Functional Characterization of ProSAAS
SIMILARITIES AND DIFFERENCES WITH 7B2

Yolanda Fortenberry, Jae-Ryoung Hwang, Ekaterina V. Apletalina, and Iris Lindberg

From the Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, New Orleans, Louisiana 70112

The prohormone convertases (PCs) are a family of serine proteinases that are believed to mediate the proteolytic cleavage of precursor proteins to mature polypeptides (for review see Ref. 1). Presently, eight members of this family have been identified based on homology to the bacterial proteolytic enzyme subtilisin. Although members of this family of proteolytic enzymes are structurally similar, their expression patterns differ. For example, furin and PACE4 are expressed ubiquitously, whereas PC4 is mainly expressed in the testes, and PC1 (also known as PC3) and PC2 are found mainly in neuroendocrine tissues (2, 3). PC1 and PC2 are involved in the processing of several neuropeptide precursors such as proinsulin (4, 5), proenkephalin (PE) (6, 7), and proopiomelanocortin (POMC) (8, 9).

The first endogenous convertase binding protein identified was the neuroendocrine protein 7B2, whose function was initially thought to consist of potent and specific inhibition of PC2 (10). In fact, recent studies show that 7B2 represents a complex bifunctional binding protein with two domains encoding opposing functions. The N-terminal domain is responsible for the production of activatable pro-PC2 (11, 12) whereas the C-terminal domain (CT peptide) represents a potent PC2 inhibitor (13, 14). The mechanism by which the N-terminal domain of 7B2 is able to effect the productive maturation of pro-PC2 to an active enzyme species is not yet clear, but it is thought to involve Golgi-mediated cellular processes rather than a direct effect on activation per se (reviewed in Ref. 15).

Recently a new endogenous convertase binding protein was identified and termed proSAAS; this protein was shown to inhibit PC1 (16). ProSAAS, like 7B2, contains two domains, an N-terminal domain and a C-terminal domain, separated by a furin cleavage site. GST-proSAAS was reported to inhibit PC1 with an IC_{50} of 2.7 μM (16). The sequence responsible for the inhibitory potency of PC1 was narrowed to a hexapeptide, LLRVKR, located in the proSAAS C-terminal domain (17), and previously identified by combinatorial library peptide screening (18) as a tight-binding competitive inhibitor against PC1.

Prohormone convertases (PC) 1 and 2, enzymes found primarily in neuroendocrine tissues, are thought to mediate the proteolytic cleavage of many peptide precursors. To date, endogenous binding proteins for both PC2 (7B2) and PC1 (proSAAS) have been identified. Although 7B2 represents a potent inhibitor of PC2, the most important function of 7B2 as regards this enzyme appears to be the absolute requirement of PC2 for 7B2 in the generation of active enzyme, recently corroborated through production of a null animal that lacks PC2 activity. The purpose of the present study was to determine whether proSAAS exerts effects on PC1 other than inhibition, and to establish functional similarities and differences between 7B2 and proSAAS. We first asked whether the N-terminal domain of proSAAS (proSAAS-(1–180)) could stabilize PC1 activity, similar to the effect of the N-terminal domain of 7B2 on PC2. Recombinant His-tagged proSAAS-(1–180) had no effect on PC1 activity in vitro and was unable to protect PC1 from thermal denaturation. Transient cotransfection of proSAAS-(1–225) cDNA with PC1 cDNA into HEK 293 cells reduced the amount of PC1 activity detected in the medium. Surprisingly, cotransfection of proSAAS-(1–180) cDNA, encoding a protein that lacks the inhibitory C-terminal domain peptide, also reduced the activity of PC1 detected in the medium, but the mass of PC1 secreted into the medium was increased, suggesting a proSAAS-mediated activation reaction. Similar results were observed in CHO/PC1 cells stably transfected with proSAAS-(1–180). Stable transfection of SAAS cDNAs into AtT-20 cells was used to examine the role of proSAAS in a neuroendocrine setting. Unlike 7B2, proSAAS-(1–225) was able to slow convertase-mediated processing of proopiomelanocortin and proenkephalin; however, similarly to 7B2, proSAAS expression did not result in any accumulated differences in the content of cellular processed peptide. In summary, although both proSAAS and 7B2 potently inhibit PC enzymes via a C-terminal peptide, their intracellular interactions with PCs appear to differ significantly, with each binding protein exhibiting unique properties.

* This work was supported by National Institutes of Health Grants DK49703 and DA05084 (to I. L.), by K award DA00204 (to I. L.), and by Predoctoral Fellowship DK49703S2 (to Y. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, 1901 Perdido St., New Orleans, LA 70112. Tel.: 504-568-4799; E-mail: ilindb@LSUHSC.edu.

‡ This was supported by National Institutes of Health Grants DK49703 and DA05084 (to I. L.), by K award DA00204 (to I. L.), and by Predoctoral Fellowship DK49703S2 (to Y. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
with nanomolar potency (17–19). Thus both 7B2 and proSAAS contain inhibitory CT peptides. Although the 7B2 CT peptide is highly specific for PC2, the proSAAS CT peptide is apparently not as specific for PC1. The peptide containing the first 24 residues of the proSAAS C-terminal domain, SAAS CT 1–225, was then subcloned into the hygromycin-encoding pcDNA 3.1 vector containing mutations from 208KR209 to SS in the C-terminal region. The eukaryotic expression vector encoding this peptide was subcloned directly from a zeocin-encoding pcDNA 3.1 vector at the Kpn I site. For the eukaryotic vectors, proSAAS-(1–225) was subcloned into the pcDNA 3.1 vector at the Kpn I site for subcloning into the pQQE30 vector (Qiagen); this vector adds an N-terminal hexahistidine tag as well as 8 additional residues. ProSAAS-(1–225) was also used to compare cell amounts per well in CHO/PC1 cells as well as transiently transfected HEK cells.

**MATERIALS AND METHODS**

**Construction of Prokaryotic and Eukaryotic Vectors—** Both full-length proSAAS-(1–225) and the N-terminal domain proSAAS-(1–180) were amplified via PCR. The following primers were used: 5′-CGG GGT ACC GCC AGG CCC GTG AAG GAG CCC-3′, directed toward the 5′-end (used for construction of both plasmids); the two primers directed toward the 3′-end were 5′-CGG AAG CCT ATG ATT GAG GCC GTA GGC-3′ (for proSAAS-(1–225)) and 5′-CGG GGT ACC TCA TCA GAG GCC GCG CCC-3′ (for proSAAS-(1–180)). The 5′-primer for proSAAS-(1–180) contains an engineered Kpn I site for subcloning into the pQE30 vector (Qiagen); this vector adds an N-terminal hexahistidine tag as well as 8 additional residues. ProSAAS-(1–225) was subcloned between the Kpn I and HindIII sites, and proSAAS-(1–180) was subcloned into the pQE30 site. For the eukaryotic vectors, proSAAS-(1–225) was subcloned directly from a zeocin-encoding pCDNA 3.1 vector into a hygromycin-encoding pcDNA 3.1 vector (both from Invitrogen) between the EcoRV and HindIII sites. Truncated proSAAS-(1–180) was amplified via PCR from the plasmid pBluescript supplied by J. Douglass toward the 3′-end. The following primers were used: 5′-CGG CCT GTC GGA GCC GCA GCC-3′ directed toward the 5′-end and 5′-GCC TAC GCC TAC GGA AGC AGC CTC-3′ directed toward the 3′-end. The PCR-amplified product was ligated into the pcDNA 3.1 zeocin-encoding vector between the EcoRI and XhoI sites. The cDNA encoding SAAS prokaryotic vector encoding a protein containing a mutations from 208KR209 to SS in the C-terminal region were generated in a similar fashion using mouse proSAAS-(1–225) as a template. The primers used for generating the first PCR fragment were 5′-CTGCTACGCGTCAGTAGCCTGGAGAACCCCTCGC-3′ and 5′-GGC-GAACGGTATTATTAGGGCTCAGG-3′. The product from the first round of PCR was used as a C-terminal primer with the 5′-primer (5′-CGGCGTAGCCGAGGCCCTGTAGGAGGACC-3′) for generating the second PCR fragment. The PCR fragment was then cloned into the pQE30 vector at the Kpn I and HindIII sites. All cDNA sequence generated by PCR was verified by DNA sequencing.

**Cell Culture, Transfection, and Selection—** Parental AtT-20, and AtT-20 cells stably expressing proenkephalin (PE) (AtT-20PE (21)) were used in these studies. AtT-20 cells were cultured at 37 °C in 5% CO2. They were maintained in DMEM high glucose medium containing 10% Fetal Bovine Serum (FBS) (Invitrogen, Gaithersburg, MD, 50% active) and 10 μg/ml hygromycin (Sigma Chemical Co., St. Louis, MO). Chinese hamster ovary (CHO) cells expressing PC1 were transfected (22, 23). CHO/PC1 cells were grown in minimal essential medium lacking nucleosides (α-minimal essential medium, Invitrogen) containing 10% dialyzed heat-inactivated FBS, with 5 μM methotrexate and 200 μg/ml G418. HER 293 cells were grown in DME high glucose medium containing 10% FBS.

Both AtT-20/PE and CHO cells were transfected with the above-mentioned eukaryotic vectors encoding either proSAAS-(1–225) or proSAAS-(1–180) using the Lipofectin method described previously (11). Approximately 1 × 10^6 cells were plated into 10-cm dishes and allowed to grow overnight. The following day the cells were washed twice with phosphate-buffered saline (PBS) and once with Opti-MEM (Invitrogen) followed by a single incubation with 3 μl of Opti-MEM containing 30 μl of Lipofectin DNA, 30 μl of Lipofectin, and 15 μl of gentamycin (100 μg/ml). Following incubation, 7 ml of growth medium containing 100 μg/ml hygromycin (Sigma) was added. The medium was changed twice a week, and after ~4–6 weeks hygromycin-resistant clones were selected and subcloned into 24-well plates using the soft agar method previously described (23). Cells were allowed to grow in 24-well plates for 7 days. After semiconfluence was reached, 1 ml of Opti-MEM was added to each well; in the case of AtT-20 cells phosphor 12-myristate 13-acetate (PMA) was added as a secretagogue, and cells were incubated overnight. The overnight medium was collected, and 100 μl was used to screen for expression of proSAAS by radioimmunassy (RIA). The LEP RIA used to detect expression of proSAAS has been previously described (17). The three highest-expressing clones were used for subsequent experiments.

For transient transfections, ~500,000 HER 293 cells were plated into 6-well plates on the day prior to use. The next day cells were incubated for 5 h with 1 ml of Opti-MEM containing 2 μg of cDNA vector encoding PC1 alone or doubly transfected with PC1 and either proSAAS-(1–225) or proSAAS-(1–180), respectively, and 10 μl of Lipofectamine (Invitrogen). The cells were then incubated for 24 h with 3 ml of complete medium, after which the wells were washed with Opti-MEM and incubated with 1 ml of Opti-MEM overnight. The conditioned Opti-MEM was collected, and PC1 activity was determined using a fluorogenic substrate (see below). Actual expression of proSAAS-(1–225) and proSAAS-(1–180) was verified by QERA RIA (see below). To verify the expression of PC1 and to compare amounts secreted, the conditioned medium was trichloroacetic acid-precipitated, or cells were solubilized in sample buffer, and samples were subjected to electrophoresis on 8.8% SDS-PAGE gel followed by Western blotting using antisera 2B6, directed against the N terminus of PC1 (24). The eukaryotic expression vector encoding 66-kDa PC1 and CHO cells expressing this C-terminally truncated form of PC1 have both been previously described (25). Western blotting using tubulin antisera (a kind gift of Dr. Kevin Brown, Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center) was also used to compare cell amounts per well at the time of harvest.

**Enzyme Assay—** PC1 and PC2 activity were measured using 200 μM fluorogenic substrate, pERK-TKFK-methylcoumarin amide (MCA), in an assay previously described (12). Briefly, each assay was performed in 50 μl containing 50 μM CaCl2, 50 μM ATP, 1 mM dithiothreitol, 0.1 mM Kemptide, 0.1% octyl glycoside in the presence of a protease inhibitor-mixture composed of 1 μM pepstatin, 0.28 mM TLCK, 1 μM trans-eosphysentic acid, and 0.14 mM TPCK. The assay for furin was performed using the same substrate at a pH of 7.0 in 100 mM HEPES, 5 mM CaCl2, and 0.1% Brij 35. All assays were performed at 37 °C in a 96-well plate fluorometer (LabSystems) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The fluorogenic substrate was incubated with the cell samples in 50 μl of complete medium. The absorbance at 5176 nm was measured in duplicate or triplicate every minute for a period of 2 h, and the rates of hydrolysis over this time were calculated; the first 10 min were excluded from the rate calculation. Enzyme activity is given in fluorescence units per minute (FU/min) where one FU corresponds to 8.6 pmol of MCA. Recombinant His-tagged proSAAS-(1–225) and SAAS CT 1–24 were preincubated for 30 min at room temperature with enzyme before substrate was added. Inhibition constants were determined using the following equation: \( K_i = K_{i,app}(1 + |S/K_m|) \), where \( K_m \) values for PC1, PC2, and furin were 11, 42, and 8 μM, respectively.

**Metabolic Labeling and Immunoprecipitation—** Approximately 500,000 AtT-20/PE cells were seeded into 6-well plates and allowed to grow overnight or until 70–80% confluent at 37 °C. The cells were washed twice with PBS and starved for 20 min at 37 °C in 1 ml of methionine- and cysteine-free DMEM (ICN Biomedicals, Irvine, CA) containing 10 mM HEPEs, pH 7.4. Cells were then labeled (pulsed) with 1 ml of methionine-and cysteine-free medium containing 0.5 μCi of [35S]methionine and cysteine (ProMix, Amersham Biosciences, Inc., Arlington Heights, IL) for 20 min. For steady-state labeling, cells were incubated for 6 h in [35S]methionine and cysteine-containing medium following 10 min of 0.1% HEPES, pH 7.4. After 10 min, the cells were either homogenized immediately in 1 ml of acid mix (1 n M acetic acid, 20 mM HCl, and 0.1% (β-mercaptoethanol) or further incubated in 1 ml of warm chase medium (DMEM high glucose medium containing 10 mM HEPES, pH 7.4, and 2% FBS) for 2 h and then homogenized in acid mix. The homogenized cells were frozen at −70 °C, thawed, centrifuged for 10 min at 4 °C, and the supernatants were removed and lyophilized.
Functional Characterization of ProSAAS

overnight. The dry cell extract was resuspended in 500 μl of AG buffer (0.1 M sodium phosphate, pH 7.4, 1 mM EDTA, 0.1% Triton, 0.5% Nonidet P-40, 0.9% NaCl, and 0.02% sodium azide) and centrifuged prior to use.

For immunoprecipitation, either 250 or 500 μl of cell extract was incubated with 100 μl of prehydrated 20% protein A-Sepharose beads (Amersham Biosciences, Inc., Piscataway, NJ) at 4 °C with constant rocking. The samples were centrifuged for 3 min; the supernatants were then transferred to fresh tubes and incubated overnight at 4 °C with 10 μl of polyclonal rabbit antiseraum LS41 (directed against ACTH 1-24 coupled to keyhole limpet hemocyanin); or 5 μl of antiseraum Xandra (directed against p53-de B) (29). The following day the samples were centrifuged for 5 min, and the supernatant was removed, placed in fresh tubes, and incubated with 100 μl of 20% protein A-Sepharose beads at 4 °C for 1 h with constant rocking. The samples were centrifuged, and beads were washed twice with 1 ml of AG buffer, once with 0.5 M NaCl in PBS, and once with PBS alone. Immunoprecipitated proteins were extracted from the beads by adding 40 μl of 1 M acetic acid and 80 μl of 8 M urea in 32% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA). This mixture was incubated at room temperature for 15 min and centrifuged, and the supernatant was removed. Supernatants were either frozen at −70 °C, or 100 μl was immediately size-fractionated by high pressure gel permeation chromatography (HPGPC) in 32% ACN, 0.1% TFA as previously described (27). Radioactivity in each fraction was determined directly by on-line liquid scintillation spectroscopy using a β-RAM system (INUS, Tampa, FL). The flow rate of the scintillant was 4 ml/min; that of the eluant was 0.5 ml/min.

Expression of PC1: SDS-PAGE and Western Blotting—Approximately 500,000 CHO/PC1 or CHO/PC1 cells expressing either proSAAS-(1-225) or proSAAS-(1-180) were plated into 6-well plates and allowed to grow overnight. The next day cells were washed twice with PBS and incubated with Opti-MEM medium. Conditioned medium was removed after 3 h, 6 h, 9 h, and overnight. Lømmel sample buffer (5×) was added to 40 μl of conditioned medium, boiled for 5 min, and subjected to electrophoresis on 8.8% polyacrylamide SDS gels. Proteins were transferred from gels to nitrocellulose membranes, and the membranes were preincubated in 5% nonfat milk in TBS for 30 min at room temperature prior to incubation overnight at 4 °C with antiseraum 2B6 (anti-PC1 antiseraum directed toward the N-terminal region) diluted 1:1000 in milk. The membrane was washed three times with TBS containing 0.05% Tween followed by incubation at room temperature for 1 h with secondary antibody (goat anti-rabbit IgG coupled to alkaline phosphatase) diluted 1:10,000 in milk. The membrane was then washed once with TBS containing 0.05% Tween and twice with TBS alone prior to development with 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt/p-nitro blue tetrazolium chloride).

Radioimmunoassay—RIAs for the midregion of proSAAS, directed toward the sequence QERARAAEQEAD (QERA), and the C-terminal region of proSAAS, directed toward the sequence LENPSQPAPA (LENP) were carried out by procedures described previously (17). Briefly, samples were incubated with 10,000 cpm of iodinated peptide and separated by gel permeation chromatography as previously described (17).

Preparation of the proSAAS mutant protein containing an inactivating mutation in the inhibitory peptide (LLRVKR to LLRVS) was accomplished in a similar fashion; reverse-phase high performance liquid chromatography was performed. The fractions that contained the His-tagged proSAAS SS mutant were pooled, lyophilized, and resuspended in water containing 10 mM acetic acid. This preparation (∼50% pure) was used to assess effects on PC1 activity in Golgi- and ER-enriched membrane fractions obtained from CHO/PC1 cells.

Stimulation of Peptide Release—AtT-20/PE cells expressing either proSAAS, proSAAS-(1-225), or proSAAS-(1-180) were plated into 6-well plates and allowed to grow until 70% confluent. Cells were washed three times with TBS and then incubated with 1 ml of Opti-MEM medium for 1 h. The cells were then washed once with PBS and incubated in 1 ml of Opti-MEM for an additional 3 h (basal secretion). The medium was collected, and cells were incubated in the presence of stimulating agent, which consisted of 1 ml of Opti-MEM plus 100 mM phorbol 12-myristate 13-acetate (PMA) for 3 h at 37 °C. A 100-μl aliquot from basal and stimulated medium was assayed for proSAAS expression using the LENP and QERA RIAs.

Effect of ProSAAS-(1-180) on the Thermal Stability of PC1—Recombinant mouse PC1, obtained from purification from CHO/PC1 cells, was incubated at 45 °C for various times in 100 mM sodium acetate buffer, pH 5.0, containing 5 mM CaCl2 and 0.1% octyl glucoside in the presence or absence of 6.8 μM purified recombinant His-tagged proSAAS-(1-180). Aliquots were removed at specific times and kept on ice for subsequent determination of residual PC1 enzymatic activity under standard conditions. The experiment was repeated once with a different preparation of proSAAS-(1-180) with identical results.

Preparation of Golgi- and ER-enriched Subcellular Fractions—Golgi- and ER-enriched subcellular fractions were prepared from CHO cells overexpressing mPC1 (CHO/PC1 cells) as previously described for CHO/PC2 cells (30). Briefly, cells were grown in four 850-cm2 rollers for each preparation. The cells were washed with calcium-free PBS then scraped in the same buffer. After centrifugation for 10 min at 1500 rpm, cells were resuspended in 6 ml of 0.25 M sucrose in 10 mM Tris-HCl, pH 7.4, and homogenized with a ball-bearing homogenizer. The homog- enate was centrifuged for 20 min at 10,000 rpm, and the supernatant was pelleted at 56,000 rpm in a Beckman TL-100.4 rotor at 4 °C. The pellets were resuspended in 1.15 ml of 10 mM Tris-HCl, pH 7.4, and loaded on the bottom of a discontinuous sucrose gradient consisting of each 1.5 ml of 0.86 M sucrose and 0.25 M sucrose in 10 mM Tris-HCl, pH 7.4. The gradient was centrifuged in the same rotor at 46,000 rpm for 140 min, and five fractions were collected. Each fraction was assayed individually, as described previously (30).
FIG. 1. Recombinant His-tagged proSAAS-(1–225) is inhibitory against PC1 but is not as potent as smaller peptides. Various concentrations of either recombinant His-tagged proSAAS-(1–225) or the synthetic peptide proSAAS-(1–24) were assayed for their potency against PC1. Squares, recombinant His-tagged proSAAS-(1–225); triangles, the synthetic peptide SAAS CT (1–24). Enzyme assays were performed in duplicate in polystyrene microtiter plates; duplicate values agreed to within 15% of each other.

µg of protein) were incubated either in the absence of proSAAS or in the presence of 1 µM of purified His-tagged proSAAS-(1–225), proSAAS-(1–180), proSAAS SS mutant, or the synthetic peptide, LLRVRK, in 100 mM sodium acetate, pH 5.0, containing 5 mM CaCl₂, 0.1% Triton X-100, and a protease inhibitor mixture composed of 1 µM trans-epoxy-succinic acid, 1 µM pepstatin, 280 µM TPCK, and 140 µM TLCK. Incubations were conducted at 37 °C for 2 h. PC1 activity was estimated in aliquots of the Golgi reactions using 290 µM Pyr-Glu-Arg-Thr-Lys-Arg-methylcoumarin amide as a substrate as described above. Experiments were performed with Golgi and ER fractions obtained from two different preparations.

RESULTS

Recombinant His-tagged proSAAS-(1–225) Is a Potent PC1 Inhibitor in Vitro—To determine the potency of full-length proSAAS-(1–225) on PC1 activity, we expressed and purified His-tagged recombinant proSAAS-(1–225). Fricker et al. (16) reported that full-length GST-proSAAS-(1–225) is inhibitory against PC1 with an IC₅₀ in the micromolar range. By contrast, reported that full-length GST-proSAAS-(1–24) performed with Golgi and ER fractions obtained from two different preparations.

Previous studies suggested that GST-proSAAS associates

with the 66-kDa form of PC1 and not with the 87-kDa form (19). To determine whether our lack of effect of proSAAS-(1–180) was due to its lack of association with the 87-kDa form of PC1, we also tested the effect of this protein on the activity of 71- and 66-kDa forms of PC1 in vitro. There was similarly no effect of proSAAS-(1–180) on these forms of PC1 (data not shown). Thus, proSAAS-(1–180) appears to differ from 7B2 in being unable to stabilize any form of PC1 activity in vitro.

We have previously shown that the N-terminal domain of 7B2 is capable of protecting PC2 from thermal denaturation (12). Because the N-terminal domain of proSAAS contains proline-rich sequences, as does the N-terminal domain of 7B2, we speculated that the two neuroendocrine proteins might exert the same protective effect on their respective convertases. However, unlike 7B2, the N-terminal region of proSAAS was unable to protect thermally denatured PC1 from inactivation (data not shown).

FIG. 2. Recombinant proSAAS-(1–180) has no effect on PC1 activity in vitro and is unable to protect PC1 from thermal denaturation. Recombinant His-tagged proSAAS-(1–180) (6.8 µM) and purified 87-kDa PC1 (1 µM) were incubated with 200 µM fluorogenic substrate in 100 mM sodium acetate buffer containing 5 mM CaCl₂ and 0.1% octylglucoside at 37 °C. PC1 activity was determined at various time points. Triangles, PC1 assayed in the presence of recombinant His-tagged proSAAS-(1–180); squares, PC1 assayed in the absence of proSAAS-(1–180).

Full-length ProSAAS-(1–225) and Its N-Terminal Domain, ProSAAS-(1–180), Inhibit PC1 Activity Derived from Both HEK 293 Cells and CHO/PC1 Cells—Because both GST-proSAAS (16) and His-tagged recombinant proSAAS-(1–225) (this study) were able to potently inhibit recombinant purified PC1, we asked whether proSAAS-(1–225) is able to inhibit PC1 secreted by cells. To address this issue we transiently transfected HEK 293 cells with vectors encoding PC1 alone or with proSAAS-(1–225). Fig. 3A shows that expression of full-length proSAAS-(1–225) in HEK293 cells was associated with a reduction in the amount of PC1 activity. Western blotting was used to demonstrate that cellular PC1 levels were similar in both cells expressing and not expressing full-length proSAAS-(1–225) (not shown).

To further confirm the ability of proSAAS-(1–225) to affect PC1 activity secreted from cells, we stably transfected cDNA encoding proSAAS-(1–225) into CHO/PC1 cells (Fig. 3B). Three proSAAS-expressing clones were analyzed; results from two are shown. These results indicate that PC1 activity present in the conditioned medium was reduced by ~50% by coexpression of full-length proSAAS-(1–225) (Fig. 3B). The inability of this potent inhibitor to completely inhibit PC1 can perhaps be related to inactivation via internal cleavage, which occurs extensively in this cell line (see below).

The N-terminal domain of 7B2 has been shown to facilitate the maturation of pro-PC2 and the production of activatable pro-PC2 from CHO cells; in the absence of intracellular encour-
with 7B2, pro-PC2 is secreted as a zymogen form that is inactivatable (11). However, PC1 is secreted from CHO cells in an already active form (25), although only \( \frac{1}{100} \) g of active PC1 can be obtained from the CHO/PC1 cell expression system (25) versus milligram quantities of PC2 from CHO/PC2/7B2 cells (12); large amounts of PC1 are expressed in an apparently inactive form (25). We therefore asked whether the N-terminal domain of proSAAS, proSAAS-(1–180), could facilitate the generation and/or the secretion of active PC1, thus potentially allowing the production of milligram quantities of PC1.

To investigate the effect of proSAAS-(1–180) on PC1 activity in vivo we transiently transfected PC1 alone or with proSAAS-(1–180) into HEK 293 cells. Quite surprisingly, in view of its lack of the C-terminal inhibitory domain, expression of proSAAS-(1–180) not only did not increase PC1 activity in the conditioned medium but reduced the activity severely (Fig. 3A).

This experiment was performed on six independent occasions; the average inhibition of PC1 activity by proSAAS-(1–180) was 46% \( \pm \) 16% (mean \( \pm \) S.D.), whereas the average inhibition of PC1 by proSAAS-(1–225) in three independent experiments was 57% \( \pm \) 11% \( (p < 0.05 \) compared with PC1 controls for each protein). The effect was observed using two independent constructs encoding proSAAS-(1–180).

We confirmed the apparent inhibitory effect of proSAAS-(1–180) by stably transfecting constructs into CHO/PC1 cells, which yielded similar results (Fig. 3C). To rule out the artifactual possibility that our cells were actually erroneously expressing proSAAS-(1–225) rather than proSAAS-(1–180), we performed RIAs for both the N-terminal domain (QERA RIA) and the C-terminal domain (LENP RIA) on overnight-conditioned medium. These RIAs confirmed good expression of SAAS but complete lack of expression of any C-terminal domain immunoreactivity (data not shown).

In summary, these experiments show that the N-terminal domain of proSAAS cannot be used to facilitate active PC1 production in CHO/PC1 cells; furthermore, in vivo, expression of this domain apparently results in the secretion of either inhibited or intrinsically inactive PC1.

Decreased C-terminal Cleavage of PC1 Occurs in CHO/PC1 and HEK 293 Cells Expressing Full-length proSAAS-(1–225) but Not in Cells Expressing ProSAAS-(1–180)—To establish any potential effect of proSAAS-(1–225) on the biosynthesis of PC1, we subjected overnight-conditioned medium from CHO/PC1 cells stably expressing full-length proSAAS-(1–225) but not in CHO/PC1 cells stably expressing full-length proSAAS-(1–225) to Western blotting. Fig. 4A (16-h lanes) shows that there is a decrease in processing of 87-kDa PC1 to its 66-kDa form in
the interpretation of proSAAS-(1–225)-mediated enzyme inhibition, because a lower amount of PC1 in the medium would be expected to exhibit less activity.

To confirm the effect of proSAAS-(1–225) on decreased secretion of PC1, HEK 293 cells transiently expressing PC1 and PC1/proSAAS-(1–225) were subjected to pulse/chase analysis. These data indicated that proSAAS-(1–225) expression was not associated with a substantially decreased rate of secretion of newly synthesized secreted PC1 from radiolabeled HEK 293 cells, as assessed after a 2-h chase period (data not shown). However, it is possible that a slight decrease in the rate of secretion not detectable in a 2-h chase might have accumulated over the course of the longer collection periods used for the Western blot study. These pulse-chase experiments did, however, confirm proSAAS-mediated (1–225) inhibition of C-terminal cleavage of PC1 to its 66-kDa form (data not shown).

ProSAAS-(1–180) Enhances Expression and Secretion of PC1 in CHO/PC1 and HEK 293 Cells—To investigate the effect of proSAAS-(1–180) on PC1 biosynthesis, conditioned medium was similarly collected at various time points and subjected to Western blotting. Paradoxically, this experiment showed that cells expressing proSAAS-(1–180) exhibited increased PC1 secretion (Fig. 5A). Similar results were obtained upon independent repetition of the experiment and also using an independent clone of CHO/PC1-SAAS-(1–180) cells.

To further confirm these results we repeated this experiment using transiently transfected HEK 293 cells. These experiments also consistently showed increased secretion of PC1 in cells transfected with proSAAS-(1–180) (Fig. 5B). We were again unable to detect effects of proSAAS on PC1 secretion in labeling studies (not shown).

The data showing enhanced secretion of PC1 forms in the presence of proSAAS-(1–180) are of interest in light of the fact that PC1 activity is substantially reduced in medium taken from cells expressing this proSAAS domain (shown in Figs. 3, A and C). The increased amounts of secreted PC1 (Fig. 5, A and B) are apparently associated with much less enzymatic activity (Fig. 3, A and C), supporting the idea that expression of proSAAS-(1–180) in CHO/PC1 and HEK cells results in the secretion of enzymatically incompetent PC1.

To determine the effect of proSAAS-(1–180) on the 66-kDa form of PC1, we transiently transfected cDNAs encoding 66-kDa PC1 and proSAAS-(1–225) into CHO 293 cells (Fig. 5C). Although based upon the interaction study of Qian et al. (19) and our own in vitro results, we expected to see good inhibition of secreted 66-kDa PC1 by proSAAS-(1–225), in three separate experiments no inhibition was observed. Thus the effects of proSAAS-(1–225) on the 87- and 66-kDa forms of PC1 appear to differ markedly in vitro, suggesting an important role for the C-terminal tail of PC1.

Fig. 4A indicates that proSAAS expression affects secretion of PC1; cells expressing full-length proSAAS-(1–225) appear to exhibit decreased secretion of all PC1 forms. Note that immunoreactive PC1 was consistently detected in the conditioned medium after 3 h in control cells; however, in cells expressing proSAAS-(1–225), PC1 was only detected at the 6-h time point (Fig. 4A). These results suggest that expression of proSAAS-(1–225) decreases the rate of secretion of PC1. The same number of cells was present in each condition (as determined by Western blotting of cells with PC1 antiserum as well as with tubulin antiserum; data not shown). These results confound
Fig. 5. CHO/PC1 and HEK 293 cells expressing proSAAS-(1–180) exhibit increased expression of PC1. A, CHO/PC1 cells stably transfected with proSAAS-(1–180) were incubated at 37 °C in Opti-MEM medium for 3, 6, 9 h, and overnight. Conditioned medium was collected at the various time points, and aliquots were subjected to electrophoresis prior to Western blotting with anti-PC1 antisera. The experiment was independently repeated three times using three different clones with similar results. B, HEK 293 cells were transiently transfected with PC1 alone or doubly transfected with 87-kDa PC1 and proSAAS-(1–180). Overnight-conditioned medium was collected, and aliquots were subjected to electrophoresis prior to Western blotting for PC1. C, HEK 293 cells were transiently transfected with cDNAs encoding either 66-kDa PC1 alone, or 66-kDa PC1 and proSAAS-(1–180). Overnight-conditioned medium from two wells of each type of transfection was collected, and PC1 activity was determined as described in Materials and Methods. The experiment was repeated four times with similar results.

Fig. 6. The C-terminal inhibitory domain is efficiently removed from proSAAS in both CHO/PC1 and AtT-20/PE cells expressing full-length proSAAS-(1–225). Overnight-conditioned medium from either CHO/PC1 cells or AtT-20/PE cells stably transfected with proSAAS-(1–225) was collected, acidified, and concentrated using a C4 disposable column (see Materials and Methods). Peptides were eluted with 60% isopropanol and lyophilized, and the cell extracts were resuspended in 32% ACN and 0.1% TFA. Samples were size-fractionated by HPGPC, and proSAAS expression per fraction was determined by LENP RIA. Top panel, schematic diagram of proSAAS; the epitope used for RIA is in boldface. A, CHO/PC1 cells expressing proSAAS-(1–225); B, AtT20/PE cells expressing proSAAS-(1–225).
proenkephalin (PE), by stably transfecting proSAAS-(1–225) into AtT-20/PE cells, which similarly targeted proSAAS to the regulated pathway (Table I). Although PC1-mediated processing of PE to peptide B was also slowed under pulse/chase conditions (Fig. 8A), under steady-state conditions no effect of proSAAS expression was observed, and the cellular content of peptide B remained similar in SAAS-transfected and control cells (Fig. 8B).

Collectively our results indicate that proSAAS expression is unable to effect alterations in the steady-state profile of PC1-derived prohormone peptide products, possibly because of compensation during long residence times with enzyme in secretory granules.

**Distribution of PC1 and PC1 Activity in Golgi-enriched Subcellular Fractions**—Golgi-enriched fractions (G2–G3) contained more than 87-kDa PC1 than ER-enriched subcellular fractions (P3) when the same amount of protein was subjected to Western blotting using an antiserum against the N terminus of PC1 (Fig. 9A). The PC1 enzymatic activity of each fraction obtained from Golgi-enriched subcellular preparations was measured using the same amount of protein per tube. As shown in Fig. 9B, the G3 fraction exhibited the highest PC1 activity, most likely because the G3 fraction contains slightly more PC1 than G2 (see Fig. 9A). Much less PC1 activity was detected in the ER-enriched fraction (P3 fraction; Fig. 9B); however, the amount of PC1 protein present in this fraction was also lower (panel A). These data contrast with the same experiment performed with CHO/PC2 cells and 7B2, in which enzymatic activity derived from PC2 was detected almost exclusively in the Golgi whereas the pro-PC2 protein was abundantly present in both Golgi and ER (30).

**His-tagged ProSAAS-(1–225), but Not His-tagged ProSAAS-(1–180), Inhibits PC1 Activity in Golgi- and ER-enriched Fractions**—The N-terminal domain of 7B2 is able to facilitate the production of active PC2 in Golgi membranes from CHO/PC2 cells (in which pro-PC2 is otherwise enzymatically inert (30)). To investigate whether the N-terminal domain of pro-SAAS can activate or inhibit PC1 activity in ER- and/or Golgi-enriched subcellular fractions, we measured PC1 enzymatic activity in ER- and Golgi-enriched fractions in the presence and absence of purified pro-SAAS-(1–180). As shown in Fig. 10, pro-SAAS-(1–180) slightly inhibited PC1 activity in the Golgi but not in the ER fraction, whereas pro-SAAS-(1–225) potently inhibited PC1 activity in both Golgi- and ER-enriched fractions. However, the slight inhibition observed in this experiment with pro-SAAS-(1–180) could not be reproduced upon repetition of the experiment, leading us to conclude that pro-SAAS-(1–180) is not inhibitory in this assay system. The analogous protein 21-kDa 7B2 represents a potent activator of pro-PC2 in Golgi, but not ER, membranes (30).

We hypothesized that inhibition of PC1 by pro-SAAS-(1–225) might mask an initial facilitatory effect that requires the full-length protein; however, a pro-SAAS mutant containing a non-inhibitory CT peptide (by mutation of KR to SS) was also unable to activate PC1 in either cellular fraction (Fig. 10B, SAAS SS mut). This again contrasts with the situation regarding 7B2 and PC2 where a similarly located mutation resulted in a fully active molecule regarding the production of active PC2 in Golgi membranes (34). We conclude that pro-SAAS and 7B2 do not appear to exhibit similar effects on Golgi convertase maturation processes.

**DISCUSSION**

PC1 and PC2 are the only two members of the PC family of enzymes for which endogenous binding proteins have been identified thus far. The first convertase binding protein identified was 7B2, a bifunctional protein with an N-terminal activating domain and a C-terminal inhibitory domain separated by a furin cleavage site (for review see Ref. 15). Both full-length (“27-kDa”) 7B2 and the 7B2 CT peptide (the C-terminal domain) are potent and specific inhibitors of PC2 with Ki values in the nanomolar range (15). Pro-SAAS, the second endogenous convertase binding protein identified (16), also contains two domains separated by a furin cleavage site. Fricker et al. (16) reported that full-length GST-pro-SAAS is an inhibitor of PC1 with a Ki in the micromolar range. It was suggested that GST-pro-SAAS-(1–225) may be improperly folded in bacteria, and that this may account for its decreased potency against PC1 (16). However, the present data indicate that His-tagged pro-SAAS-(1–225) is able to potently inhibit recombinant PC1 (Ki = 143 nm); it is thus likely to be folded in the correct conformation for association with this enzyme. Studies from our laboratory have shown that the LLRVRK hexapeptide sequence is responsible for inhibition (20); this precise sequence was previously identified by combinatorial peptide library screening for potent PC1 inhibitors (18).

**TABLE I**

| Clone no. | Basal release | Stimulated releasea | Stimulated releasea |
|-----------|--------------|---------------------|---------------------|
|           | QERA-ir b    | LENP-ir             | QERA-ir (pmol)      |
|           |              |                     | LENP-ir (pmol)      |
| 4         | 0.03         | 2.8                 | 0.12 (4.0)          |
| 7         | 0.04         | 1.5                 | 0.13 (3.3)          |
| 8         | 0.02         | 0.6                 | 0.09 (4.5)          |
| Average   |              |                     | 3.9 (1.8)           |

a Cells were stimulated in the presence of 1 μM PMA for 3 h.
b ir, immunoreactivity.
(1–225) is, however, not nearly as potent as the LLRVKR hexapeptide (\(K_i = 5 \text{ nM}\)), a relative order that is consistent with recent studies comparing GST-proSAAS-(1–225) and other synthetic peptides (19). The reduced potency of proSAAS-(1–225) against PC1 compared with the hexapeptide differs considerably from the situation with 7B2 and PC2, because full-length 7B2 is 10-fold more potent against PC2 than is the 7B2 CT peptide (13, 10). Additionally, sequences shorter than 7B2 CT-(1–18) do not represent inhibitors of PC2 (35), indicating a smaller effective binding site for natural inhibitors for PC1 as opposed to PC2.

In addition to differences in length requirements, the convertase specificity of each natural convertase inhibitor also differs. The SAAS hexapeptide LLRVKR and SAAS CT-(1–24) represent micromolar inhibitors of both PC2 and furin (20), unlike the 7B2 CT peptide, which shows no inhibitory activity against PC1 at any concentration. On the other hand, His-tagged proSAAS-(1–225) is not inhibitory against either PC2 or furin (data not shown; 19). These data suggest that sequences within the N-terminal domain of proSAAS-(1–225) are likely to be responsible for conferring additional specificity for PC1. Cleavage of the SAAS protein to the CT peptide and its various fragments during transit through the secretory pathway may therefore result in loss of specificity, implying that other convertases (and potentially also convertases not tested in this study, such as PC5) may also become targets for inhibition during cellular processing of proSAAS. While this paper was in revision, another study confirmed that smaller proSAAS CT-derived peptides exhibit higher potency but lower specificity compared with extended peptides (36).

Although it has been shown that PC1-mediated cleavage of POMC is inhibited in AtT-20 cells expressing proSAAS-(1–225) (16), a direct effect of proSAAS on PC1 activity secreted from cells has not yet been demonstrated. We have here presented data to indicate that secreted 87-kDa PC1 activity is reduced by proSAAS expression in two different cell lines, CHO/PC1 and

\[ \text{\(2\)} \text{. Lindberg, unpublished data.} \]

**FIG. 8.** PC1-mediated processing of proenkephalin is inhibited in AtT-20/PE cells expressing full-length proSAAS-(1–225) under pulse/chase conditions but not under steady-state conditions. AtT-20/PE cells expressing a mutant form of proSAAS-(1–225) with an internal methionine (E. Apletalina, unpublished data) were labeled with 0.5 mCi of [\(^{35}\)S]methionine for either 20 min (pulse-chase) or for 6 h (steady state). For pulse-chase experiments, cells were incubated for an additional 30 min in chase medium. Cell extracts were immunoprecipitated with anti-peptide B antiserum (Xandra (26)) and size-fractionated by HPGPC. Pulse/chase experiment: A, AtT-20/PE cells; B, AtT-20/PE cells expressing methionine-proSAAS-(1–225). Steady-state labeling: C, AtT-20 cells; D, AtT-20/PE cells expressing methionine-proSAAS-(1–225).

**FIG. 9.** Distribution of PC1 protein and activity in subcellular fractions derived from CHO/PC1 cells. A, characterization of Golgi-enriched subcellular fractions from CHO/PC1 cells. Western blot analysis of each fraction obtained from Golgi-enriched membrane preparation using anti-PC1 antiserum (LSU2B6). The same amount of protein (3 μg) was loaded for each fraction. G2, G3, G4, and P3 represent fractions obtained from the Golgi-membrane preparation, where G3 corresponds to the fraction enriched in Golgi membranes and P3 corresponds to the ER-enriched fraction. B, assay of PC1 enzymatic activity. 3 μg of protein from each fraction were used for measuring PC1 activity in the standard assay buffer containing protease inhibitors. FU represents fluorescence units.

\[ \text{\(2\)} \text{. Lindberg, unpublished data.} \]
HEK293. These results support the idea that the inhibitory CT peptide is able to function when expressed as intact proSAAS. Interestingly, 66-kDa PC1 secreted by transfected HEK 293 cells was not inhibited by proSAAS-(1–225) expression; however, in vitro, this form of PC1 was potently inhibited by recombinant proSAAS-(1–225). The reason for the inability of cotransfected proSAAS to inhibit 66-kDa PC1 activity is unclear, and this finding underscores the need for examination of all inhibitory effects of convertase binding proteins in a cellular setting.

Studies on the N-terminal domain of 7B2 in constitutive cell lines have shown that this domain is responsible for the generation of enzymatically competent PC2 by facilitating the secretion of proSAAS proteins in an ER-enriched fraction prepared from CHO/PC1 cells. Purified His-tagged proSAAS proteins were used at 1 μM final concentration. B, PC1 enzymatic activity was measured in a Golgi-enriched fraction obtained from CHO/PC1 cells. No inhibitor represents ER- or Golgi-enriched fractions lacking any additional proSAAS proteins. The synthetic peptide LLRVKR, a potent PC1 inhibitor, was used as a positive control for inhibition of PC1. FU represents fluorescence units. Each experiment was repeated independently using two different Golgi subcellular preparations as well as different proSAAS protein preparations.

FIG. 10. PC1 activity in subcellular fractions is inhibited by proSAAS-(1–225) but not by proSAAS-(1–180).

A. PC1 enzymatic activity was measured in the presence of various recombinant proSAAS proteins in an ER-enriched fraction prepared from CHO/PC1 cells. Purified His-tagged proSAAS proteins were used at 1 μM final concentration. B, PC1 enzymatic activity was measured in a Golgi-enriched fraction obtained from CHO/PC1 cells. No inhibitor represents ER- or Golgi-enriched fractions lacking any additional proSAAS proteins. The synthetic peptide LLRVKR, a potent PC1 inhibitor, was used as a positive control for inhibition of PC1. FU represents fluorescence units. Each experiment was repeated independently using two different Golgi subcellular preparations as well as different proSAAS protein preparations.

However, this was not found to be the case. Instead, we observed reduced activity for secreted PC1 coexpressed with proSAAS-(1–180). These data suggest that in vivo the N-terminal domain of proSAAS does not function as an activator of PC1, as does the N-terminal domain of 7B2, but instead functions as an apparent inhibitory domain. This was quite surprising, because no inhibitory effect was observed when recombinant proSAAS-(1–180) was added to PC1 in vitro. This requirement for cells suggests that the apparent inhibitory effect of proSAAS-(1–180) on PC1 activity may occur via a negative intracellular interaction rather than through direct inhibition. Unlike the situation with 7B2 and PC2, these presumed intracellular interactions could not be reproduced in the Golgi assay, in which proSAAS-(1–180) exhibited little or no effect.

If proSAAS-(1–180) actually functions as a direct inhibitor, the site conferring such inhibition is unclear. Two furin consensus sequences in the N-terminal domain could potentially represent inhibitory sequences (Fig. 11). However, the synthetic peptide proSAAS-(42–59), which contains the most N-terminal furin site (#1 in Fig. 11), was not found to be inhibitory to PC1 in a previous study (19). The second furin consensus sequence RQER (see Fig. 11) could potentially be responsible for direct inhibition of PC1, although the consensus sequence RXKR is generally preferred by PC1 and PC2 for inhibition (reviewed in Ref. 32). We tested the inhibitory activity of AERQER, the synthetic hexapeptide containing this internal furin consensus site, and found that this peptide was only able to inhibit PC1 with a Kᵢ in the high micromolar range (data not shown). These data, taken together with the complete lack of inhibition of the proSAAS-(1–180) domain in vitro, support the alternative hypothesis that the N-terminal domain of proSAAS may not represent a direct inhibitor of PC1 but instead might interact with PC1 or proPC1 intracellularly to generate inactive enzyme. This is precisely opposite of the effect of the N-terminal domain of 7B2 on proPC2.

The effects of proSAAS expression on PC1 biosynthesis are also clearly not analogous to those of 7B2 on PC2 biosynthesis, most likely due to the pronounced differences in the cell biology of maturation of these two convertases. 7B2 expression affects the rate of conversion of proPC2 to PC2 in the late secretory pathway (11); proSAAS expression has no apparent effect on the extremely rapid conversion of proPC1 to the 87-kDa active form in the endoplasmic reticulum. Unlike PC2, 87-kDa PC1 undergoes extensive autocatalytic, intermolecular C-terminal truncation in neuroendocrine cells (24, 25); coexpression of proSAAS-(1–225) reduces the extent of this auto-truncation event, possibly via direct inhibition of secreted PC1. It has been shown that the truncated 66-kDa form of PC1 is more active than the 87-kDa form (25, 31, 38). We did not test whether

FIG. 11. Sequence of mouse proSAAS-(1–225). Potential furin cleavage sites are in boldface and numbered; the CT inhibitory domain is underlined, and the inhibitory hexapeptide is boxed.

3 Y. Fortenberry, unpublished results.
proSAAS-mediated interruption of C-terminal PC1 processing also occurs in neuroendocrine cells; if so, it is not reflected in a decreased content of PC1-mediated intracellular cleavage of prohormone precursors.

We also observed that CHO/PC1 and HEK293 cells expressing proSAAS-(1–180) consistently exhibited the paradoxical finding of increased expression of PC1 associated with decreased enzymatic activity. In other words, in the presence of this proSAAS domain, a greater amount of PC1 in the conditioned medium was secreted than in the absence of proSAAS-(1–180), but much of the secreted PC1 appeared to be enzymatically inert. These data further support the possibility that the apparent inhibition of PC1 by proSAAS-(1–180) is not due to a direct interaction with the catalytic site of PC1 but may instead be mediated indirectly. Potential explanations for this interesting phenomenon include an anti-chaperone mechanism, such as a decrease in the efficiency of folding of PC1 (perhaps associated with enhanced speed of transit through the secretory pathway?), or decreased retention and degradation of improperly folded PC1. Further studies are required to determine the complex intracellular cell biology of the PC1-proSAAS-(1–180) interaction.

Overexpression of either 27- or 21-kDa 7B2 in AtT-20 cells does not result in any final effect on either proenkephalin or POMC processing.4,5 However, expression of proSAAS-(1–225) in AtT-20 cells reduces the cleavage of newly synthesized POMC to ACTH (16). We have repeated this experiment with similar results and have additionally shown that the cleavage of newly synthesized proenkephalin is reduced in AtT-20/PE cells expressing proSAAS-(1–225). Despite this finding, under steady-state conditions, the amounts of stored cellular PC1-mediated peptide products, ACTH and peptide B, are identical in AtT-20 cells either expressing or not expressing proSAAS-(1–225), most likely because of the ability of PC1 to complete cleavages during the relatively long residence time in granules. This finding implies that, similarly to 7B2, proSAAS-(1–225) expression does not affect the cellular content of PC1-mediated processing products. We speculate that proSAAS is used to control the intracellular timing of PC1 activity rather than its total level of activity.

The proteolytic processing of proSAAS has been the investigation of several previous studies. Fricker et al. (16) have used mass spectroscopy of brain tissue extracts to show that proSAAS-(1–225) is cleaved at its N-terminal furin cleavage site RRRR (Fig. 11); our recent data support this finding (17). We show here that proSAAS-(1–225) obtained from overnight-conditioned media is readily cleaved at the furin consensus site separating the N-terminal domain from the CT peptide (site #3, Fig. 11), thereby liberating this inhibitory peptide. An internal cleavage event, followed by carboxypeptidase action, then inactivates the inhibitory peptide. In the studies presented here, the extent of this internal cleavage correlated with the extent of expression of PC1, further supporting the idea that PC1 performs the inhibitory internal cleavage event (20).

In summary, the convertase binding protein proSAAS appears to exhibit some similarities, but many distinct differences, from the convertase binding protein 7B2. Obvious structural similarities include the presence of a highly potent inhibitory peptide at the C terminus of the protein; both proteins also contain a proline-rich sequence located in the middle of the protein. In addition, overexpression of both proteins does not affect the contents of processed peptide products in AtT-20 cells, indicating that neuroendocrine cells possess a level of control over peptide content that supersedes the inhibitory action of these binding proteins. Differences between 7B2 and proSAAS include the inability of the N-terminal domain of proSAAS, spatially equivalent to 21-kDa 7B2, to stabilize PC enzyme activity, and the apparent ability of this domain to facilitate the secretion of enzymatically inactive PC1 in vivo by an as-yet obscure mechanism. These results hint at novel intracellular roles for proSAAS, which will require further investigation.

The profound differences in the cell biology of PC1 and PC2 are likely to influence the roles of their respective binding proteins. Evolutionary differences between the two convertases may also account for certain differences between the proSAAS/PC1 and 7B2/PC2 interactions. PC2, but not PC1, has been identified in Caenorhabditis elegans and Drosophila, although hydra and Aplysia possess PC1 homologs (39, 40). ProSAAS homologs have not yet been identified in any invertebrates; it is likely that proSAAS, like 7B2, is conserved only over small segments, thus rendering homologs undetectable. Further studies on the ability of proSAAS forms to affect PC1 synthesis, action, and secretion in neuroendocrine cells should lead to a better understanding of the physiological regulation of PC1, an enzyme with a primary role in the production of many biologically important peptide hormones.

Acknowledgments—We thank Joelle Finley for assistance with cell culture and Jim Douglass for the mouse proSAAS cDNAs used for vector constructions.

REFERENCES

1. Zhou, A., Webb, G., Zhu, X., and Steiner, D. F. (1999) J. Biol. Chem. 274, 20745–20749
2. Seidah, N. G., Gaspar, L., Mion, P., Marcinkiewicz, M., Mbiyak, M., and Chretien, M. (1990) DNA Cell Biol. 9, 415–424
3. Day, R., Schafer, M. K., Watson, S. J., Chretien, M., and Seidah, N. G. (1992) Mol. Endocrinol. 6, 485–497
4. Bennett, D. L., Bailleys, E. M., Nielsen, E., Guest, P. C., Rutherford, N. G., Arden, S. D., and Hutton, J. C. (1992) J. Biol. Chem. 267, 15229–15236
5. Sneathens, S., Montag, A. G., Thomas, G., Albige-Rizo, C., Carroll, R., Benig, M., Phillips, L. A., Martin, S., Ohagi, S., Gardner, P., Swift, H. H., and Steiner, D. F. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8822–8826
6. Breslin, M. B., Lindberg, I., Benjannet, S., Mathis, J. P., Lazure, C., and Seidah, N. G. (1993) J. Biol. Chem. 268, 27084–27093
7. Johanning, K., Mathis, J. P., and Lindberg, I. (1996) J. Neurochem. 66, 898–907
8. Benjannet, S., Rondeau, N., Day, R., Chretien, M., and Seidah, N. G. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3564–3566
9. Zhou, A., and Mains, R. E. (1994) J. Biol. Chem. 269, 17440–17447
10. Mettens, G. J., Brals, J. A., Eib, D. W., Zhou, Y., and Lindberg, I. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5784–5785
11. Zhu, X., and Lindberg, I. (1999) J. Cell. Biol. 142, 1641–1650
12. Lamano, N. S., Zhu, X., and Lindberg, I. (1996) Arch. Biochem. Biophys. 330, 238–250
13. Lindberg, I., Van den Hurk, W. H., Bai, C., and Batie, C. J. (1995) Biochemistry 34, 5486–5493
14. Van Horssen, A. M., Van den Hurk, W. H., Bailleys, E. M., Hutton, J. C., Martens, G. J. M., and Lindberg, I. (1995) J. Biol. Chem. 270, 14292–14296
15. Muller, L., and Lindberg, I. (1999) Proc. Nucleic Acids Res. 27, 69–108
16. Fricker, L. D., McKinzie, A. A., Sun, J., Curran, E., Qian, Y., Yan, L., Patterson, S. D., Courchesne, P. L., Richards, B., Levin, L., Mzhavia, N., Devi, L. A., and Douglas, J. (2000) J. Neurosci. 20, 639–648
17. Sayah, M., Fortenberry, Y., Cameron, A., and Lindberg, I. (2001) J. Neurosci. 21, 1833–1841
18. Apletalina, E., Appel, J., Lamano, N. S., Houghten, R. A., and Lindberg, I. (1998) J. Biol. Chem. 273, 26285–26295
19. Qian, Y., Devi, L. A., Mzhavia, N., Munzer, S., Seidah, N. G., and Fricker, L. D. (2000) J. Biol. Chem. 275, 23776–23781
20. Cameron, A., Fortenberry, Y., and Lindberg, I. (2000) FEBS Lett. 473, 135–138
21. Mathis, J., and Lindberg, I. (1992) Endocrinology 131, 2287–2296
22. Lindberg, I., Shaw, E., Finley, J., Leone, D., and Deininger, P. (1991) Endocrinology 129, 1849–1856
23. Lindberg, I., and Zhu, Y. (1995) in Methods in Neuroscience, (Smith, A. I., ed.), Vol. 25, pp. 94–108, Academic Press, Orlando, FL
24. Vindrola, O., and Lindberg, I. (1992) Mol. Endocrinol. 6, 1088–1094
25. Zhou, Y., and Lindberg, I. (1994) J. Biol. Chem. 269, 18408–18413
26. Lindberg, I., and White, L. (1986) Biochem. Biophys. Res. Commun. 139, 1024–1032
27. Fortenberry, Y., Liu, J., and Lindberg, I. (1999) J. Neurochem. 73, 994–1003
28. Zhou, Y., and Lindberg, I. (1993) J. Biol. Chem. 268, 5615–5623
29. Cameron, A., Appel, J., Houghten, R. A., and Lindberg, I. (2000) J. Biol. Chem. 275, 36741–36749
5186

Functional Characterization of ProSAAS

30. Muller, L., Zhu, X., and Lindberg, I. (1997) J. Cell Biol. 139, 625–638
31. Boudreault, A., Gauthier, D., Rondeau, N., Savaria, D., Seidah, N. G., Chretien, M., and Lazure, C. (1998) Protein Expr. Purif. 14, 353–366
32. Cameron, A., Apletalina, E. V., and Lindberg, I. (2001) in The Enzymes (Dalbey, R. E., ed) Vol. XXII, pp. 291–332, Academic Press, New York
33. Zhu, X., Rouille, Y., Lamango, N. S., Steiner, D. F., and Lindberg, I. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4919–4924
34. Hwang, J. R., and Lindberg, I. (2001) J. Neurochem. 79, 1–9
35. Apletalina, E. V., Juliano, M. A., Juliano, L., and Lindberg, I. (2000) Biochem. Biophys. Res. Commun. 267, 940–942
36. Basak, A., Koch, P., Dupelle, M., Fricker, L. D., Devi, I. A., Chretien, M., and Seidah, N. G. (2001) J. Biol. Chem. 276, 32720–32728
37. Muller, L., Cameron, A., Fortenberry, Y., Apletalina, E. V., and Lindberg, I. (2000) J. Biol. Chem. 275, 39213–39222
38. Rufaut, N. W., Brennan, S. O., Hakes, D. J., Dixon, J. E., and Birch, N. P. (1993) J. Biol. Chem. 268, 20291–20298
39. Oliva, A. A., Chan, S. J., and Steiner, D. F. (2000) Biochim. Biophys. Acta 1477, 338–348
40. Gorham, E. L., Nagle, G. T., Smith, J. S., Shen, H., and Kurosky, A. (1996) DNA Cell Biol. 15, 339–345