The C-terminal Region of proSAAS Is a Potent Inhibitor of Prohormone Convertase 1*

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ProSAAS is a recently discovered 26-kDa neuroendocrine protein that was previously found to inhibit prohormone convertase (PC) 1 and not PC2. In the present study, the specificity of proSAAS toward other members of the prohormone convertase family was determined. Two µM proSAAS selectively inhibits PC1 but not furin, PACE4, PC5A, or PC7. The PC1 inhibitory region of proSAAS was mapped to an 8–12-residue region near the C terminus that includes a critical Lys-Arg sequence. Synthetic peptides corresponding to this region are competitive inhibitors of PC1 with apparent Kᵢ values of 14–40 nM. The inhibition becomes more effective with incubation time, indicating that the inhibitor is slow binding. A fusion protein containing the inhibitory region of proSAAS linked to the C terminus of glutathione S-transferase binds the 71-kDa form but not the 85-kDa form of PC1. This binding, which occurs at pH 5.5 and not at pH 7.4, is stable to incubation at room temperature for 1 h in the presence or absence of 0.5% Triton X-100 and/or 0.5 M NaCl. The removal of Ca²⁺ with chelating agents partially releases the bound PC1. High concentrations of the inhibitory peptide quantitatively release the bound PC1. Taken together, these data support the proposal that proSAAS functions as an endogenous inhibitor of PC1.

Most bioactive peptides are produced from larger precursors by selective cleavage at specific sites (1). Typically, these sites contain basic amino acids with the consensus KᵢXᵢR where n = 0, 2, 4, or 6 and X indicates any amino acid(s) except cysteine (1, 2). Processing at these sites is accomplished by an endopeptidase that cleaves to the C-terminal side of the consensus site and then a carboxypeptidase to remove the C-terminal basic residue(s). The prohormone/proprotein convertases (PCs)¹ are a family of mammalian endopeptidases that are related to bacterial subtilisin and to the yeast KEX2 endopeptidase (1, 2). The PCs can be divided into two subgroups based on their distribution within cells. One group, which includes furin, PACE4, PC5B (also known as PC6B), and PC7 (also known as PC8 and LPC), is primarily localized to the trans-Golgi network in a variety of cell types (1, 2). The other group, which includes PC1 (also known as PC3), PC2, and PC5A (also known as PC6A), is enriched in secretory vesicles in neuroendocrine cell types (1, 2). In contrast to the multitude of PCs, only two carboxypeptidases are thought to be involved in processing proteins in the secretory pathway. Carboxypeptidase D is primarily localized to the trans-Golgi network, and carboxypeptidase E is enriched in secretory vesicles (3, 4). The enzymes localized to the trans-Golgi network are thought to process proteins that transit the constitutive secretory pathway, such as the insulin receptor and some growth factors (5–7). The secretory vesicle enzymes are primarily involved in the processing of neuroendocrine peptides (1, 2).

A large number of enzyme systems have endogenous inhibitors that serve to regulate the enzyme activity. All of the PCs are initially produced as zymogens that must be proteolytically activated (1, 2). In some cases, the pro-segment is still an effective inhibitor even after initial proteolysis and additional cleavages are required to fully activate the enzyme (8, 9). In addition to being produced as a pro-form, PC2 is also co-expressed with an endogenous chaperone/inhibitor, 7B2 (10). 7B2 was initially discovered as a granin-like protein present in the secretory pathway of neuroendocrine cells (11). This protein is initially produced as a 27–30-kDa protein that is proteolytically processed by furin (12) into a 31-residue C-terminal peptide (13). Although co-expression of 7B2 is necessary for the production of active PC2 (14), the C-terminal peptide of 7B2 is also a potent inhibitor of PC2 (13, 15). PC2 slowly cleaves at a Lys-Lys site within this C-terminal peptide, and then, following the action of a carboxypeptidase, the cleaved peptide is no longer inhibitory toward PC2 (13).

Recently, a novel secretory pathway protein was detected while analyzing peptides that were not properly processed in mice lacking carboxypeptidase E activity (16). The Cpe∥/Cpe∥ mice contain a point mutation in the carboxypeptidase E gene that renders the enzyme inactive (17). Without active carboxypeptidase E, these mice accumulate peptides with C-terminal Lys and/or Arg extensions (17–19). A large number of improperly processed peptides were detected using an affinity

¹ The abbreviations used are: PC, prohormone convertase; GST, glutathione S-transferase; AMC, 7-amino-4-methylcoumarin.

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column to isolate peptides with C-terminal basic residues from Cpe \textsuperscript{rat} /Cpe \textsuperscript{rat} tissues (16). Mass spectrometry-based sequence analysis revealed that five of these peptides were encoded by a novel protein, designated proSAAS (16). Although there is no amino acid sequence homology between proSAAS and TB2, they are both similar sizes, they both contain a relatively high percentage of proline as well as several pairs of basic amino acids, and they both have a broad neuroendocrine tissue distribution (10, 11, 16, 20). Based on this similarity and on the finding that overexpression of proSAAS in AtT-20 cells inhibited the processing of the endogenous prohormone, proopio- melanocortin, proSAAS was directly tested with PC1 and PC2 (16). This analysis revealed that proSAAS inhibited PC1 but not PC2. The specificity of proSAAS for other members of the PC family was not previously investigated. In this study, we report that proSAAS is highly specific for PC1 and does not inhibit other members of this gene family. In addition, we map the inhibitory region to a short 8–12-amino acid segment near the C terminus of proSAAS. Peptides corresponding to this region inhibit PC1 with a slow onset of action that is partially competitive with substrate. Finally, this region of proSAAS is able to bind PC1 tightly with a slow dissociation rate. Taken together, these data support the proposal that one of the physiological functions of proSAAS is to inhibit PC1.

**MATERIALS AND METHODS**

Production and Assay of Prohormone Convertases for Testing with GST-proSAAS—BSC40 cells were infected as described previously at 2 plaque-forming units/cell with the following vaccinia virus constructs: full-length mouse PC1, human furin, rat PC7-TMDD, mouse PC5A, human PACE4, and mouse PC2/TB2 (21–24). The serum-free culture media were collected after an 18-h incubation, concentrated 40–50-fold using a Centriprep-30 microfiltration unit (Amicon), and stored at −20 °C. All PC assays were conducted with the substrate pyroglycaminyl-Arg-Thr-Lys-Arg-4-methyl-coumaryl-7-amide (Peptides International). For the analysis of PC1 with GST and GST-proSAAS (in comparison with other PCs), the assay buffer contained 50 mM sodium acetate, pH 5.5, 5 mM CaCl\textsubscript{2}, 0.1% Triton X-100, 0.1 mM 2-mercaptoethanol, and 100 μM substrate. PC2 was assayed in an identical buffer, and for the other PCs the substrate concentration was replaced by 550 mM glutathione and 100 μM substrate. Substrates were used to give approximately the same hydrolytic activity in an aliquot of 10 μl GST or GST-proSAAS were added in a volume of 10 μl. Fluorescence was monitored approximately every 45 min for up to 4 h using a Perkin-Elmer LS-50B spectrophotometer (excitation, 370 nm; emission, 460 nm). Enzymatic activities were calculated from the linear portion of the activity versus time plots.

**Purification and Assays of PC1**—The purification of PC1 was essentially as described in Boudreau et al. (25) with a few modifications. Briefly, 200 ml of medium were collected from Sf9 cells 42 h after infection with baculovirus expressing mouse PC1 (25). Cells were pelleted, supernatant containing enzymatically active PC1 was adjusted to 10 mM CaCl\textsubscript{2} and proteins were bulk precipitated with 30 μl/100 ml of polyethylene glycol 3350 (Sigma). Following gentle rotation for 4 h at 4 °C the precipitated proteins were collected by centrifugation at 8000 × g for 15 min. The precipitate was resuspended in 20 ml of 25 mM sodium acetate, pH 6.0, containing 1 mM CaCl\textsubscript{2}. The insoluble material was removed by centrifugation at 27,000 × g for 15 min and washed once with buffer A. The combined supernatants were clarified by pas- through a 0.45-μm filter and incubated with 2 ml of concavalin A-Sepharose (Amersham Pharmacia Biotech) for 60 min with gentle agitation at 4 °C. The slurry was poured into a plastic column and washed with 10 volumes of 25 mM sodium acetate, pH 6.0, containing 1 mM CaCl\textsubscript{2} and the protein-bound proteins were eluted with 7 volumes of the same buffer containing 250 mM α-methylglucopyranoside (Sigma). The eluted material was then concentrated using an Amicon concentrator with YM10 membranes and Centricon 30 and 50-kDa cut-off membranes (Millipore). The concentrate was adjusted and collected at 50 mM sodium acetate, pH 6.0, and 50 mM sodium chloride and fractionated on a Superdex-75 column (Amersham Pharmacia Biotech). The flow rate was 0.5 ml/min, and 400-μl fractions were collected. The PC1 activity was determined as described below. The fractions containing PC1 activity were subjected to electrophoresis under denaturing conditions and visualized by silver staining. Aliquots of the fractions were analyzed by Western blotting using 1:1000 dilution of a rabbit antiserum raised against the N-terminal region of PC1, prepared using the peptide and procedure described by Vindrola and Lindberg (26). Bound antiserum was visualized using the enhanced chemiluminescence detection kit (Pierce).

For the analysis of PC1 with GST-proSAAS deletion mutants, the assay was performed with 5 μM substrate in a final volume of 2 ml of 0.1 M sodium acetate, pH 5.5, with 0.01% Triton X-100 and 10 mM CaCl\textsubscript{2}. Fluorescence was determined in a Perkin-Elmer LC-3 spectrofluorimeter (excitation: 370 nm; emission: 460 nm) following incubation at room temperature. For the analysis of PC1 with the 8–16-residue peptides, the assay was performed in 96-well flat bottom white microplates (Dyntact) with 100 μM substrate in a final volume of 50 μl of 0.1 M sodium acetate pH 6.0 containing 10 mM CaCl\textsubscript{2} and 50–500 ng of purified PC1. The fluorescence of the released 7-amino-4-methylcouma- rin (AMC) was measured on-line with a Fluoro Max-2 Spectrofluorimeter (Jobin Yvon-Speex Instruments) using an excitation wavelength of 360 nm (slit width, 5 nm) and an emission wavelength of 480 nm (slit width, 5 nm). The fluorescence units were quantitated using AMC as a standard.

**Expression of proSAAS Deletion Mutants**—Various rat proSAAS fragments were amplified by polymerase chain reaction using oligonucleotide pairs with a BamHI or BglII site in the upstream oligonucleo- tide and a stop codon followed by an EcoRI site in the downstream sequence. Then the processing sequence region was amplified into the BamHI-EcoRI sites of the pGEX-2T vector. All se- quences were verified by the DNA sequencing facility of the Albert Einstein College of Medicine.

To express GST-proSAAS fusion proteins, the plasmids were transformed into the protease deficient Escherichia coli strain BL21, and colonies expressing high levels of the protein were selected. Protein expression was induced by adding 0.2–0.5 mM isopropyl-β-D-thia-galactoside (Life Technologies, Inc.) at 37 °C for 2–3 h. The proSAAS fusion proteins were separated on SDS-polyacrylamide gels and identified by Western blot analysis using antisera against either the N-terminal or C-terminal regions of proSAAS. To purify the GST-proSAAS fusion proteins, the cell pellets were suspended in phosphate-buffered saline buffer with 0.5% Triton X-100 and disrupted by sonication. The homo- geneous mixture was centrifuged at 50,000 × g for 1 h, and the supernatant was mixed with glutathione-agarose at room temperature for 1 h, and then the mixture was poured into an Econo-Pak column (Bio-Rad). After washing with phosphate-buffered saline buffer, the GST-proSAAS fusion proteins were eluted with 10 mM glutathione in 50 mM Tris-Cl pH 8.0 buffer and then dialyzed against a large volume of 50 mM Tris-Cl pH 7.0 buffer for 3–4 h.

**Binding Assays**—To examine whether the PC1 binds to proSAAS-derived peptides, 10–20 μg of GST-proSAAS fusion proteins were mixed with 20 μl of glutathione-agarose in phosphate-buffered saline pH 7.4 buffer at room temperature for 30 min and centrifuged, and then the pellet was washed with phosphate-buffered saline. Typically, 0.5 ml of medium from Sf9 cells infected with PC1-expressing baculovirus was mixed with glutathione-agarose at room temperature for 1 h, and then the mixture was centrifuged, and the pellet was washed twice with 1 ml of the same binding buffer. In some experiments, the pellet was additionally incubated with buffer containing 0.5 mM NaCl and 0.5% Triton X-100 and other additions (peptides, EDTA, as described in figure legends) for the indicated time, the mixture was centrifuged, and the supernatant was removed for analysis on a Western blot. The bound PC1 was dissociated by boiling in 1% SDS gel loading buffer for 5 min. A fraction of the extract was analyzed on a Western blot using an antisera to the N-terminal region of PC1 (described above) and the enhanced chemiluminescence detection method (Pierce). All binding experiments were repeated 2–5 times with comparable results.

**RESULTS**

Previously, rat proSAAS produced as a GST fusion protein in bacteria was found to inhibit PC1 (16). To assess the specificity of proSAAS for PC1, the GST-proSAAS fusion protein was tested with other members of the PC family. At 2 μM concen-
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To further map the inhibitory region, a series of peptides was synthesized. A 17-residue peptide corresponding to residues 231–246 of proSAAS with an additional Tyr on the N terminus inhibited PC1 with an IC_{50} of approximately 35 nM (Fig. 2, YE17). The 14- and 12-residue peptides showed comparable potency toward PC1, whereas the 10- and 8-residue peptides had IC_{50} values around 70 nM (Fig. 2). To test whether this inhibition is due to a nonpeptide contaminant present in the peptide, the peptides were treated with trypsin, the trypsin was then heat-inactivated, and the peptides were tested with PC1. This trypsin treatment eliminated the inhibitory activity of the peptides toward PC1, indicating that the inhibition was due to a peptide component (and also supporting the data shown in Fig. 1 illustrating the importance of the intact Lys-Arg sequence for PC1 inhibition). The specificity of YE-17 for various members of the PC family was evaluated. As found with full-length proSAAS, low concentrations (0.25–2 mM) of YE-17 selectively inhibit PC1. However, at 25 μM YE-17 some inhibition of PC7 and furin were detected and to a much lesser extent PC5 and PACE4 (not shown). It is possible that this inhibition observed with 25 μM YE-17 is due to competition with the 100 μM substrate.

Kinetic analysis of PC1 in the presence of various concentrations of YE-17 showed a mixture of noncompetitive and competitive-type inhibition with a K_{I} of 14 nM (Fig. 3). Similar analysis of LE-8 showed pure competitive inhibition kinetics (not shown). The portion of inhibition that appears to be noncompetitive may be due to slow dissociation of YE-17 from the active site rather than true noncompetitive inhibition. Analysis of the time course of PC1 activity in the presence of various amounts of YE-17 showed that relatively high concentrations of peptide did not immediately inhibit PC1 (Fig. 4). Instead, the progress curves for the reaction followed typical slow binding kinetics, with greater inhibition observed at later reaction.

Table 1

| Enzyme | Activity* | % control ± S.D. |
|--------|-----------|------------------|
| PC1    | GST alone | 76 ± 4           |
| PC2    | GST-proSAAS | 20 ± 1          |
| Furin  | GST alone | 83 ± 10          |
| PACE4  | GST-proSAAS | 70 ± 8          |
| PC5A   | GST alone | 103 ± 2          |
| PC7    | GST-proSAAS | 101 ± 3         |

*Values are the averages of three separate experiments, each performed in duplicate using 100 μM pyroGlu-Arg-Thr-Lys-Arg-AMC substrate.

Fig. 1. Deletion mutational analysis of the PC1 inhibitory region of proSAAS. The proSAAS-derived peptides previously identified in Cpe^iso/Cpe^iso mouse tissues and in AtT-20 cells are indicated by shading; the names of several of these peptides are indicated on the top line and refer to internal amino acid sequences (KEP, SAAS, PEN, and LEN). Single (R) and dibasic (RR or KR) processing sites are indicated. Fusion constructs consisting of GST with C-terminal extensions of the indicated portions of rat proSAAS were created in the pGEX-2T vector (Amersham Pharmacia Biotech) as described under "Materials and Methods." Protein was expressed in bacteria and purified on glutathione-agarose. The amount of protein was quantitated from the absorbance at 280 nm, using the calculated extinction coefficient for each construct. The construct 231–246 was created as both a GST fusion protein and as a synthetic peptide, and the constructs 221–242, 245–260, 245–254, 34–59, 34–40, and 42–59 were created only as synthetic peptides. The PC1 assay was performed with 5 μM substrate, using media from PC1-expressing baculovirus as described under "Materials and Methods." All constructs were tested at 1 μM in at least two separate experiments, with similar results. Yes indicates greater than 50% inhibition, whereas no indicates less than 10% inhibition; none of the constructs showed partial inhibition.

tation the GST-proSAAS does not significantly inhibit PC2, furin, PACE4, PC5, or PC7 (Table 1). Although a small decrease in PC2 activity was observed, this decrease was not significantly different from the activity in the presence of GST alone (Table 1). In addition, kexin activity was not significantly affected by this concentration of GST-proSAAS (not shown).

To map the inhibitory region of rat proSAAS, a series of deletion mutants was expressed as GST fusion proteins and tested with baculovirus-expressed PC1. Deletion of the C-terminal 28 residues abolished the effectiveness of proSAAS as a PC1 inhibitor (Fig. 1, 34–232). Conversely, constructs that contained the C-terminal 30 residues (Fig. 1, 231–260 and others) were inhibitory. Further deletion analysis indicated that the 16-residue region from residues 231–246 of rat proSAAS was inhibitory (Fig. 1); this region contains an internal Lys-Arg sequence that was previously found to be cleaved in AtT-20 cells and in mouse brain (16). Synthetic peptides corresponding to the cleavage products were ineffective as PC1 inhibitors (Fig. 1). The inhibitory activity of the 16-residue proSAAS C-terminal peptide is not dependent on attachment to GST; cleavage with thrombin did not alter the inhibitory potency of the construct. Mutation of both the Lys243 and Arg244 sequence (KEP, SAAS, PEN, and LEN).

Table 1: Effect of 2 μM GST-proSAAS or GST on the activity of various members of the prohormone convertase family

| Enzyme | Activity* | % control ± S.D. |
|--------|-----------|------------------|
| PC1    | GST alone | 76 ± 4           |
| PC2    | GST-proSAAS | 20 ± 1          |
| Furin  | GST alone | 83 ± 10          |
| PACE4  | GST-proSAAS | 70 ± 8          |
| PC5A   | GST alone | 103 ± 2          |
| PC7    | GST-proSAAS | 101 ± 3         |

*Values are the averages of three separate experiments, each performed in duplicate using 100 μM pyroGlu-Arg-Thr-Lys-Arg-AMC substrate.
To test whether PC1 is able to bind tightly to the proSAAS C-terminal peptides, several GST-proSAAS constructs were incubated with media from Sf9 cells infected with PC1-expressing baculovirus and then the GST purified on glutathione-agarose. The resin was washed briefly with binding buffer, and the bound material was eluted by boiling in 1% SDS. When the binding was performed at pH 5.5, the 71-kDa form of PC1 in the baculovirus media bound to the full-length proSAAS construct (Fig. 5, lanes 2) and the construct containing proSAAS 221–260 (Fig. 5, lanes 3). No binding was detected to GST alone (Fig. 5, lanes 1) or to the construct containing proSAAS 34–174 (Fig. 5, lanes 4), which lacks the C-terminal PC1 inhibitory region. When binding was performed at pH 7.4, no binding was detected for any of the constructs or forms of PC1 (Fig. 5, bottom panel). Longer exposures of the Western blot revealed that in addition to the 71-kDa form, the less abundant 75-kDa form of PC1 in the media (but not the 85-kDa form) showed some binding to the full-length proSAAS and to proSAAS 221–260 at pH 5.5 but not to the other inactive constructs (not shown).

To evaluate whether the binding is competitive with peptide YE-17, PC1 was incubated with GST-proSAAS construct 221–246, and the material was isolated on glutathione-agarose and then incubated for 1 h at room temperature under various conditions. In the presence of buffer alone, all of the PC1 remained bound to the GST-proSAAS construct (Fig. 6). The addition of 0.5 M NaCl and 0.5% Triton X-100 did not release the bound PC1 (Fig. 6, lanes Tx). The calcium chelator EDTA was partially effective at releasing the bound PC1 (Fig. 6). The peptide YE-17 quantitatively released the bound PC1 when tested at either 100 or 300 μM (Fig. 6), but 1 μM peptide was ineffective (not shown). Approximately 50% of the bound PC1 was released by the peptide within 10 min of incubation (Fig. 6C). However, incubation for 70 min with 100 μM of the PC1 substrate pGlu-Arg-Thr-Lys-Arg-MCA was not effective in releasing bound PC1 (Fig. 6C).

**DISCUSSION**

One of the major findings of the present study is that proSAAS is selective for PC1. Although relatively high concentrations of the C-terminal inhibitory peptide YE-17 do inhibit some of the other PCs, this could simply be competition with substrate and not tight binding as found for proSAAS and PC1. The low nM $K_i$ for the C-terminal proSAAS peptides with PC1 is likely to be within the physiological range based on the relatively high abundance of proSAAS-derived peptides detected upon mass spectrometry of mouse brain (16).

Another important finding is that the inhibitory region of
proSAAS is within a short 8–12-residue sequence near the C terminus. This region of proSAAS (VLGALLRVKRLE) is completely conserved among human, rat, and mouse (16) and has sequence similarity (5 matches over 12 residues) to both the C-terminal region and the pro-region of rat PC1. Interestingly, both the pro-region (8) and the C-terminal region of PC1 (27) have been shown to be inhibitory toward PC1. However, the inhibitory sequences within these regions have not been defined, and it is premature to speculate that the regions in PC1 with amino acid similarity to proSAAS are functional. Interestingly, this region of proSAAS contains the LLRVKR sequence previously identified from a screen of a peptide combinatorial library (28). The hexapeptide acetyl-LLRVKR-amide was the most potent inhibitor of PC1 found from this analysis (28). Consistent with our finding that LE-8 inhibits PC1 in a purely competitive fashion, the hexapeptide was also found to be a competitive fast-binding inhibitor (28). Thus, the additional N- and/or C-terminal residues in proSAAS and in YE-17 contribute to the slow tight binding inhibition of PC1.

Surprisingly, the short proSAAS peptides are more potent than full-length proSAAS as PC1 inhibitors. This may be due to improper folding of the majority of GST-proSAAS fusion protein produced in bacteria. Although the short 8–16-residue inhibitory peptides are not likely to be present in neuroendocrine tissues, a 4-kDa peptide that includes this 16-residue region has been detected upon overexpression of proSAAS in AtT-20 cells (16). This 4-kDa C-terminal fragment is the major form of C-terminal immunoreactive peptide secreted from the proSAAS-expressing AtT-20 cells (16). In addition to the 4-kDa fragment, shorter C-terminal fragments were detected in the media of these AtT-20 cells, indicating that cleavage had occurred at the critical Lys-Arg site within the inhibitory region (16).

Another major finding of the present study is that proSAAS binds tightly to PC1. Although other investigators have not detected proSAAS during searches of proteins that bind to PC1 (27), proSAAS is within a short 8–12-residue sequence near the C terminus. This region of proSAAS (VLGALLRVKRLE) is completely conserved among human, rat, and mouse (16) and has sequence similarity (5 matches over 12 residues) to both the C-terminal region and the pro-region of rat PC1. Interestingly, both the pro-region (8) and the C-terminal region of PC1 (27) have been shown to be inhibitory toward PC1. However, the inhibitory sequences within these regions have not been defined, and it is premature to speculate that the regions in PC1 with amino acid similarity to proSAAS are functional. Interestingly, this region of proSAAS contains the LLRVKR sequence previously identified from a screen of a peptide combinatorial library (28). The hexapeptide acetyl-LLRVKR-amide was the most potent inhibitor of PC1 found from this analysis (28). Consistent with our finding that LE-8 inhibits PC1 in a purely competitive fashion, the hexapeptide was also found to be a competitive fast-binding inhibitor (28). Thus, the additional N- and/or C-terminal residues in proSAAS and in YE-17 contribute to the slow tight binding inhibition of PC1.
PC1,2,3 these studies used [35S]Met-labeled cell extracts and would therefore not have detected proSAAS because this protein lacks Met (except for the initiation methionine, which is cleaved off with the rest of the signal peptide). Also like 7B2 (11), proSAAS stains extremely weakly with Coomassie Blue and silver staining procedures and has an extremely low extinction coefficient at 280 nm because of the low abundance of aromatic residues (16). Thus, proSAAS has presumably been undetected in previous studies because of the inadequacy of tools to visualize this protein.

The tight binding and potent inhibition of PC1 by proSAAS raises the issue as to how this inhibition is regulated. A likely mechanism is cleavage at the Lys-Arg sequence within the inhibitory region; as mentioned above, forms of smaller proSAAS peptides resulting from cleavage at this site have been detected in mouse brain and proSAAS-expressing AtT-20 cells (16). The protease responsible for this cleavage is not known. It is possible that PC1 itself is able to slowly cleave this proSAAS C-terminal inhibitory peptide, as is the case for PC2-mediated cleavage of the 7B2 inhibitory peptide (13). Alternatively, another member of the PC gene family may cleave at the Lys-Arg site in the inhibitory region of the longer 4-kDa peptide to generate the shorter, noninhibitory peptides detected in cells and in mouse brain. Although the presence of a Leu in the P1' position would be expected to reduce its affinity for most of the PCs (23, 29, 30), the presence of an Arg in the P4 position would greatly improve its affinity for furin and related PCs (31).

There are several similarities between 7B2 and proSAAS despite the lack of amino acid sequence similarity. As described in the Introduction, both of these proteins are broadly expressed neuroendocrine proteins of similar sizes, and they both contain many acidic residues with several pairs of basic residues. Furthermore, the inhibitory domains of both proteins are located near the C terminus, and these domains involve pairs of residues. Furthermore, the inhibitory domains of both proteins contain many acidic residues with several pairs of basic residues. Cleavage at these pairs of basic amino acids eliminates the inhibitory activities of the peptides. One difference is that 7B2 is also a chaperone for PC2 and is required for the expression of active enzyme (14), whereas PC1 is expressed quite well in non-neuroendocrine expression systems that lack proSAAS (21, 25). In addition to functioning as an endogenous inhibitor/chaperone of PC2, it is likely that 7B2 has other functions; mice lacking PC2 are viable, whereas those lacking 7B2 die during adolescence (32). It is possible that proSAAS also performs other functions in addition to inhibiting PC1.

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