2-S and mSPC3-S (derived from the neuroendocrine cell-specific hSPC2 and mSPC3) were very poor inhibitors with $K_i$ values greater than 1 μM. The reason for this lack of inhibitory potency may be due to the lack of basic residues at the P4 or P8 positions (Fig. 3). In general, these data suggest that the short
propeptides are not very specific SPC inhibitors. There are several reasons that explain the lack of inhibitory specificity of each short propeptide. Some of the molecular determinants may be absent in the chosen sequences. In fact, some specificity could potentially be found in the P/H position of the prodomain primary cleavage site (Fig. 3). We therefore conducted a similar analysis on our three enzyme preparations using long propeptides (L) extended at the C-terminal. Even though the long propeptides have six amino acids in the P/H position as compared with the short propeptides, identical results were obtained, both in terms of potency and specificity (Table IV). Thus, our results suggest that the amino acids in the P/H positions do not contribute to the potency and specificity of inhibition of SPCs. However, it is noted that since these extended propeptides are equipotent to the short propeptides, they could in fact be useful in strategies in which such inhibitors are used ex vivo or in vivo. The amino acids in the P' positions would thus serve to protect the peptide from carboxypeptidases that would remove the critical C-terminal basic residues required for SPC inhibition. Indeed, our assays using the short propeptides were all conducted in vitro, without the presence of contaminating carboxypeptidases. The addition of carboxypeptidase E or carboxypeptidase B to our assays resulted in a completely abolished inhibition of the short propeptides (data not shown). This is consistent with previous reports showing that substitution of the C-terminal basic amino acids results in a complete loss of inhibitory potency by the prodomains (33, 45). The lack of specificity of our short propeptides could also be due to the absence of an extended N-terminal sequence. We therefore tested six different complete prodomains on the three enzyme preparations. Our results demonstrate that complete
The low nanomolar range (0.1–25 nM). However, no significant inhibitory specificity is not determined by the N-terminal region of the prodomains. However, in comparison to the short propeptides, complete prodomains were more potent inhibitors, sometimes 100-fold better. Two possible reasons could explain the higher potency of the complete prodomains. First, the short prodomain peptides (i.e. only 12 amino acids) adopt a random coil structure (analysis by NMR, data not shown), and thus addition of the N-terminal region may add structure and conformation to the polypeptide to provide a better fit at the enzyme subsites (i.e. improves affinity, but not necessarily specificity). In support of this we have shown by CD analysis that most of the prodomains, except for hSPC2 prodomain, do have extensive secondary structure (Fig. 8). Furthermore, the recent studies on the stability and global fold of the mSPC3 prodomain (1, 27–29) suggested that an N-terminal β-strand can stabilize the C-terminal region into a β-strand through the formation of a β-sheet. Secondly, secondary binding sites could be present in the N-terminal region of the prodomains. These prodomains are highly potent inhibitors, with $K_i$ values often in the low nanomolar range (0.1–25 nM). However, no significant improvement in specificity was observed as compared with the short prodomain peptides (Fig. 7). Our data indicate that inhibitory specificity is not determined by the N-terminal region of the prodomains. However, in comparison to the short propeptides, complete prodomains were more potent inhibitors, sometimes 100-fold better. Two possible reasons could explain the higher potency of the complete prodomains. First, the short prodomain peptides (i.e. only 12 amino acids) adopt a random coil structure (analysis by NMR, data not shown), and thus addition of the N-terminal region may add structure and conformation to the polypeptide to provide a better fit at the enzyme subsites (i.e. improves affinity, but not necessarily specificity). In support of this we have shown by CD analysis that most of the prodomains, except for hSPC2 prodomain, do have extensive secondary structure (Fig. 8). Furthermore, the recent studies on the stability and global fold of the mSPC3 prodomain (1, 27–29) suggested that an N-terminal β-strand can stabilize the C-terminal region into a β-strand through the formation of a β-sheet. Secondly, secondary binding sites could be present in the N-terminal region of the prodomains. These

**Fig. 5.** Short propeptides are potent but nonspecific inhibitors of hSPC1Δ, hSPC6A, and hSPC7Δ. The rate of hydrolysis of pERTKR-MCA by hSPC1Δ (A), hSPC6A (B), and hSPC7Δ (C) was determined in the presence of increasing concentrations of each SPC respective short propeptides. $K_i$ values for the propeptides against the three enzymes are plotted on a logarithmic scale.

**Table IV**

| Short/Long propeptide | Enzyme—$K_i$ (nM) |
|-----------------------|------------------|
|                       | hSPC1Δ | hSPC6A | hSPC7Δ |
| hSPC1-S               | 184    | 27     | 850    |
| hSPC1-L               | 175    | 20     | >1000  |
| hSPC2-S               | >1000  | >1000  | >1000  |
| hSPC2-L               | >1000  | >1000  | >1000  |
| mSPC3-S               | >1000  | >1000  | >1000  |
| mSPC3-L               | >1000  | >1000  | >1000  |
| hSPC4-S               | 145    | 12     | 930    |
| hSPC4-L               | 120    | 7      | 914    |
| mSPC5-S               | 123    | 13     | 228    |
| mSPC5-L               | 180    | 23     | 117    |
| hSPC6-S               | 166    | 23     | 892    |
| hSPC6-L               | 152    | 27     | 926    |
| hSPC7-S               | >1000  | 20     | 69     |
| hSPC7-L               | >1000  | 22     | 62     |

**Table V**

| Complete prodomain | Enzyme—$K_i$ (nM) |
|--------------------|------------------|
| hSPC1Δ             | hSPC6A | hSPC7Δ |
| hSPC1               | 2      | 0.6   | 150   |
| hSPC2               | >1000  | >1000 | 150   |
| hSPC3               | 1.6    | 1.6   | 25    |
| hSPC5               | 8.5    | 5.3   | 117   |
| hSPC6               | 3.3    | 0.8   | 16    |
| hSPC7               | 12.2   | 0.1   | 1.3   |

**Fig. 6.** Inhibitory potency of Ala-scan-substituted short propeptides. Ala-substituted hSPC1 and hSPC7 short propeptides were synthesized and tested on hSPC1Δ and hSPC7Δ enzyme preparations, respectively. The $K_i$ values were plotted on a logarithmic scale. $A$ shows the inhibition potency of each modified hSPC1 short propeptide on hSPC1Δ. Basic residues are essential for hSPC1Δ inhibition by the hSPC1 prodomain. $B$ shows the inhibition potency of each modified hSPC7 short propeptides on hSPC7Δ. Hydrophobic and basic residues are essential for hSPC7Δ inhibition by the hSPC7 propeptide.
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sites may produce allosteric interactions that could improve the affinity of the interaction of the C-terminal region within the catalytic pocket. Alternatively, a portion of the N-terminal extension could be involved in binding to an exosite that provides additional interactions. Indeed, the determined experimental structure of the bacterial prodomain in complex with the subtilisin BPN’ shows interactions between the β-sheet of the prodomain and two helices near the catalytic domain (46, 47). In sum, the present data do not permit us to distinguish between these different possibilities; however, we can state that specificity was not improved by the addition of the N-terminal extension of the prodomains.

The sum of our results indicates that the C-terminal region contains the primary information necessary to produce potent inhibition of SPCs. We therefore conducted an Ala-scan experiment of two of the short propeptides, hSPC1-S and hSPC7-S, in an attempt to delineate the molecular determinants important for their action, other than the essential C-terminal basic residues. We chose to study these two peptides since they showed a significant difference in specificity when comparing their actions on hSPC1Δ and hSPC7Δ. Ala-substitutions of the hSPC1-S propeptide revealed that the basic residues in the P4 (Ala-9), P5 (Ala-8), or P6 (Ala-7) positions are critical to inhibit hSPC1Δ. These data are in good agreement with recent data showing that polyarginine peptides are very potent inhibitors of mSPC1 (41). Indeed the C-terminal hSPC1 prodomain contains five basic residues out of six in the P1–P6 positions. Ala-substitutions of the hSPC7-S propeptide also revealed that the basic residues in the P4 (Ala-9) and P5 (Ala-8) positions were very important. However, we also noted that the hydrophobic residues (Leu) in the P6 (Ala-7) and P7 (Ala-6) positions had a significant effect on the inhibitory potency for hSPC7Δ. These two hydrophobic residues most likely provide a certain degree of specificity between the hSPC1-S and hSPC7-S propeptides for the two parent enzymes. Further support for the importance of hydrophobic residues at the P6 or P7 position is provided by our data showing that the short propeptide mSPC5-S is the second best inhibitor of hSPC7Δ (Fig. 5C). In contrast to the other short propeptides, the mSPC5-S short propeptide has a Leu at the P7 position.

Taken together, the experiments described demonstrated that although prodomains were very potent inhibitors (low nM range), they did not display highly specific properties within the SPC family of enzymes. In light of our present understanding of the importance of the C-terminal regions of the prodomains, this is not necessarily surprising since these regions have the highest degree of similarity. While our studies were entirely conducted in vitro, the data suggested that the use of prodomains as inhibitors in trans, possibly using expression systems, could result in inhibition of more than one SPC at a time. This is particularly important since most, if not all, cell lines examined to date express at least two or more SPCs endogenously (1, 27–29). Inhibition of an endogenous SPC using an expressed prodomain will also be difficult to control since various cellular expression systems do not control the exact levels of expression nor necessarily control the intracellular targeting of the expressed prodomain. It is clear that having a specific inhibitor for each SPC would have a tremendous usefulness to better dissect the function of each convertase within cellular systems or in vivo. Having the ability to inhibit a specific convertase with at least three log units more potency than another convertase would resolve the issue of cellular expression levels. The development of more specific inhibitors may be possible using the C-terminal sequences; however, various modifications would be required, and the success of this approach is not assured especially if SPCs are in fact very similar in their catalytic functionality. Alternatively, such potent inhibitors with broader specificity profiles may be more appropriate to target enzymes directed to the constitutive secretory pathway. This could be advantageous in the case where redundant SPC cleavage functions have been reported, such as that recently described for the β-secretase enzyme (48).

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Inhibitory Potency and Specificity of Subtilase-like Pro-protein Convertase (SPC) Prodomains

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