The SAAS granin exhibits structural and functional homology to 7B2 and contains a highly potent hexapeptide inhibitor of PC1

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Abstract Prohormone convertases (PCs) 1 and 2 are thought to mediate the proteolytic cleavage of many peptide precursors. Endogenous inhibitors of both PC1 and PC2 have now been identified; the 7B2 protein is a nanomolar inhibitor of PC2, while the novel protein proSAAS was recently reported to be a micromolar inhibitor of PC1 [Fricker et al. (2000) J. Neurosci. 20, 639–648]. We here report evidence that 7B2 and proSAAS exhibit several elements of structural and functional homology. Firstly, 26 kDa human, mouse and rat proSAAS, like all vertebrate 7B2s, contain a proline-rich sequence within the first half of the molecule and also contain a C-terminal 40 residue peptide (SAAS CT peptide) separated from the remainder of the protein by a furin consensus sequence. The SAAS CT peptide contains the precise sequence of a hexapeptide previously identified by combinatorial peptide library screening as a potent inhibitor of PC1, and the vast majority of the inhibitory potency of proSAAS can be attributed to this hexapeptide. Further, like the 7B2 CT peptide, SAAS CT-derived peptides represent tight-binding competitive convertase inhibitors with nanomolar potencies. Lastly, recombinant PC1 is able to cleave the proSAAS CT peptide to a product with a mass consistent with cleavage following the inhibitory hexapeptide. Taken together, our results indicate that proSAAS and 7B2 may comprise two members of a functionally homologous family of convertase inhibitor proteins.

Key words: Prohormone convertase 1 and 2; Furin; SAAS granin; 7B2; Inhibitor

1. Introduction

The prohormone convertases (PCs) are a family of eukaryotic subtilisins thought to be involved in the synthesis of a wide variety of secreted proteins (reviewed in [1]). Certain of these enzymes, such as furin, are expressed ubiquitously, while other members of the family, such as PC1 and PC2, are found primarily in neuroendocrine tissues, where they accomplish the biosynthetic cleavage of peptide hormone precursors such as proinsulin and proopiomelanocortin (reviewed in [1]).

Most proteinases are physiologically controlled by endogenous inhibitor systems. However, until this year, only one natural inhibitor of a member of the eukaryotic subtilisins was known, the neuroendocrine protein 7B2, which contains a 31 residue peptide with highly potent and specific inhibitory activity against PC2 (reviewed in [2]). Recently Fricker et al. [3] identified a new neuroendocrine protein known as proSAAS, which was shown to inhibit PC1 with micromolar inhibitory potency. In this report, we present evidence to support the notion of structural and functional similarities between 7B2 and proSAAS and we identify the PC1-inhibitory hexapeptide within proSAAS [4].

2. Materials and methods

2.1. Peptide synthesis

Peptide synthesis was performed by Louisiana State University Health Sciences Center Core Laboratories; mass spectroscopy was used to verify identity.

2.2. Enzyme assays

The enzyme assays for PC1 and PC2 were performed at pH 5.0 using pGlu-Arg-Thr-Lys-Arg-methylcoumarinamide (pERTKR-MCA) (Peptides International, Louisville, KY, USA) as described in [4]; the assay for furin was performed using the same substrate at pH 7.0 in 100 μM HEPES, 5 mM $CaCl_2$ and 0.1% Brij 35. All assays were performed at 37°C in a 96 well fluorometer (Labsystems) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. When used, inhibitory peptides were preincubated with enzyme for 30 min prior to addition of substrate. All assays were performed in duplicate or triplicate. For the determination of IC_{50}'s, the inhibitor concentration was varied from 1×10^{-4} to 1×10^{-11} M according to the method previously described [4]. K_i's were determined as previously described [4]. Kinetics were analyzed using EnzFitter (BioSoft, Cambridge, UK) using K_m's of 11, 42 and 8 μM for PC1, PC2 and furin, respectively.

2.3. Production of recombinant convertases

Purified recombinant mouse PC1 and PC2 was obtained from CHO cells as previously described [4,5]. Recombinant soluble mouse furin was similarly purified from the conditioned medium of CHO cells stably transfected with truncated mouse furin (D108-H771) and amplified to 50 μM methotrexate (Cameron et al., in preparation).

2.4. Cleavage of SAAS Tyr^0CT by PC1

SAAS Tyr^0CT (residues S219–P258 in intact mouse proSAAS with the addition of an N-terminal tyrosine) was iodinated by the chloramine T method and purified by reverse-phase chromatography on a C-4 column. Iodinated SAAS Tyr^0CT (10 000 cpm) was incubated with 94 ng of PC1 in 50 μl at 37°C for varying times. Reaction products were separated by high-performance liquid chromatography (HPLC) using a Biosil TSK 125 (Bio-Rad), a Protein Pak 300SW (Waters) and a TSK A0085 (Bio-Rad) column in series. The flow rate was 0.5 ml/min and 0.5 min fractions were collected and counted by gamma spectroscopy.

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Abbreviations: PC, prohormone convertase; pERTKR-MCA, pGlu-Arg-Thr-Lys-Arg-methylcoumarinamide; SAAS CT, residues S219–P258 in intact mouse proSAAS; SAAS Tyr^0CT, residues S219–P258 in intact mouse proSAAS with the addition of an N-terminal tyrosine; SAAS CT1–24, residues S219–R242 in intact mouse proSAAS

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^{1}SVDQDLGPEVPPENVLGALLRVKR↓LENPSPQAPARRLLPP^{40}

Fig. 1. Sequence of SAAS CT. The sequence of SAAS CT is shown; SAAS CT1–24 is underlined. The hexapeptide previously identified as a potent inhibitor of PC1 by combinatorial peptide library screening (4) is double-underlined. The putative cleavage site is indicated by an arrow.

Table 1
Inhibition constants for proSAAS-derived peptides against PC1, PC2 and furin

	K_i (nM) ± S.D.		
	PC1	PC2	furin
SAAS Tyr^{0}CT	7.4 ± 0.5	10 500 ± 700	< 20 000
SAAS CT1–24	1.5 ± 0.02	990 ± 20	10 000 ± 700
LLRVKR	5.4 ± 0.2	3 700 ± 170	420 ± 14

3. Results

3.1. Fragments of SAAS are tight-binding or slow-binding competitive inhibitors of PC1

We noted that proSAAS contains the precise sequence of a hexapeptide previously identified by combinatorial peptide library screening as a potent inhibitor of PC1 [4]. In order to verify that the inhibitory activity observed by Fricker et al. [3] against PC1 was indeed due to this hexapeptide [4], synthetic peptide fragments of proSAAS were tested against purified recombinant mouse PC1, PC2 and furin. We used SAAS Tyr^{0}CT (corresponding to residues S219–P258 in intact mouse proSAAS with the addition of an N-terminal tyrosine), SAAS CT1–24 (corresponding to residues S219–R242 in intact mouse proSAAS) and the LLRVKR hexapeptide previously identified but lacking the terminal blocking groups; sequences are shown in Fig. 1. As double-reciprocal plots revealed curvature at high substrate and inhibitor concentrations (results not shown), we established whether or not the SAAS CT fragments were tight or slow-binding according to the methods described in [6] and then deduced the nature of the interaction between PC1 and the inhibitors. The data in Fig. 2a,c show that for both SAAS CT1–24 and its C-terminal hexapeptide, the IC_{50} increased with enzyme concentration, a defining characteristic of tight and slow-binding reversible inhibitors [6,7]. In Fig. 2b,d, the linear increase of IC_{50} with substrate concentration demonstrates that both peptides behaved as strictly competitive inhibitors.

3.2. Fragments of proSAAS are specific nanomolar inhibitors of PC1

The K_i's of the peptides against PC1, PC2 and furin are shown in Table 1; all were nanomolar inhibitors of PC1 and exhibited at least two orders of magnitude less inhibitory activity against both PC2 and furin. It is interesting to note that the K_i's of the SAAS CT fragments against PC1 and PC2 exhibited increased potency with increased peptide length (i.e. a 3.6- to 3.7-fold decrease in K_i); in contrast, the short hexapeptide was a 24-fold better inhibitor of furin than was the longer SAAS CT 1–24. In all cases the potency of SAAS Tyr^{0}CT was less than that of the shorter peptides. When 50 μg of SAAS CT1–24 was incubated in the presence of 150 ng of carboxypeptidase B (Worthington) for 30 min at 37°C, the inhibitory action of the peptide against PC1 was abolished.

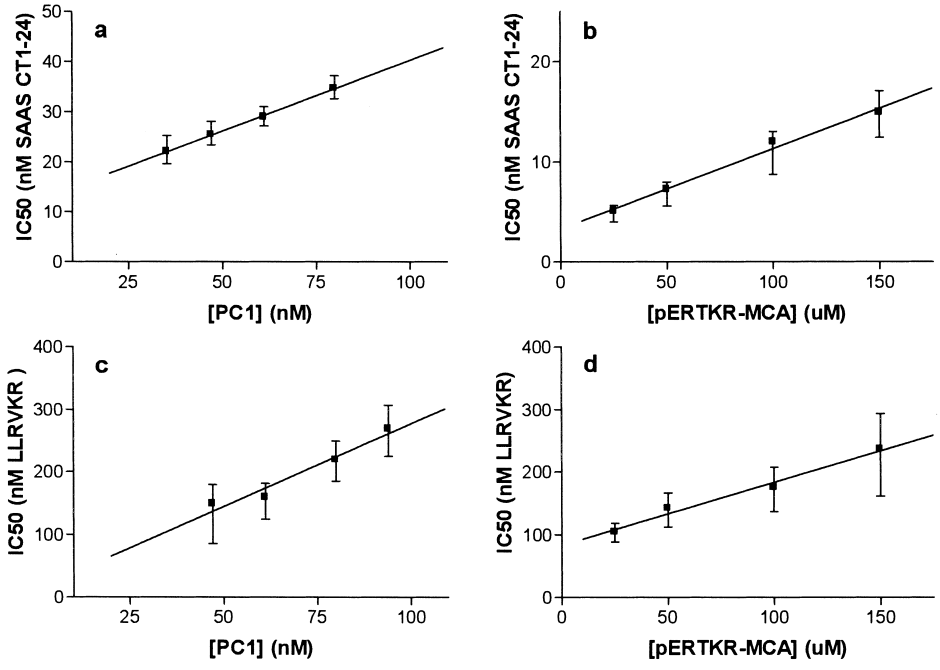

Fig. 2. Effect of enzyme and substrate concentration on the IC_{50} of proSAAS derived peptides against PC1. IC_{50}'s were determined at varying enzyme and substrate concentrations using SAAS CT1–24 (a and b) or LLRVKR (c and d). In (a) and (c) the substrate concentration was fixed at 200 μM pERTKR-AMC. In (b) and (d) the enzyme concentration was fixed at 47 and 94 nM, respectively. All assays were performed in duplicate at 37°C in 0.1 M sodium acetate buffer, pH 5, 0.1% octyl glucoside and 5 mM calcium chloride. The error bars represent the 95% confidence intervals.

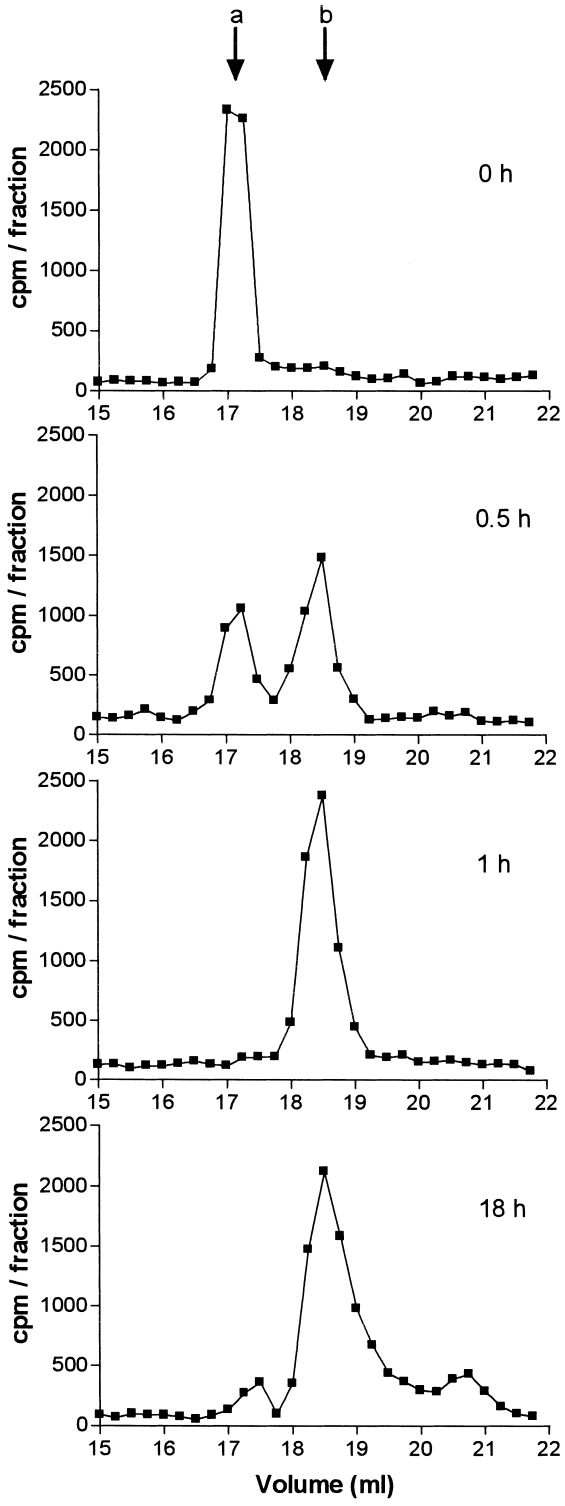

Fig. 3. Cleavage of SAAS Tyr^{0}CT by PC1. Iodinated SAAS Tyr^{0}CT (10 000 cpm) was incubated at 37°C with 94 ng PC1 in 50 μl of 0.1 M sodium acetate buffer, pH 5, 0.1% octyl glucoside and 5 mM calcium chloride for 0, 0.5, 1 or 18 h. The reaction products were separated by HPLC and 0.25 ml fractions counted by gamma spectroscopy. The column was calibrated using peptides of known molecular mass with UV detection at 214 nm. The elution volumes of SAAS Tyr^{0}CT and SAAS CT1–24 are labeled (a) and (b), respectively.
←

3.3. PC1 cleaves SAAS Tyr^{0}CT to produce a fragment with a mass corresponding to that of the inhibitory sequence SAAS CT1–24

The 7B2 CT peptide is known to be internally cleaved by PC2 at a paired basic site; the resulting peptide remains inhibitory until removal of the carboxy-terminal basic residues by carboxypeptidase action [8]. In order to determine whether a similar mechanism might hold for PC1/proSAAS, we incubated SAAS Tyr^{0}CT with recombinant PC1 and separated products by gel permeation HPLC. The data shown in Fig. 3 clearly indicate the production of a smaller PC1-generated cleavage product that had a mass corresponding to SAAS CT1–24.

4. Discussion

The first inhibitor of a member of the family of eukaryotic subtilisins to be identified, the neuroendocrine protein 7B2, contains a carboxy-terminal peptide which represents a nanomolar tight-binding inhibitor of PC2, with no inhibitory activity against the related enzyme PC1 [9,10]. The second neuroendocrine convertase inhibitor, proSAAS, was recently described by Fricker et al. [3]. Our data indicate that the great majority of the inhibitory activity of proSAAS against PC1 is borne by a hexapeptide sequence which, like 7B2, is located within the carboxy-terminal peptide. Interestingly, this precise hexapeptide was previously identified by our group in a combinatorial peptide library screen of PC1 inhibitors [4]. The ability of the peptide library screen to predict the sequence of a naturally occurring protein is remarkable, and demonstrates the power of combinatorial library screens in the identification of natural compounds.

Both SAAS CT-derived peptides and the 7B2 CT peptide represent very potent inhibitors of convertases, exhibiting nanomolar K_i's. The micromolar K_i of a GST–SAAS fusion protein against PC1 previously obtained [3] may be due to a destabilizing influence of the amino-terminal domain, or to the use of unpurified enzyme; these data are difficult to interpret given that the GST moiety alone also appeared to be inhibitory. Our data also imply that SAAS CT-related peptides may not be as enzyme-specific as the 7B2 CT peptide, as SAAS-derived CT peptides also exhibited some inhibitory potency against PC2 and, to a lesser extent, against furin. The inhibition of PC1 and PC2 by SAAS-derived CT peptides displays a similar response, increasing inhibition, to amino-terminal extension of the hexapeptide inhibitor, while the more distantly related enzyme furin is affected oppositely, suggestive of significant structural similarities within the binding pockets of the two neuroendocrine enzymes as opposed to the constitutively-expressed enzyme furin.

The mechanism of inhibition of PC1 by proSAAS is not yet clear. Fricker et al. [3], using GST-fusion proteins containing full-length proSAAS, observed double-reciprocal plots indicative of either tight-binding competitive or of non-competitive kinetics against PC1. In our hands, SAAS-derived CT peptides were found to exhibit strictly competitive kinetics against PC1; tight-binding competitive kinetics have also been reported for the inhibition of PC2 by the 7B2 CT peptide [9]. Recent data indicate that inhibition of PC2 by the 7B2 CT peptide requires two distinct sites within the peptide: the highly conserved heptapeptide VNPYLQG as well as the

hexapeptide containing the inhibitory dibasic pair [13]. A similar two-site mechanism is apparently not required for inhibition of PC1 by the SAAS CT peptide, as the hexapeptide containing the dibasic pair is a highly potent inhibitor in its own right. Despite these differences, our data indicate that both the SAAS and the 7B2 CT peptides represent nanomolar inhibitors of PCs and are thus likely to function in the physiological control of convertase activity within the secretory pathway.

Inactivation of the 7B2 CT peptide is thought to be accomplished by PC2-mediated hydrolysis at the inhibitory dibasic pair, followed by carboxypeptidase action [8]. Our data indicate that a similar mechanism might hold for the inactivation of the SAAS CT peptide by PC1, as iodinated SAAS CT peptide was efficiently cleaved by PC1 in vitro to yield a peptide product with a mass corresponding to that of SAAS CT1–24. In addition, incubation of SAAS CT1–24 with carboxypeptidase B abolished inhibitory potency. While further work will be required to establish that cleavage of SAAS CT is indeed specific to PC1 in vivo, the ability of PC1 to accomplish this cleavage in vitro lends credence to the idea that inactivation of the two CT peptides might occur similarly.

While the primary sequences of 7B2 and proSAAS are not homologous, the overall structures of these two proteins exhibit a striking resemblance, with the inhibitory sequences located at the carboxyl terminus following a furin consensus sequence (RXXR). Further, proSAAS contains many conserved proline residues located in the middle of the protein, a feature and location reminiscent of the proline-rich sequence (PPNPCP) present in all known 7B2s [11] which is known to be absolutely required for biological activity [12]. While it is difficult to search for proline-rich sequences in the database due to their relatively high abundance, we speculate that as-yet-to-be discovered inhibitors of other members of the vertebrate convertase family will exhibit the same elements of structural homology as do proSAAS and 7B2: a proline-rich sequence in the middle of the protein; a furin consensus site approximately 30–40 residues from the carboxyl terminus and a carboxyl-terminal peptide containing a potent inhibitory sequence.

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