Inhibitory specificity and potency of proSAAS-derived peptides towards proprotein convertase 1*

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SUMMARY

Prohormone Convertase 1 (PC1) mediating the proteolytic processing of neural and endocrine precursors is thought to be regulated by the neuroendocrine protein proSAAS. The PC1 inhibitory sequence is mostly confined within a 10-12 amino acid segment near the C-terminus of the conserved human proSAAS and contains the critical KR244 dibasic motif. Our results show that the decapeptide proSAAS235-244 235VLGALLRVKR244 is the most potent reversible competitive PC1-inhibitor (Ki ~9 nM). The C-terminally extended proSAAS235-246 exhibits a 5-6 fold higher Ki (~51 nM). The additional LE sequence at P1’-P2’, resulted in a competitive substrate cleaved by PC1 at KR244↓LE246. Systematic alanine-, and in some cases lysine-, scanning tested the contribution of each residue within proSAAS235-246 towards the PC1-inhibition’s specificity and potency. The amino acids P1 Arg, P2 Lys and P4 Arg are all critical for inhibition. Moreover, the aliphatic P3 Val & P5, P6 and P1’ Leu significantly affect the degree of enzyme inactivation and PC1-specificity. Interestingly, a much longer N- and C-terminally extended endogenous r (rat) proSAAS221-254 called little PenLen, was found to be a 3-fold less potent PC1-inhibitor with reduced selectivity but a much better substrate than proSAAS235-246. Molecular modeling studies and circular dichroism analysis indicate an extended and poly-L-proline II type structural conformation for proSAAS235-244, the most potent PC1 inhibitor, a feature not present in poor PC1 inhibitors.
Proprotein Convertases (PCs) - a family of Ca\(^{2+}\)-dependent mammalian subtilases are known to mediate the proteolytic processing at selected sites of many precursor proteins into their functionally active forms (1,2). These sites are generally composed of a pair of basic amino acids within the consensus sequence of R/K/H-(X)\(n\)-R\(\downarrow\) where \(n=0,2,4\) or 6 and X= any amino acid except cysteine. Numerous potential substrates have so far been identified for PCs. These include hormonal peptides and growth factors, their receptors, cell surface proteins, bacterial toxins, envelope viral glycoproteins, enzymes, transcription factors and others (1,2). The delicate balance between cleaved functional proteins and their precursors is critical for normal growth, function, metabolism and development as well as in pathophysiologic conditions (1-5).

All PCs are initially synthesized as inactive zymogens that must be proteolytically activated through the autocatalytic cleavage of their inhibitory N-terminal prosegment. A number of studies have already revealed this unique property of prodomains in the regulation of enzymatic activity (6-9). However, at least for the neuroendocrine convertases PC1 and PC2, their cellular activity is controlled by endogenous inhibitors. Thus, co-localization, \textit{in situ} hybridization and other biochemical studies revealed that the production of enzymatically active PC2 requires the presence of a binding protein 7B2 (10), which also serves as a specific temporal endogenous inhibitor of this enzyme (for reviews see 1,2,11,12). Subsequent deletion and alanine-scan studies identified the inhibitor segment as a 16 aa fragment of the 31 aa C-terminal domain of 7B2 (13).

Recently, a granin-like 26 kDa (258-260 aa) neuroendocrine secretory protein, called proSAAS, was identified as a specific PC1 inhibitor (14). It is interesting to note that while proSAAS and 7B2 are not homologous, they are of similar sizes, with an N-terminal proline-rich region, both contain several pairs of basic amino acids and are broadly expressed in neural and endocrine tissues. Very recently, proSAAS was shown to specifically inhibit PC1 and not furin, PC2, PC5, or PC7 (15). The processing profile of proSAAS was recently reported and revealed that it is cleaved in a tissue-specific fashion at its C-terminus into smaller inhibitory peptides (15-17). The inhibitory segment was mapped to a short 6-12 aa sequence near its C-terminus that contains a critical Arg\(^{244}\).
PC1 inhibition by proSAAS peptides

(human proSAAS nomenclature) located at the processing site 235VLGALLRVKR↓LE246 (15,17). Interestingly, the peptide LLRVKR was previously identified from a combinatorial peptide library screen aimed at identifying specific PC1 inhibitors (18). The in vitro inhibition of PC1 by these short peptides was at least 17 fold better than with the full length proSAAS (15). Furthermore, it was reported that the 66 kDa processed form of PC1 is better inhibited by proSAAS than is its 87 kDa precursor (15) (Fig. 1).

In this article, we present detailed kinetic studies on the specificity and potency of PC1-inhibition by the above 12-mer proSAAS peptide and its mutants. Data obtained for the wild type sequence as well as from alanine- and lysine-scans allowed the identification of the critical aa within this sequence. The specificity and potency of these peptides was also tested against other PCs such as the mammalian furin, PACE4, PC5, PC7 and yeast kexin. The 12mer proSAAS peptides were compared with that of r (rat) proSAAS221-254, also known as little PenLen, which is one of the major processing forms of proSAAS in AtT20 cells (14). Finally, circular dichroism and molecular modeling studies were used to correlate the secondary structure of the inhibitory peptides to their potency of PC1 inhibition.
EXPERIMENTAL PROCEDURES

Peptide synthesis - All Fmoc protected amino acids (L-configuration), the coupling reagents and the solvents were purchased from PE Biosystems Inc. (Framingham, Ma, USA), Calbiochem-Novabiochem (San Diego, Ca, USA) and Chem Impex International, (Wood Dale, IL, USA). The peptides, prepared with carboxy-terminal in the amide form (CONH₂) are listed in Table I. The synthesis was accomplished on a solid phase automated peptide synthesizer instrument (Pioneer, PE Biosystems, Framingham, Ma, USA), following the HATU / DIEA-mediated Fmoc chemistry (19). The peptides were purified by RP-HPLC on C₁₈ semipreparative (0.94 x 25cm) and analytical columns (0.46 x 25cm, Jupitor, Phenomenex) using conditions previously described (19). The “little PenLen” peptide, r (rat) proSAAS₂₂₁⁻₂₅₄, AVDQDLGPEVPENVLGALLRVKR LENSSPQAPA was synthesised for us by Research Genetics Inc.

MALDI-TOF Mass Spectrometry - The identity of all peptides and their digests were confirmed by MALDI-TOF mass spectrometry using Voyageur DE-Pro instrument (PE Biosystems, Framingham, Ma, USA) with CHCA and DHB (Aldrich Chemical Co) as matrices (7,19).

Production of recombinant PCs - All the recombinant forms of PCs were produced by using the vaccinia virus constructs of soluble before transmembrane domain (BTMD) human (h)furin-BTMD, hPACE4, mouse (m)PC1, mPC5A, rat (r)PC7-BTMD, and yeast (y)kexin-BTMD. The enzymes were recovered from serum-free culture media as reported previously (15,19). The recombinant mPC1 used in the present study is obtained from expression of its full-length cDNA but it contains mostly the C-terminal truncated and enzymatically more active 66 kDa form and some 74 and 87 kDa as well.

Enzyme assay - All enzyme assays were performed with the fluorogenic substrate pyroglutamyl-Arg-Thr-Lys-Arg-4-methyl-coumaryl-7-amide (Peptides International, Louisville, KY, USA) at pH either 7.4 (furin) or 6.5 (for other PCs).
assay buffer in all cases comprised of 25 mM Tris + 25 mM Mes + 2.5 mM CaCl₂. The concentration of the substrate was maintained at 100 µM unless otherwise mentioned. The amounts of enzymes used were adjusted so as to give approximately the same hydrolytic activity (3.9–4.5 nmol of AMC released per hour of incubation) in an aliquot of 5 µL. The release of fluorescence was monitored for 6 h using a spectrofluorometer (Gemini, Molecular Probes, Ca, USA) at excitation and emission wavelengths of 370 and 460 nm respectively. Enzymatic activities were measured either from raw fluorescence readings in end time assay or from the progress curves.

**Progress curve for enzyme inhibition** - The fluorescence of released AMC was measured on-line with a spectrofluorometer every 60 s up to 60 min and the slope of each curve was assessed with the computer generated highest point fit.

**Determination of inhibition constant, Kᵢ and IC₅₀** - For Kᵢ determination, various peptide concentrations (0.12 nM to 700 µM) were incubated at 37°C with respective enzyme (5 µL) in the above described buffer (100 µL) in presence of at least two different concentrations of fluorogenic substrate, pERTKR-MCA (100, 50, 25 or 12.5 µM) (7, 15). The precise concentration range was selected so as to produce an inhibition of 20–80% of initial activity. For all kinetic measurements, the peptides were pre-incubated with enzyme for 15 min prior to addition of the substrate. All assays were performed in duplicate for two independent experiments on a 96-well microplates (flat bottom, black, Dynatec). Kᵢ was estimated from Dixon plots while for IC₅₀ value, a double reciprocal Lineweaver-Burke plot was used (15, 17, 19). Both initial rate and end-time assays were used and the values obtained for the measured parameter were averaged.

**Cleavage of proSAAS peptides by PCs** Each proSAAS peptide including the little PenLen, (20 µM) was incubated for 6 h or overnight at 37°C with PC1, furin, PACE4, kexin, PC5 or PC7 (5 µL) in buffer (100 µL) as described. The enzyme digests were separated by RP-HPLC using a C₁₈ analytical column with a diode-ray detector (Varian, Prostar, Ca, USA) and
the peaks were analysed by MALDI-TOF MS.

**Comparative analysis of PC-inhibition by proSAAS peptides** - Each proSAAS peptide (750 nM or 25 µM) was pre-incubated with enzyme (5 µL) for 15 min in buffer (100 µL) before addition of fluorogenic substrate, pERTKR-MCA (100 µM). Fluorescence readings were measured following overnight incubation and compared with the control experiment run in parallel without the peptide.

**Effect of pre-incubation time on enzyme inhibition** - For this study a representative peptide (proSAAS235-244, Table I) (20 µM) was pre-incubated with PC1 (5 µL) for 0, 5, 10, 15 and 30 min in buffer (100 µL) before addition of substrate pERTKR-MCA (100 µM). Control experiments were run in parallel without the peptide and the fluorescence readings were measured after 6h of reaction.

**Circular dichroism (CD) analysis** - All CD measurements were carried out with a JASCO J-810 spectropolarimeter instrument (Easton, MD, USA) using a 100 µL solution of each peptide in distilled water (concentration 0.2-0.5mg/mL) in a quartz cell (1mM path length) as described (20). The final corrected CD spectra were obtained by subtracting the spectrum obtained with control water from those of crude samples.

**Molecular modelling study** - Three dimensional theoretical structures of proSAAS peptides and their mutants were generated by computer software hyperchem (v5.0, Hypercube) with Robek-Polard energy minimisation carried out at ambient temperature.

**RESULTS**

**Inhibition constant (K_i) of proSAAS peptides against PCs** – Based on earlier studies (15), we selected the 12 mer human/rat proSAAS 235VLGALLRVRKL246 [same as in (233-242) in mouse sequence] as a model peptide for detailed kinetic analysis and positional scanning. As shown in Fig.1, the N-terminal 10aa of this inhibitory peptide
represent the C-terminus of the natural processing product obtained by PC1-cleavages at PRRLRR$^{220}$ and LLRVKR$^{244}$ and contains the critical Arg$^{244}$ (15,17). Table I lists all the 19 peptides synthesized including little PenLen and analyzed in this work. These include the wild type sequence and its 11 Ala derivatives, 3 Lys mutants at P1, P4 and [P1 +P4] and a [P3+P5] double Ala mutant. Finally, in order to compare the effects of P’ residues on inhibition, we also synthesised the wild type proSAAS$^{235-244}$ 235VLGALLRVKR$^{244}$ as well as its all dextro-derivative.

The measured $K_i$ of all the above proSAAS peptides against PC1 and in some cases against furin, PC5 and PC7 are presented in Table II. The competitive nature of inhibition of PC1 by both wild type proSAAS$^{235-246}$ (Fig. 2A) and its mutants (not shown) as well as proSAAS$^{235-244}$ (Fig. 2B), was demonstrated by Dixon plots conducted at four different concentrations of the fluorogenic substrate pERTKR-MCA. The near linear regression and the presence of a single point of intersection are evident. However a close examination of the graphs revealed a slight hyperbolic nature. This may perhaps be due to some artefact, to the presence of multiple PC1-forms in the enzyme preparation or alternatively to the adsorption of the peptide or enzyme to the sample tubes. The competitive nature of this inhibition was further confirmed by an observed linear increase of IC$_{50}$ with substrate concentration (not shown) (21-23).

Overall, the data indicated that the 10mer 235VLGALLRVKR$^{244}$ lacking any P’ residues was the most powerful PC1 inhibitor ($K_i$~ 9 nM). This value is 5.7-fold lower than the ~50.5 nM calculated for the $K_i$ of the C-terminally extended 12mer 235VLGALLRVKRL$^{246}$ (Table II). Although both peptides are selective PC1-inhibitors, the C-terminal Leu-Glu extension resulted in an enhancement of selectivity for PC1 inhibition by 27 and 57 fold as compared to furin and PC5, respectively. Interestingly, the ~100 fold lower inhibitory potency towards PC7 was not affected by the dipeptide insertion. Noticeably, Ala mutation of either the P1’ Leu or the P2’ Glu resulted in a drastic loss of potency and selectivity towards PC1 inhibition. This means that the nature
of these residues is critical for the observed high PC1-selective inhibitory property of proSAAS\textsuperscript{235-246}. Finally, it is worth mentioning that the Ala mutation of P1’ Leu resulted in a complete loss of PC1 selectivity as compared to furin, whereas the P2’ Glu to Ala mutation had a 14 fold lesser effect (Table II). This is reminiscent of the observed critical importance of P1’ Leu for the processing of prerenin that is cleaved by PC1 but not at all by furin (24). Finally, we note that the loss of selectivity towards PC1 vs PC5 and PC7 is somewhat similar for either Ala mutants, but not as drastic as the P1’ mutation for furin.

As indicated in Table II, PenLen or rproSAAS\textsuperscript{221-254} inhibits PC1 with $K_i \approx 120\text{nM}$, nearly 3- and 13-fold less efficiently than proSAAS\textsuperscript{235-246} and proSAAS\textsuperscript{235-244} respectively. It also displayed a reduced level of selectivity towards PC1-inhibition as compared to furin, PC7 and especially PC5 (Table II).

In order to extend those data and include other convertases such as human PACE4 and yeast kexin, in Fig. 3 are presented in a bar graph format the extent of enzyme inhibition by 750 nM proSAAS peptides and their selected mutants. For this purpose, we used a 4h stop time assay in which the level of each enzyme used was adjusted so as to give similar initial pERTKR-MCA activity. In agreement with the $K_i$ data (Table II) the shorter 10mer is more potent than the 12mer proSAAS peptide on PC1, furin, PC5 and PC7 (Fig. 3, first row). In contrast, PACE4 is not inhibited by either peptide even at this high concentration, whereas kexin is $\sim 50\%$ inhibited by the 12mer peptide and not at all by the 10mer one. The chirality of inhibition is evident by the fact that the all-dextro derivative of the 10mer peptide does not significantly affect the activity of any enzyme tested. Although not shown Ala mutation at P1’ and P2’ follow the expected pattern from Table II, i.e., they are both important for the selective inhibition of PC1.

The critical importance of the three basic residues at P1, P2 and P4 is also evident, since their Ala mutants lost most of their inhibitory properties against all enzymes (Table II, Fig. 3, second row). Furthermore, Arg to Lys mutations at either P1, P4 or [P1 + P4] resulted in a drastic loss of PC1-inhibition, especially for the P4 or [P1 + P4] mutations.
These data attest to the critical importance of Arg at the P1 and P4 positions and a basic residue at P2.

In addition, the aliphatic residues Val at P3 and P10, and Leu at P5, P6 and P9 also contribute to the inhibitory potency and selectivity of proSAAS235-246 towards PC1, with the P9 Leu and P3 Val positions being the most critical (Table II, Fig. 3, third and fourth rows). We also tested whether double P3 Val and P5 Leu to Ala mutations could further influence the inhibitory selectivity of proSAAS235-246 towards PC1 versus other convertases. Interestingly, the double mutant is a better inhibitor of furin (K_i ~127 nM) as compared to PC1, PC5 and PC7 (Table II).

**Digestion of proSAAS^{235-246} and its Ala mutants by PCs**—Previously it was demonstrated that radioiodinated [Tyr+mSAAS^{219-258}] was internally cleaved by PC1 (17), suggesting that PC1 is inhibited by proSAAS by a mechanism similar to that of PC2 by 7B2 (13, 25). However, the exact cleavage site of the PenLen peptide was not determined in this previous study (17). To determine this cleavage site, as well as to examine whether human proSAAS^{235-246} behaves as a competitive substrate, it was incubated for 18h with PC1 in the presence of the fluorogenic substrate pERTKR-MCA. The RP-HPLC of the crude digest and the mass spectral data of the isolated peaks are shown in Fig. 4. Unlike the fluorogenic peptide which was partially cleaved by PC1, the peptide proSAAS^{235-246} (R_t=33.7 min; [M+H]^+=1369) was completely digested giving the expected decapeptide VLGALLRVK\textsubscript{R}^{244}\downarrow (R_t=21.7 min; [M+H]^+=1123). The C-terminal dipeptide LE product eluted with the injection peak and was not analysed. The two other peaks at R_t=23.1 and 15.1 min were characterized by mass spectrometry as the unreacted fluorogenic substrate pERTKR-MCA [(M+H)^+=830] and its N-terminal product pERTKR-OH [(M+H)^+=668].

Using a similar approach we tested the cleavage of the Ala mutants of the 12 mer proSAAS^{235-246} peptides as well as the PenLen at 20 µM by PC1, furin, PC5, PACE4,
and PC7, under identical initial pERTKR-MCA activities (Table III). Overall, the data show that, following 6h incubation, PCs cleaved the individual proSAAS235-246 derivatives only at amino acid P1 Arg244 (except for its Ala mutant) with varying efficiencies. The wild type 12-mer sequence is best cleaved by PACE4 (100%) and least so by PC1 (20%) but PenLen containing this peptide in its internal sequence is most efficiently cleaved by PC1, poorly (15%) by PACE4, and not at all by either furin, PC5 or PC7. Interestingly the P2’ Ala mutant was found to be a better substrate compared to the wild type for all PCs except PACE4, while the P1’ Ala mutant was a worst substrate for all enzymes. As expected, the P1 Arg to Ala mutant was not cleaved by any convertase. In contrast, the P4 Arg to Ala mutant although not cleaved by PC1, and less cleaved by furin, PC5, and PC7, is still quite efficiently so by PACE4 (Table III). The latter observation is in accord with a report that shows that a basic residue at P4 (e.g., Arg), is not an essential requirement for PACE4’s ability to cleave fluorogenic substrates (26). The P2 Lys to Ala mutant although leading to a better substrate for PC1 and furin, is paradoxically a worse one for the other enzymes.

The Ala mutations of the P8 Gly and the aliphatic residues at P3, P5, P9 and P10 demonstrated that, except for PC5, some of them are critical for the ability of the other PCs to selectively process these peptides (Table IV). Particularly PACE4 is very sensitive to these Ala mutations at all the above residues, including a double P3, P5 one. Furthermore, P3 Val, P5 Leu, P8 Gly generally enhance processing by PC1, furin and PC7, while the P9 Leu is not favorable for PC1 processing and has no effect on either furin, PC5 or PC7. Finally P10 Leu enhances processing by furin. Thus for PC1, among the aliphatic and/or neutral residues only three selectively affect its ability to process proSAAS235-246, with P3 Val and P8 Gly enhancing processing and P9 Leu diminishing it as compared to their Ala mutants.

Slow and tight binding inhibition of PCs by proSAAS235-244 - The extent of PC1 inhibition by proSAAS235-244 is sensitive to the pre-incubation time preceding substrate addition. Accordingly, for a fixed inhibitor concentration, a 15-30 min pre-
incubation period of enzyme and proSAAS^{235-244} is necessary before achieving maximal PC1 inhibition (Fig. 5 and inset). This indicates a slow and tight binding inhibition of PC1 by proSAAS^{235-244} (7,8). Using a 15 min preincubation, progress curves of PC1, furin, PACE4 and PC7 activity in the presence of various concentrations of the 10mer proSAAS^{235-244} are depicted in Fig. 6. Accordingly, at a fixed inhibitor concentration, the degree of PC1, furin and PACE4, PC7 (Fig. 6), as well as PC5 and kexin (not shown) inhibition increases linearly with time. The slow tight binding inhibition is also deduced from the characteristic observation that IC_{50} of proSAAS^{235-244} increased linearly with the enzyme amount (not shown) (22,23).

Circular dichroism and molecular modelling - The secondary structures of proSAAS^{235-244} and proSAAS^{235-246} was investigated by circular dichroism (Figs. 7A and 7B). The data indicated that these potent PC1 inhibitors (Table II) exhibited a predominantly poly-L-proline II type conformation (27) in aqueous solution at pH 6.8 whereas this structure was completely destroyed in both the inactive P1 Arg to Ala mutant (Fig. 7C) and in the all dextro-proSAAS^{235-244} peptide (Fig. 7D). The presence of some helical structure is also noticeable. Studies with the other Ala mutants of proSAAS^{235-246} revealed varying degrees of poly-L-proline II type conformations nearly in proportion to the extent of PC1-inhibitory potency deduced from the K_i values in Table II (not shown).

In order to extend this conclusion, we performed molecular modelling studies, using minimum energy calculations (Hyperchem software, version 5) aimed at predicting the possible three dimensional structures of the various proSAAS peptides. The theoretical structures obtained suggest that while proSAAS^{235-246} is predominantly found in an extended conformation, the inactive P1, P2 and P4 mutants mostly exhibit β-turn structures (not shown). The above structural features, namely poly-L-proline II type and extended conformations may thus be important parameters affecting the ability of proSAAS to inhibit PC1.
The most important finding of this study is that the highly conserved 10- or 12-mer proSAAS peptides, namely proSAAS235-244 or proSAAS235-246 encompassed within the sequence 235VLGALLRVKRLE246 are highly potent and selective inhibitors of PC1. Alanine scanning of this peptide at all positions and lysine mutations at selected sites revealed the inhibitory profile played by the various P and P' aa. For Ala mutations, substitution of P1 Arg was most critical followed by P2 Lys and P4 Arg, respectively. These data agree with those predicted from modelling studies by Lipkind et al. (28) and Siezen et al. (29), whereby specific Glu and Asp residues in the S1, S2 and S4 subsites of PC1 are thought to intimately contact these basic residues. Among the aliphatic residues at P3, P5, P6, P9 and P10, all except P6 made significant contributions towards PC1-inhibition (Table II). Although generally agreeing with the results of positional scanning synthetic peptide combinatorial 6mer peptide library studies (18), our data differ with respect to the critical importance of P6 Leu. Thus, our data reveal that within the context of the 12mer proSAAS235-246 peptide, P6 Leu is not critical for selective PC1 inhibition and/or recognition (Fig. 3 and Table II). The difference might be attributed to the 12mer peptide used in this study which is extended both N-terminally (up to P10) and C-terminally (up to P2'), as compared to the N-acetylated 6mer used by Apletalina et al. (18). Among the aliphatic residues, the P9 Leu was found to be the most critical since its substitution by Ala led to a ~15-fold decrease in PC1-inhibition. Interestingly, alignment of the inhibitory prosegment of the PCs revealed a strict conservation of a P9 Gln among all members, possibly implicating this site in PC-inhibition (30). The next critical hydrophobic residue is the P3 Val, since its replacement by Ala led to a ~7-fold increase in $K_i$. Interestingly, the P3 position is occupied by Val in the inhibitory prosegment of PC1 and PC4 and by Ala in that of furin and PC7 (30). Ala replacements at P5 and P10 resulted in only 3-fold increase in $K_i$, suggesting a relatively lesser contribution of these residues towards PC1 inhibition. Thus, the P9 Leu in proSAAS235-
246 plays a critical discriminating role in PC1 recognition, as opposed to other PCs. In addition, our data also predict that hydrophobic/neutral residues at positions P3, P5 and even at distant positions such as P8 and P10 may further enhance the selective recognition of proSAAS235-246 by PC1.

It should be pointed out that the recombinant mPC1 used in the present study and obtained from expression of its full-length cDNA, contains mostly the C-terminally truncated and enzymatically more active 66 kDa form but that some 74 and 87 kDa forms are also present. The use of homogeneous and pure 66 kDa PC1, which is more likely the functional form in neuroendocrine granules, may lead to quantitative differences which may differ somewhat from those presented in this study.

Another interesting outcome of this study is that the elimination of P' residues from proSAAS235-246 led to an increased (~6 fold) potency but decreased selectivity of PC1 inhibition. Thus, while proSAAS235-244 inhibited PC1 780, 1980 and 94-fold more efficiently than furin, PC5 and PC7, proSAAS235-246 inhibited PC1 29, 35 and 103-fold more efficiently than the above convertases, respectively (Table II). This suggested that the aa occupying the P1' and P2' positions as well as the P9 one (see above) play major discriminatory roles in the potent selective inhibition of PC1. In addition, our data suggest that the P1' Leu in proSAAS235-246 is very critical to discriminate between furin and PC1 (Table II and Fig. 3), an observation already reported for human pro-renin processing (24) and would be predicted for the leptin pro-receptor (1). This information may be further exploited in the future in order to engineer more potent and specific PC1 inhibitors and/or substrates.

The other interesting observation is that the little PenLen peptide which comprises aa 221-254 of rat proSSAS sequence exhibited a strong but less efficient inhibition of PC1 compared to the conserved shorter proSAAS235-244 and proSAAS235-246. Moreover PenLen was found to be less discriminatory towards PC1 than either proSAAS235-244 or proSAAS235-246. Thus, whereas PenLen is selectively cleaved by PC1 (Table III), it
is not as selective an inhibitor of PC1 as compared to the 12mer proSAAS\textsuperscript{235-246}, especially with respect to PC5 (Table II). Finally, the observation that the 34mer proSAAS\textsuperscript{221-254} (PenLen) is more efficiently processed than the 12mer proSAAS\textsuperscript{235-246} (Table III) suggests that residues before and after the 12mer sequence may enhance the ability of PC1 to process the KR\textsuperscript{244}. This reinforces the notion that the potency and selectivity of PC1 inhibition of proSAAS is mostly located around the dibasic site KR\textsuperscript{244} representing the junction between Pen and Len (16). Interestingly this motif is found between two other dibasic sites RR\textsuperscript{220} and RR\textsuperscript{256}, the processing of which leads to the formation of PenLen. The latter peptide was found to be one of the processed forms of proSAAS in AtT20 cells (14), and it is likely that PenLen is also a processing intermediate in rat brain and pituitary (16). Recent data showed that in \textit{Cpefat/Cpefat} mice the processing of proSAAS is slightly impaired relative to wild-type mice, resulting in the accumulation of partially processed peptides (16). Our \textit{in vitro} data showing the inhibitory function of PenLen towards PC1 may rationalise the observed reduction in the level of PC1 activity in \textit{Cpefat/Cpefat} mice.

Another important outcome of the present study is derived from circular dichroism data on various proSAAS peptides that suggested that a distinguishing poly-L-proline type II-like conformation could be critical for the selective and potent inhibition of PC1. The implication of this secondary structural motif in PC1 inhibition in relation to pH and possible association with trace metal ions found in the secretory pathway such as Zn\textsuperscript{2+} and Cu\textsuperscript{2+} are currently under investigation.

The identification of the highly specific and potent PC1 inhibitors proSAAS\textsuperscript{235-246} or proSAAS\textsuperscript{235-244} may be useful in the development of an effective affinity procedure for the purification of PC1. Methodologies using peptide based inhibitors have been applied in the past to purify a number of serine proteases (31). So far, PC1 has only been purified partially using commonly used multiple chromatographic steps (15, 32). Other applications of these inhibitors may include derivatization with either fluorescent or
radioactive moieties as specific molecular markers of PC1 (33, 34). In view of the potential implications of PC1 in early embryogenesis, pre-implantation and in obesity (35,36), development of selective inhibitors may help to define the role of PC1 in vivo. This is especially needed, since so far no viable PC1 (-/-) mice could be obtained (M. Mbikay et al., in preparation). In this respect, more work is needed to improve the cellular permeability and/or delivery of the designed inhibitors. Future work on the targeting of proSAAS peptides or expression of hybrid constructs inside the cell should provide further insights on the biological functions of this regulated critical convertase.

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Abbreviations: PC, proprotein/prohormone convertase; aa, amino acid; HATU, O-hexafluorophospho-[7-azabenzotriazol-1-yl]-N,N,N',N'-tetramethyluronium; DIEA, Diisopropyl ethyl amine; Fmoc, fluorenyl 9-methoxy carbonyl; RP-HPLC, reverse-phase high performance liquid chromatography; MALDI-TOF MS, matrix assisted laser desorption ionization time-of-flight mass spectroscopy; CHCA, α-cyano-4-hydroxycynnamic acid; DHB, 2,3-dihydroxy benzoic acid; h, human; m, mouse; r, rat; BTMD, before transmembrane domain; Tris, Tris[hydroxymethyl] amino methane; Mes, 2-[N-morpholino] ethane sulfonic acid; AMC, 7-amino 4-methyl coumarin
FIGURE LEGENDS

Fig. 1: Schematic representation of the PC1-specific inhibitor proSAAS. The presence of two PC-like cleavage sites in proSAAS and its site of interaction with the catalytic domain of PC1 are illustrated. The selected proSAAS\textsuperscript{235-246} peptide used in the current study is also indicated.

Fig. 2. Dixon plots for inhibition of mPC1 by proSAAS\textsuperscript{235-246} (A) and proSAAS\textsuperscript{235-244} (B). The graphical plots were obtained with data using mPC1 (5 \( \mu \)L) and pERTKR-MCA as substrate at concentrations 12.5 (S1), 25 (S2), 50 (S3) and 100 (S4) \( \mu \)M. The inhibition study was conducted with a 15 min pre-incubation between the enzyme and the inhibitor following which the substrate pERTKT-MCA was added. The fluorescence readings were measured after 6h reaction at 37\(^{\circ}\)C (see Experimental Procedures).

Fig. 3. Inhibition of proteolytic activity of PCs by proSAAS peptides at 750 nM concentration. The activity of each enzyme used was adjusted so as to produce similar pERTKR-MCA cleavage efficiency. For each group of enzymes, the peptide used was indicated on the top of each bar chart. The control enzyme activity in the absence of any inhibitor was normalized to 100\% and indicated by a horizontal dotted line. Each inhibition study was performed in duplicate in two separate experiments and the data obtained were averaged.

Fig 4. RP-HPLC chromatogram of cleavage products of proSAAS\textsuperscript{235-244} by PC1. proSAAS\textsuperscript{235-244} (5\( \mu \)g) was incubated for 18h at 37\(^{\circ}\)C with PC1 (5\( \mu \)L, containing 3.9 nmoles of AMC released/h) in 100\( \mu \)L of 25mM Tris + 25mM Mes + 2.5mM CaCl\textsubscript{2}, pH 7.4. Elution conditions were as described in experimental procedure. The arrow (\( \downarrow \)) indicates site of cleavage. The molecular mass of each isolated peptide measured by MALDI-TOF is reported in parenthesis immediately above the corresponding peak.
Fig. 5. **Time dependent inhibition of mPC1 activity by proSAAS\textsuperscript{235-244} (192nM).**
mPC1 (5\(\mu\)L, containing 3.9 nmoles of AMC released/h) was pre-incubated separately with 192nM of proSAAS\textsuperscript{235-244} for 0, 5, 15 and 30min. Following each pre-incubation period, pERTKR-MCA (100\(\mu\)M) was added and the progress curves were followed by fluorescence for 30min. The enzyme inhibition was determined by initial slope of various progress curves.

Fig. 6. **Progress curves over a period of 1000 second showing inhibition of PC1, furin, PACE4 and PC7 activity in the presence of varying concentrations of proSAAS\textsuperscript{235-244}.**
The enzyme activity was measured against the fluorogenic substrate pERTKR-MCA (100\(\mu\)M) in the presence of increasing concentrations of proSAAS\textsuperscript{235-244} (as indicated in the right side of each panel). The release of fluorescence was followed over a period of 10 min. The inset in the PC7 panel is the progress curve monitored over a period of 1h.

Fig. 7. **Circular dicroism spectra of various proSAAS peptides.**
The spectra were recorded in 1mM path length of quartz cell in water (100\(\mu\)L), pH 6.8.
Table I

List of various proSAAS derived and related peptides

All amino acids are in L-configuration except for peptide #14 where all amino acids are in D-configuration (indicated by the front letter “d” and the small case letters for each amino acid residue♦), all mutations of amino acids were indicated in bold and underlined.

| No | Peptide name     | Amino acid sequence       | MW (M+H)+ |
|----|------------------|---------------------------|-----------|
| 1  | h/rSAAS235-246   | VLGALLRVKRLE246          | 1364      |
| 2  | h/rSAAS235-246P2A| VLGALLRVKRLE             | 1306      |
| 3  | h/rSAAS235-246P1A| VLGALLRVKRAE             | 1322      |
| 4  | h/rSAAS235-246P1A| VLGALLRVKALE             | 1279      |
| 5  | h/rSAAS235-246P2A| VLGALLRVARLE             | 1307      |
| 6  | h/rSAAS235-246P3A| VLGALLRAKRLE             | 1336      |
| 7  | h/rSAAS235-246P4A| VLGALLAVKRLE             | 1279      |
| 8  | h/rSAAS235-246P5A| VLGALARVKRLE             | 1322      |
| 9  | h/rSAAS235-246P6A| VLGALVRVKRLE             | 1322      |
| 10 | h/rSAAS235-246P8A| VLAGALLVRKRE             | 1378      |
| 11 | h/rSAAS235-246p9A| VLAGALLVRKRE             | 1322      |
| 12 | h/rSAAS235-246P10A| VLAGALLVRKRE             | 1336      |
| 13 | h/rSAAS235-244   | VLGALLRVKR               | 1121      |
| 14 | h/r-dSAAS235-244 | VLGALLRVK                 | 1121      |
| 15 | h/rSAAS235-246P1K| VLGALLRVKKLE             | 1337      |
| 16 | h/rSAAS235-246P4K| VLGALLKVKKLE             | 1337      |
| 17 | h/rSAAS235-246P1KP4K| VLGALLKVKKLE             | 1309      |
|   | h/rSAAS<sup>233-246</sup> P3AP5A | VLGAL<sup>ARA</sup>KRLE | 1294 |
|---|--------------------------------|------------------------|------|
| 19. | rproSAAS<sup>221-254</sup> | AVDQDLGPEVPPENVLGALLRVKRLENSSQPAPA<sup>254</sup> | 221  |
|    | (Little PenLen)                |                        | 3581 |
Table II

Inhibition constant (K_i) of proSAAS and other peptides against PC1, furin, PC5 and PC7

K_i values were determined using the linear regression of Dixon plot and Graphit software as described under “Experimental Procedures” section. N.I. = no inhibition, all data were acquired in duplicate and then averaged. S.D. = standard deviation, the lowest K_i value was indicated by an underline.

| Peptides      | PC1      | furin    | PC5       | PC7       | PC1 : furin : PC5 : PC7 |
|---------------|----------|----------|-----------|-----------|-------------------------|
| dSAAS^{235-244} | 122,000 ± 11900 |          |           |           |                         |
| SAAS^{235-244}  | 9 ± 0.5   | 261 ± 44 | 317 ± 9   | 940 ± 32  | 1 : 29 : 35 : 103       |
| SAAS^{235-246}  | 51 ± 3.8  | 39,400 ± 4300 | 100,000 ± 7000 | 4,750 ± 95 | 1 : 780 : 1980 : 94     |
| SAAS^{235-246p2'A} | 293 ± 12  | 4,399 ± 220 | 35,963 ± 1438 | 1,667 ± 67 | 1 : 15 : 123 : 6        |
| SAAS^{235-246p1'A} | 1,024 ± 40 | 1,280 ± 120 | 231,000 ± 21600 | 2,330 ± 330 | 1 : 1 : 181 : 2         |
| SAAS^{235-246p1A} | 509,000 ± 15270 |          |           |           |                         |
| SAAS^{235-246p2A} | 4,503 ± 30 |          |           |           |                         |
| SAAS^{235-246p3A} | 360 ± 72  | 92,000 ± 8000 | 335,000 ± 40,000 | 484 ± 34  | 1 : 274 : 931 : 1       |
| SAAS^{235-246p4A} | 286 ± 34  |          |           |           |                         |
| **SAAS^{235-246p5A}** | **172 ± 41** |          |           |           |                         |
| SAAS^{235-246p6A} | 58 ± 16   |          |           |           |                         |
| SAAS^{235-246p8A} | 143 ± 24  |          |           |           |                         |
| SAAS^{235-246p9A} | 737 ± 110 |          |           |           |                         |
| SAAS^{235-246p10A} | 177 ± 6.6 |          |           |           |                         |
| SAAS^{235-246p1K} | 1,530 ± 198 |          |           |           |                         |
| SAAS^{235-246p4K} | 177,500 ± 3,020 |          |           |           |                         |
| SAAS^{235-246p1Kp4K} N.I. (185 µM) | |          |           |           |                         |
| SAAS^{235-246p3AP5A} | 13,700 ± 1200 | 127 ± 30 | 27,700 ± 720 | 10,817 ± 454 | 11 : 1 : 218 : 85       |
| PenLen[tSAAS^{221-254}] | 119 ± 1.5 | 19,300 ± 1800 | 1,400 ± 200 | 4,630 ± 600 | 1 : 162 : 12 : 39       |
Table III

Comparative analysis on the cleavages of proSAAS peptides by various PCs

Each peptide (20 μM) was incubated for 6 h with respective enzyme (5 μL) and the digest was analysed by RP-HPLC as described in Experimental procedure. Rt = Retention time, NT = amino terminal, nd = not determined.

| Peptide        | HPLC Rt for % peptide cleaved by enzyme |  |  |  |  |  |
|----------------|-----------------------------------------|---|---|---|---|---|
|                | cleaved NT                               | PC1 | furin | PC5 | PC7 | PACE4 |
|                | fragment (min)                           |    |      |    |    |      |
| SAAS235-246    | 21.7                                     | 20 | 36 | 35 | 60 | 100 |
| SAAS235-246p2’A | 21.6                                     | 56 | 100 | 65 | 100 | 26 |
| SAAS235-246p1’A | 19.1                                     | 9  | 6  | 5  | 14 | 36 |
| SAAS235-246p1A  | 23.7                                     | 0  | 0  | 0  | 0  | nd |
| SAAS235-246p2A  | 19.9                                     | 65 | 60 | 17 | 12 | 40 |
| SAAS235-246p3A  | 20.3                                     | 9  | 16 | 40 | 17 | nd |
| SAAS235-246p4A  | 19.7                                     | <1 | 20 | 26 | 23 | 100|
| SAAS235-246p5A  | 18.3                                     | 25 | <1 | 41 | 0  | 68 |
| SAAS235-246p6A  | 17.2                                     | 24 | 25 | 45 | 8  | 23 |
| SAAS235-246p8A  | 22.3                                     | 8  | 16 | 40 | 40 | 15 |
| SAAS235-246p9A  | 18.2                                     | 63 | 39 | 38 | 70 | 16 |
| SAAS235-246p10A | 21.2                                     | 24 | <1 | 42 | 57 | 15 |
| SAAS235-246p3AP5A | 16.0                                     | 21 | 25 | 60 | 28 | 10 |
| PenLen/rSAAS221-254 | 31.0                                     | 100| 1  | 0  | 0  | 15 |
Inhibitory specificity and potency of proSAAS-derived peptides towards proprotein convertase 1
Ajoy Basak, Peter Koch, Marcel Dupelle, Lloyd D. Fricker, Lakshmi A. Devi, Michel Chretien and Nabil G. Seidah

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