# Prohormone convertase 1 (PC1) processing and sorting: effect of PC1 propeptide and proSAAS

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#### Abstract

Prohormone convertase 1 (PC1) is a serine proteinase responsible for the proteolytic processing of many precursor proteins within the regulated secretory pathway. The activity of PC1 is potentially regulated by two endogenous inhibitors, the PC1 propeptide and proSAAS. Here we have investigated the effect of proSAAS and propeptide-containing constructs on PC1 carboxy-terminal processing and activity. In AtT-20 cells, proSAAS expression inhibited both C-terminal PC1 processing and pro-opiomelanocortin (POMC) processing under pulse/chase conditions. SAAS CT peptide–propeptide chimeric constructs had no effect on the cleavage of PC1 and POMC under pulse/chase conditions. However, a construct

#### Introduction

Prohormone convertase 1 (PC1, also known as PC3) is a member of the family of calcium-dependent subtilisin-like endoproteases, which include furin, PC1, PC2, PC4, PACE4, PC5/6, PC7/LPC/PC8, and SKI-1/S1P (Steiner 1998, Seidah et al. 1999). PC1, unlike furin, PC4, PACE4, PC5-B, PC7 and SKI-1, is predominantly expressed in neural and endocrine cells (Seidah et al. 1990, Day et al. 1992). PC1 is thought to be involved in the post-translational processing of several prohormones and neuroendocrine peptides including proglucagon, proenkephalin, proopiomelanocortin (POMC) and proinsulin (Benjannet et al. 1991, Bennett et al. 1992, Mathis & Lindberg 1992, Breslin et al. 1993, Rouille et al. 1997). Like the other proprotein convertases, PC1 contains an N-terminal signal peptide, a proregion, a catalytic domain, a P (Homo B) domain and a C-terminal domain.

The activity of PC1 is potentially regulated by three kinds of naturally occurring intracellular inhibitors. First, similar to many secreted proteases including subtilisin (Shinde *et al.* 1993, Yabuta *et al.* 2001), furin (Anderson *et al.* 1997, 2002), SKI-1/S1P (Toure *et al.* 2000, Elagoz *et al.* 2002), PC2 (Muller *et al.* 2000) and PC5A (Nour *et al.* 2003), the 83 amino acid proregion of PC1 functions *in cis* by an intramolecular mechanism as a potent

containing the propeptide alone reduced C-terminal PC1 processing under pulse/chase conditions and also inhibited POMC processing. In contrast, experiments using HEK293 cells transiently expressing PC1 plus the respective constructs demonstrated significant inhibition of zymogen processing and decreased C-terminal processing of PC1 by the SAAS CT peptide portion of the chimera. Our results suggest that the PC1 propeptide expressed *in trans* is able to act as an endogenous inhibitor of PC1, but that SAAS CT peptide-containing/propeptide constructs cannot function as effective inhibitors of precursor maturation in the regulated pathway.

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autoinhibitor in the low nanomolar range  $(6 \pm 0.6 \text{ nM})$ (Boudreault et al. 1998). In vitro experiments also demonstrated that the PC1 propeptide is a slow tight-binding competitive inhibitor of human furin (hfurin) with a  $K_i$  of  $10 \pm 1.0$  nM, but also of PC2, albeit to a lesser extent  $(10 \pm 1.0 \,\mu\text{M})$  (Boudreault et al. 1998). PC1 is activated by an autocatalytic intramolecular cleavage of its propeptide within the endoplasmic reticulum (ER) (Goodman & Gorman 1994, Lindberg 1994, Milgram & Mains 1994). NMR analysis has shown that the PC1 propeptide consists of two  $\alpha$ -helices and four  $\beta$ -strands in a  $\beta$ - $\alpha$ - $\beta$ - $\beta$ - $\alpha$ - $\beta$ arrangement and contains a number of hydrophobic residues in the  $\beta$ -sheet as well as three His residues and five basic residues in the  $\beta$ 3- $\alpha$ 2 loop (Tangrea *et al.* 2002). It has been postulated that the hydrophobic patch may provide a binding interface with the catalytic domain of PC1 within the ER (pH 7.0), while the basic residues buried at the putative binding interface may be protonated within the trans-Golgi network (TGN) (pH 6.0) and then dissociated from the catalytic domain (Tangrea et al. 2002).

A second type of regulation of PC1 is its additional proteolytic truncation of its C-terminal tail by an autocatalytic event, presumably within immature secretory granules, which results in an activation of the enzyme (Vindrola & Lindberg 1992, Benjannet *et al.* 1993, Zhou & Lindberg 1994, Zhou *et al.* 1995). The C-terminal tail may act as an inhibitor of PC1 activity against certain substrates in the ER and Golgi apparatus (Jutras *et al.* 1997) and may also act as a sorting signal of PC1 to the secretory granules (Zhou *et al.* 1995). However, the exact role of the C-terminal tail on PC1 activity is still poorly understood. *In vitro* expression of the C-terminal tail does not yield a peptide inhibitor of PC1 (Y Zhou and I Lindberg, unpublished observations).

Lastly, proSAAS, a granin-like neuroendocrine secretory protein, is a potent endogenous PC1 inhibitor (Fricker et al. 2000, Fortenberry et al. 2002). ProSAAS, which consists of an N-terminal domain and a C-terminal domain separated by a furin cleavage site, is broadly expressed in neural and endocrine tissues (Fricker et al. 2000). A recombinant GST-proSAAS fusion protein inhibits PC1 activity, but does not inhibit PC2, furin, PACE4, PC5, or PC7 (Fricker et al. 2000). Our laboratory has shown that His-tagged proSAAS inhibits PC1 with a K<sub>i</sub> of 143 nM (Fortenberry et al. 1999). The inhibitory region of proSAAS is located in the C-terminal domain (Cameron et al. 2000, Qian et al. 2000) and includes the LLRVKR hexapeptide sequence which was previously identified as a potent PC1 inhibitor by combinatorial library peptide screening (Apletalina et al. 1998). Synthetic peptides containing this hexapeptide are slow tightbinding competitive inhibitors of PC1 with apparent  $K_i$ values in the low nanomolar range (1.5-40 nM) and also represent micromolar inhibitors of both PC2 and furin (Cameron et al. 2000, Qian et al. 2000, Basak et al. 2001). In vivo experiments showed that overexpression of pro-SAAS in AtT-20 cells substantially reduces the rate of processing of newly synthesized POMC into its smaller products (Fricker et al. 2000); however, proSAAS expression had no effect on the steady-state content of processed peptides derived from POMC (Cameron et al. 2000), indicating that its effects are solely kinetic in nature.

Because of their potent inhibitory capacities, both convertase propeptides as well as convertase-interacting proteins could represent molecules capable of inhibiting precursor maturation within cells. While inhibition of precursor maturation by the propeptides of furin and PC7 has been achieved in cell culture (Zhong *et al.* 1999), *in vivo* data on inhibitory properties of the proregion of other PCs is not available. In this study, we investigated whether the PC1 propeptide and the proSAAS CT peptide, expressed singly or together, can act *in trans* to inhibit the processing and secretion of PC1 and its substrates. Two different cell types (regulated and constitutively secreting cells) were used in order better to study both the early and the late cellular PC1 processing events.

#### Materials and Methods

#### Construction of eukaryotic vectors

For the construction of the expression vector encoding the proregion of PC1 and an additional portion of the catalytic

domain - containing two methionine sites for the labeling experiments (C1) - the mPC1ST/CMV expression vector (Zhou et al. 1995) was digested with NheI and XbaI, and then religated. To obtain the C-terminal domain of mouse proSAAS, PCR was performed using the pcDNA 3.1 vector encoding full-length mouse proSAAS as a template source (Fortenberry et al. 2002) with the following primers: the two primers with an NheI site in the upstream oligonucleotide were 5'-GCG GCT AGC AGT TCG GAG CCG GAG G-3' (the SAAT CT peptide containing the furin recognition site derived from pro-SAAS) and 5'-GCG GCT AGC TCT GTG GAC CAG GAT TTG-3' (the SAAT CT peptide lacking the furin cleavage site); the downstream primer with a ApaI was 5'- GCG GGG CCC CAG CGG TTT AAA CTT AA-3' (used for construction of both plasmids). The resulting PCR fragments were then cloned into the mPC1ST/ CMV expression vector (Zhou et al. 1995), and digested with NheI and ApaI for the construction of chimeras containing the PC1 propeptide and SAAT CT peptide separated by a furin recognition site (C1) or the same construct lacking the furin recognition site (C2) (Fig. 1).

The eukaryotic vector encoding mouse proSAAS containing a single point mutation from Q83 to M83 (Met-SAAS) was generated via a two-step PCR-mediated method (Vallette et al. 1989, Zhou et al. 1995) using the pBluescript vector encoding proSAAS supplied by J Douglass (Amgen, Thousand Oaks, CA, USA) as a template source. This residue was chosen because it is not conserved between various mammals and because it provided a methionine to proSAAS, which otherwise lacks a Met or Cys to follow during labeling. The following primers were used: 5'-GCG CGG AAT TCA GCC CGG CTC GTT GGG GCA GC-3', 5'-GGC CTC CAT CAG GTG CGC CAG CGC CCG CGC CAG CTC-3', 5'-CTG GCG CAC CTG ATG GAG GCC GAG AGA CAG GAA CGC-3', and 5'-GCG AAG CTT ATT ATT GAG GGC TCA GG-3'. The first round of PCR was carried out using the first and second primers and the third and fourth primers respectively. The resulting two fragments purified from the first round of PCR were mixed and then amplified using the first and the fourth primers. The final PCR fragment was cloned into pcDNA 3.1 (Invitrogen, Gaithersburg, MD, USA) between the EcoRI and HindIII sites. All cDNA sequences generated by PCR were verified by DNA sequencing.

#### Cell culture, transfection and selection

AtT-20 cells were cultured at 37 °C in 5% CO<sub>2</sub>. They were maintained in Dulbecco's Modified Eagle Medium (DMEM) high glucose medium containing 10% Nuserum (Becton Dickinson, Mountain View, CA, USA), 2.5% fetal bovine serum (FBS) (Irvine Scientific, Santa Ana, CA, USA) and the appropriate selection agent, 500 µg/ml



Figure 1 Fusion constructs used in this study. The furin cleavage site within construct 1 is indicated.

active G418 (Invitrogen). HEK293 cells were grown in DMEM high glucose medium containing 10% FBS.

AtT-20 cells were transfected with eukaryotic expression vectors encoding proSAAS, C1, C2 or C3 using lipofectin (Invitrogen) as described previously (Zhu & Lindberg 1995). Colonies were selected using the growth medium containing 0.5 mg/ml active G418 and subcloned into 24-well plates using the soft agar method previously described (Lindberg & Zhou 1995). Clones were allowed to grow in 24-well plates for  $\sim 2$  weeks. After semiconfluence was reached, 1 ml Opti-MEM (Invitrogen) containing 1 µM PMA (phorbol 12-myristate 13-acetate) as a secretagog, was added to each well, and cells were incubated overnight. One hundred microliters of the overnight medium were collected and used to screen for expression of C-terminal domain of proSAAS by the LENP (directed towards the sequence LENPSPQAPA) RIA as described previously (Sayah et al. 2001). The expression of PC1 propeptide was indirectly screened by the inhibition of C-terminal processing of endogenous PC1 in AtT-20 cells as compared with parental AtT-20 cells by Western blotting with the amino-terminally directed PC1 antiserum 2B6. Verification of propeptide construct synthesis was obtained using Western blotting and radiolabeling experiments. Three high-expressing clones were used for subsequent experiments.

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For double transient transfections, 100 µl Opti-MEM containing 0.5 µg pRc/CMV recombinants encoding PC1 plus pRc/CMV recombinants encoding C1, C2 or C3, or an empty pRc/CMV respectively, and 4 µl Fugene 6 (Roche, Indianapolis, IN, USA) were directly added to each well containing 70~80% confluent HEK293 cells cultured in a 12-well plate without the removal of medium. After 24 h, each well was washed with Opti-MEM and incubated with 1 ml Opti-MEM containing 100 µg/ml aprotinin in the presence or absence of 5 µM lactacystin (Calbiochem, La Jolla, CA, USA) overnight. The conditioned medium was collected and briefly centrifuged, and then used for the determination of PC1 activity. The eukaryotic expression vectors encoding 87 kDa and 66 kDa PC1 have previously been described (Benjannet et al. 1992, Zhou & Lindberg 1993, 1994).

#### Western blotting and enzyme assay

Two hundred microliters of the conditioned medium collected as described above were precipitated with trichloroacetic acid (10% final concentration) and the precipitate was dissolved in Laemmli sample buffer; cells were directly solubilized in Laemmli sample buffer. Samples were subjected to electrophoresis on 10% Criterion Tris–HCl (Bio-Rad, Hercules, CA, USA) SDS gels, followed by Western blotting using the antiserum 2B6 (Vindrola & Lindberg 1992). Proteins were transferred from gels to nitrocellulose membranes, and the membranes were preincubated in 5% nonfat milk in Tris-buffered saline (TBS) for 30 min at room temperature prior to incubation overnight at 4 °C with antiserum 2B6 diluted 1:1000 in milk. The membrane was washed three times with TBS containing 0.05% Tween followed by incubation at room temperature for 1.5 h with secondary antibody (goat anti-rabbit IgG coupled to alkaline phosphatase) diluted 1:10 000 in milk. The membrane was washed once with TBS containing 0.05% Tween and twice with TBS alone and then developed with 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt/p-nitro blue tetrazolium chloride. For Western blotting using horseradish peroxidase (HRP)-conjugate as secondary antibody, the membrane was incubated with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) for 1 min and the chemiluminescent bands were revealed using Fluor-S Max MultiImager System (Bio-Rad).

The assay for PC1 was carried out using 25 µl of each conditioned medium sample in a total volume of 50 µl containing 200 µM fluorogenic substrate, pERTKRmethylcoumarin amide (MCA) as a substrate and 100 mM sodium acetate, pH 5.5, 5 mM CaCl<sub>2</sub>, and 0.1% Brij 35 in the presence of a protease inhibitor-mixture composed of 1 µM pepstatin, 0.28 mM tosylphenylalanyl chloromethyl ketone (TPCK), 1 µM trans-epoxysuccinic acid (E-64), and 0.14 mM tosyllysyl chloromethyl ketone (TLCK). Released 7-amino-4-methylcoumarin was measured with a 96-well plate Fluoroscan Ascent fluorometer (LabSystems, Sunnyvale, CA, USA) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm for 2 h at 37 °C. Enzyme activity was measured in triplicate and is given in fluorescence units (FU) per minute in which one FU corresponds to 4.82 pmol MCA.

#### RIA

RIAs for the C-terminal region of proSAAS, directed towards the sequence LENPSPQAPA (LENP) were carried out using procedures described previously (Sayah et al. 2001). Samples were incubated with 10 000 c.p.m. iodinated peptide and the appropriate dilution of rabbit antiserum in a final volume of 300 µl at 4 °C overnight. Antisera for the LENP RIAs were used at a working dilution of 1:750 (final dilution=1:2250). Samples were digested with trypsin/carboxypeptidase B as previously described (Sayah et al. 2001) to reveal cryptic immunoreactivity. To separate the antibody-bound labeled peptide from the unbound labeled peptide, 1 ml 25% polyethylene glycol and 100  $\mu$ l 7.5% carrier bovine  $\gamma$ -globulin (in PBS) were added. The samples were then vortexed vigorously, kept on ice for 30 min, and then centrifuged for 20 min at 5000 g at 4 °C using a Sorvall RT6000B refrigerated centrifuge. The supernatant was

#### Metabolic labeling and immunoprecipitation

Approximately 500 000 wild-type AtT-20 cells and AtT-20 cells overexpressing proSAAS, C1, C2, or C3 were seeded into a 6-well plate and grown until ~70-80% confluent at 37 °C. The cells were pulsed with methionine- and cysteine-free medium containing [<sup>35</sup>S]methionine and cysteine 0.5 mCi(ProMix, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) for 20 min and chased for the indicated times. Cells were boiled in 100 µl boiling buffer (50 mM Na phosphate, pH 7.4, 1 mM EDTA, 0.1% Triton, 0.5% NP-40, and 0.9% NaCl) for 5 min and then diluted with 900 µl AG buffer (0.1 M sodium phosphate, pH 7.4, 1 mM EDTA, 0.1% Triton, 0.5% Nonidet P-40, and 0.9% NaCl). The metabolic labeling for the HEK293 cells transiently cotransfected with pRc/CMV PC1 expression vector plus the C1, C2, or C3 constructs was performed as described above except for the use of a 12-well plate for the cell culture.

For immunoprecipitation, 500 µl cell extract were incubated with 100 µl 20% protein A-Sepharose beads (Amersham Pharmacia Biotech Inc.) prehydrated with AG buffer at 4 °C for 1 h with gentle shaking and then centrifuged. The supernatants were incubated overnight at 4 °C with either 5 µl PC1 antiserum 2B6 (Vindrola & Lindberg 1992) or 5 µl adrenocorticotropin (ACTH(1-24)) antiserum (Laurent et al. 2002) containing 25 µl 10 mM PCMS and 25 µl 100 mM PMSF; the amount of antiserum required for maximal immunoprecipitation was titrated and sufficient antiserum was added to immunoprecipitate all immunoreactive forms. The samples were then centrifuged and the supernatants were incubated with 100 µl 20% protein A-Sepharose beads at 4 °C for 1 h with gentle rocking. The samples were centrifuged and washed twice with 1 ml AG buffer, once with 1 ml 0.5 M NaCl in PBS, and twice with 1 ml PBS. Immunoprecipitates were resuspended in 100 µl Laemmli sample buffer and analyzed using either 10% Criterion Tris-HCl or 16.5% Criterion Tris-Tricine/ Peptide gels. The dried gels were exposed to phosphoimage screens and analyzed with a Typhoon 9410 Variable mode Imager and ImageQuant software (Amersham Pharmacia Biotech Inc.).

#### Results

#### *Expression of full-length proSAAS in AtT-20 cells decreases C-terminal processing and secretion of PC1*

As previously reported, expression of full-length proSAAS in AtT-20 cells inhibits PC1-mediated processing of

POMC under pulse/chase conditions (Fricker *et al.* 2000, Fortenberry *et al.* 2002) but not under steady-state conditions. To investigate whether proSAAS-mediated interruption of C-terminal processing of 87 kDa PC1 into the 66 kDa form occurs in neuroendocrine cells, AtT-20 cells overexpressing Met-SAAS (mouse proSAAS containing a single point mutation from Q83 to M83) were subjected to pulse/chase analysis and immunoprecipitation (Fig. 2). We found that Met-SAAS expressed in AtT-20 cells slightly inhibited C-terminal truncation of newly synthesized PC1 into the 66 kDa form (Fig. 2A and B) and also reduced the secretion of both mature forms (Fig. 2C).

#### The PC1 propeptide expressed as an independent domain inhibits the C-terminal processing of endogenous PC1 in AtT-20 cells

Both the PC1 propeptide and proSAAS CT peptide could potentially act as endogenous convertase inhibitors in neuroendocrine cells. In order to investigate whether the two endogenous inhibitors could have an additive effect on processing and secretion of PC1 in regulated and constitutively secreting cells, we constructed chimeras containing the PC1 propeptide and SAAT CT peptide separated by a furin recognition site (C1), the same construct lacking the furin recognition site (C2) and a construct containing the PC1 propeptide alone (C3) (see Fig. 1).

We first asked whether the PC1 propeptide and the SAAS CT peptide overexpressed in AtT-20 cells could act to inhibit C-terminal processing of endogenous PC1, an enzymatic action known to be accomplished by PC1 acting in trans (Zhou & Lindberg 1994). To test this idea, AtT-20 cells expressing C1, C2, or C3 were harvested at the indicated times and subjected to pulse/chase labeling, followed by immunoprecipitation. We observed that the propeptide construct C3 significantly inhibited C-terminal processing of PC1 into the mature 66 kDa form (Fig. 2A and B), but did not affect secretion of PC1 into the medium (Fig. 2C). However, the presence of the SAAS CT peptide in the chimeric constructs did not confer an additional inhibitory effect above and beyond that observed with the propeptide construct, suggesting that the SAAS CT peptide portion of the construct does not contribute to this inhibition. We also observed that in spite of the existence of the furin recognition site the majority of the unprocessed radiolabeled C1 and C2 was retained intracellularly at 1 h of chase (Fig. 2A). We interpret these results to suggest that the majority of C1 and C2 is misfolded and/or incorrectly processed; it is therefore retained in the ER and degraded rather than trafficking through the secretory pathway. We conclude that the propeptide construct reduces C-terminal PC1 processing into the mature 66 kDa form under pulse/chase conditions.

### In trans expression of PC1 propeptide inhibits PC1-mediated processing of POMC in AtT-20 cells

The processing of the pituitary precursor POMC is known to require convertase-mediated cleavage. To examine whether C1, C2, and C3 could inhibit PC1-mediated cleavage of POMC, AtT-20 cells expressing these constructs were subjected to pulse/chase labeling, followed by immunoprecipitation with anti-ACTH antiserum. Because the expression of proSAAS in AtT-20 cells substantially slows the processing rate of newly synthesized POMC (Fricker et al. 2000, Fortenberry et al. 2002), proSAAS was included as a positive control. We observed the extent of POMC processing through pulse/chase analyses, using both cell extracts and conditioned medium collected from wild-type and Met-SAAS-overexpressing AtT-20 cells. As expected, expression of Met-SAAS in AtT-20 cells decreased cellular POMC processing (Fig. 3A); we observed increased secretion of intact POMC (Fig. 3B), possibly via increased constitutive secretion (Fernandez et al. 1997).

POMC was processed into a number of radiolabeled immunoprecipitated POMC-derived peptides, including singly and doubly glycosylated POMC precursors (30– 35 kDa), intermediates (20–25 kDa) generated by the endoproteolytic cleavage at the ACTH-lipotropin junction, glycosylated mature ACTH (13 kDa) and unglycosylated mature ACTH (4·5 kDa) (Fig. 3). As expected from the above data showing that C1 and C2 had no kinetic effects on C-terminal processing of newly synthesized PC1, C1 and C2 did not affect the rate of PC1mediated processing of POMC. However, C3, containing only the PC1 propeptide, did reduce cleavage of newly synthesized POMC into ACTH; in the case of this construct we also observed increased secretion of intact POMC (Fig. 3B).

Taken together, these data showing reduced truncation of 87 kDa PC1 in the presence of propeptide-containing constructs suggest that decreased POMC processing produced by the propeptide construct might result from decreased levels of the highly active 66 kDa PC1 form of endogenous PC1. Secondly, these results show that the PC1 propeptide can function effectively as an *in trans* PC1 inhibitor in AtT-20 cells; however, the addition of the SAAS CT peptide – a potent inhibitor of PC1 *in vitro* – confers no additional inhibitory benefits, possibly because the constructs C1 and C2 are misfolded and/or are incorrectly processed in the ER.

## When expressed in HEK293 cells, all constructs inhibit zymogen processing; the SAAS CT peptide portion of the chimera inhibits the C-terminal cleavage of PC1

To investigate the effect of propeptide constructs on endoproteolytic processing and secretion of PC1 in constitutively secreting cells, we used HEK293 cells that were transiently transfected with either proPC1 plus empty





**Figure 2** The PC1 propeptide decreases C-terminal cleavage of 87 kDa PC1 into the fully mature 66 kDa form in AtT-20 cells under pulse/chase conditions. Wild-type AtT-20 cells (control) and AtT-20 cells stably expressing either Met-SAAS or constructs C1, C2, or C3 were labeled with [<sup>35</sup>S]Met for 20 min (P) and chased in methionine-containing medium with 1% fetal bovine serum for the indicated times, and then cell extracts (A) and medium (C) were subjected to immunoprecipitation using PC1 amino-terminally directed antiserum. The immunoprecipitates were separated using 10% Tris–HCl gels, and analyzed with a phosphoimager. (B) The ratio of 66 kDa PC1 to 87 kDa PC1 was determined by densities of digital images which were calculated using ImageQuant software. Values are shown as the ratio of 66 kDa PC1 to 87 kDa PC1 at 0 time. The experiment was independently repeated three times with similar results.

#### A. Cell extracts



**B.** Medium



**Figure 3** The PC1 propeptide decreases PC1-mediated processing of POMC in AtT-20 cells. Wild-type AtT-20 cells and AtT-20 cells stably expressing either Met-SAAS or constructs C1, C2, or C3 were subjected to pulse/chase experiments, and then cell extracts (A) and medium (B) were subjected to immunoprecipitation using ACTH(1–24) antiserum. The immunoprecipitates were separated using 16.5% Tris-Tricine/peptide gels and analyzed with a phosphoimager. Bottom panels of (A) and (B) Image modified by ImageQuant software. (C) The structure of POMC and known PC1-mediated cleavage sites ( $\downarrow$ ); N-POMC, N-terminus of POMC; JP, joining peptide;  $\downarrow$ , N-glycosylation sites. The asterisks indicate glycosylated (upper asterisk) and unglycosylated ACTH (lower asterisk).

vector (control) or with proPC1 plus constructs containing C1, C2, or C3. HEK293 cells expressing the respective constructs were incubated overnight in the presence or absence of  $5 \,\mu$ M lactacystin, and cell extracts and

overnight conditioned medium were then subjected to Western blotting.

In contrast with wild-type AtT-20 cells, HEK293 cells expressing 87 kDa PC1 together with the various



C. 66 kDa PC1, Western Blot

A. 87 kDa PC1, Western Blot

**Figure 4** HEK293 cells expressing either the PC1 propeptide with SAAS CT peptide or the PC1 propeptide alone exhibit decreased zymogen processing of proPC1. (A) and (C) HEK293 cells were transiently transfected with either proPC1 vector plus empty vector (control), or proPC1 vector plus constructs containing C1, C2, or C3 and incubated in the absence and presence of 5  $\mu$ M lactacystin for 16 h. Overnight conditioned medium was collected, concentrated and analyzed on 10% Tris–HCl gels, followed by Western blotting with anti-PC1 antiserum. (B) and (D) Conditioned medium was used to determine PC1 activity as described in Methods. FU, fluorescence units. Tubulin was used as a marker.

constructs contained decreased amounts of 87 kDa PC1; this effect was particularly pronounced for C1 and C2 (Fig. 4A). To investigate whether this decreased level of PC1 expression was related to ER-mediated degradation, we treated cells with 5 µM lactacystin. Treatment with lactacystin resulted in a slight increase in the amount of 94 kDa proPC1 in the controls, supporting the idea that a portion of proPC1 may naturally undergo degradation in HEK293 cells. Lactacystin differences in total PC1 expression were more pronounced in HEK293 cells expressing C1, C2 or C3 as opposed to control cells, as shown by the reduced density of PC1 (Fig. 4A); this result is potentially artifactual, i.e. due to a reduction in total PC1 expression in the presence of coexpression of other cDNAs. However, in the presence of lactacystin, the expression of all constructs clearly

resulted in significantly decreased zymogen processing, i.e. conversion of 94 kDa proPC1 to the active 87 kDa species (Fig. 4A), indicating that the PC1 propeptide is an effective inhibitor of autocatalysis when expressed *in trans.* In the case of HEK293 cells expressing either C1 or C2 containing the SAAS CT peptide, unlike AtT-20 cells, we observed decreased processing of 87 kDa PC1 to its 66 kDa form in the overnight-conditioned medium, indicating that the SAAS CT peptide portions of C1 and C2 inhibit C-terminal processing of PC1 (Fig. 4A).

To investigate whether the C1, C2, and C3 propeptide constructs could affect endoproteolytic processing and secretion of 66 kDa PC1, we performed a similar experiment with this truncated form of PC1 and observed similar results (Fig. 4C).





**Figure 5** Both the PC1 propeptide with SAAS CT peptide and the PC1 propeptide alone are processed in HEK293 cells. HEK293 cells transiently cotransfected with either recombinant vector containing proPC1 plus empty vector (control), or proPC1 vector plus constructs containing C1, C2, or C3 were labeled with [<sup>35</sup>S]Met for 20 min and chased in methionine-containing medium with 1% fetal bovine serum for the indicated times, and subjected to immunoprecipitation using PC1 amino-terminally directed antiserum. The immunoprecipitates were separated using 16-5% Tris–Tricine/peptide gels and analyzed with a phosphoimager. The asterisks indicate the product peptides expected by the cleavage of the propeptide portion of the respective constructs.

To confirm the ability of the PC1 propeptide and the SAAS CT peptide to affect PC1 activity secreted from HEK293 cells expressing C1-C3, PC1 assays and Western blots were performed on overnight-conditioned medium. As shown in Fig 4B, the quantity of 87 kDa PC1 in the media collected from HEK293 cells expressing the various constructs (detected by Western blotting) correlated well with enzyme activity, suggesting that constructs do not affect the activity of secreted PC1 *per se* but rather inhibit their secretion. We obtained a similar result in HEK293 cells expressing 66 kDa PC1 and the various constructs (Fig. 4D) although the total amount of activity was much smaller than that observed for 87 kDa PC1. This could be a result of the known instability of the 66 kDa form at the neutral pH of the collection medium.

To investigate whether the C1, C2, and C3 propeptide constructs are correctly processed, HEK293 cells expressing C1, C2, or C3 were harvested at the indicated times and subjected to pulse/chase labeling, followed by immunoprecipitation. We observed that all constructs were internally cleaved into the radiolabeled immunoprecipitated peptide products at the cleavage sites expected in Fig. 1. The size of the product peptides corresponded to the peptides expected (both glycosylated and unglycosylated forms), and the major bands depicted by asterisks corresponded to the glycosylated peptides generated by the cleavage of the propeptide portion of the chimera (Fig. 5). These results suggest that all constructs were correctly trafficked and processed in HEK293 cells, unlike in AtT-20 cells. Taken together, our results indicate that in HEK293 cells the PC1 propeptide can function as an inhibitor of proPC1 autocatalysis, and that the SAAS CT peptide portion of the chimera can decrease C-terminal processing of PC1 into the mature 66 kDa form.

#### Discussion

The pan-neuronally distributed protein proSAAS has been characterized in vitro as a potent inhibitor of PC1 (Fricker et al. 2000, Fortenberry et al. 2002). Fricker et al. (2000) reported that full-length proSAAS expressed in AtT-20 cells substantially inhibits the PC1-mediated cleavage of POMC when the kinetics of cleavage of newly synthesized POMC are examined. Our previous data indicate, however, that the steady-state accumulation of POMC processing products is unaffected by the coexpression of proSAAS (Fortenberry et al. 2002). We have also observed that secreted 87 kDa PC1 activity, but not 66 kDa PC1 activity, is reduced by proSAAS expression in constitutively secreting cell lines (Fortenberry et al. 2002). However, a direct effect of proSAAS on the processing and secretion of PC1 in neuroendocrine cells has not yet been examined. Here, we have used three different types of PC1 propeptide constructs, containing or lacking the presence of a second potent inhibitor, the proSAAS CT peptide, to investigate the potential inhibition of the processing and secretion of PC1 in regulated and constitutively secreting cell lines.

The data presented above demonstrate that under pulse/chase conditions, overexpression of proSAAS in AtT-20 cells inhibits C-terminal processing of newly synthesized endogenous PC1 and also reduces secretion of both mature forms. It is therefore possible that proSAAS-mediated interruption of POMC processing may be an indirect result of this inhibition of C-terminal PC1 cleavage, i.e. by prevention of the premature production of the more active 66 kDa form. By contrast, under steady-state conditions, overexpression of intact proSAAS does not inhibit C-terminal processing of PC1; this is similar to the situation with POMC (Fortenberry et al. 2000). We hypothesize that proSAAS proteins interact with PC1 and prevent premature activity of this enzyme until the PC1-proSAAS complex arrives at the TGN, where processing of POMC is thought to begin (Fernandez et al. 1997) and proSAAS may become inactivated. In this scenario the inhibitory protein pro-SAAS is used to control the intracellular timing of PC1 activity rather than its total level of activity (Fortenberry et al. 2002). It has been shown that proregion or proregionrelated peptides of Kex2p, furin, PC1, PC5A, SKI-1/S1P, or PC7 can inhibit their cognate enzymes in vitro with nanomolar Kis (Anderson et al. 1997, Boudreault et al. 1998, Zhong et al. 1999, Toure et al. 2000, Lesage et al. 2001, Fugere et al. 2002, Nour et al. 2003). PC1 is synthesized as an inactive zymogen that must be activated by the autocatalytic intramolecular cleavage of its propeptide within the ER (Goodman & Gorman 1994, Lindberg 1994, Milgram & Mains 1994). Although it has not been directly shown for PC1, by analogy with furin (Thomas 2002), the cleaved proregion is likely to remain bound to the catalytic domain until the complex arrives at the *trans*-Golgi network and is separated from the catalytic domain by acid-induced dissociation and/or additional cleavage at a secondary processing site (Boudreault *et al.* 1998). However, for PC1 this may be a ternary complex, consisting of propeptide, PC1, and proSAAS.

We found that overexpression of the PC1 propeptide alone results in significantly decreased C-terminal PC1 processing in AtT-20 cells under pulse/chase conditions. C-terminal processing of PC1 into the final 66 kDa form presumably occurs within immature secretory granules (Benjannet *et al.* 1993, Lindberg 1994, Milgram & Mains 1994). Therefore, overexpression of the PC1 propeptide in AtT-20 cells results in its independent entry into the TGN/immature secretory granules, where it is able to inhibit C-terminal auto-processing of 87 kDa PC1 into the fully active 66 kDa species.

Overexpression of constructs containing the PC1 propeptide containing an additional inhibitory module, the proSAAS CT peptide, did not appear to affect C-terminal PC1 processing under pulse/chase conditions. Our results showing that the majority of the unprocessed radiolabeled C1 and C2 remained in the cell at 1 h of chase support the idea that trafficking of these molecules through the regulated secretory pathway is impaired. It has been reported that mutation or deletions within convertase propeptides affect zymogen folding and prevent or significantly decrease autocatalytic processing within the ER (Creemers et al. 1995, 1996, Taylor et al. 1997). Based on these reports, it is possible that the overexpressed C1 and C2 constructs are (i) improperly folded in AtT-20 cells; (ii) are incapable of internal cleavage; or (iii) are cleaved at an abnormal site within the PC1 propeptide. In any of these scenarios, these constructs are predicted to remain in the ER and to undergo partial degradation in the ER/cytosol. Consequently, the newly synthesized, misprocessed, C1 and C2 proteins are most likely not efficiently routed to the later compartments of the secretory pathway to affect C-terminal PC1 processing.

We observed that under pulse/chase conditions the PC1 propeptide construct also reduced PC1-mediated cleavage of newly synthesized POMC into ACTH; the above-mentioned data on PC1 truncation effects support the idea that this decrease in POMC processing may be due, in part, to inhibited conversion of 87 kDa PC1 into the more active 66 kDa form. However, PC1 propeptide constructs containing the proSAAS CT peptide had no effect on PC1-mediated POMC processing, again, possibly because these constructs are inefficiently trafficked through the secretory pathway.

Somewhat different effects were observed in HEK293 cells, a constitutively-secreting cell line which lacks a regulated secretory pathway. All constructs expressed in HEK293 cells significantly decreased processing of proPC1 into enzymatically competent PC1. These results imply that the PC1 propeptide is an effective inhibitor of autocatalysis, and that nonprocessed proPC1 is subject to ER degradation. Consequently, both the amount and the enzymatic activity of secreted PC1 decreased. The SAAS CT peptide portions of C1 and C2 also contributed to decreased cellular C-terminal processing of PC1, supporting the idea that the SAAS CT peptide can interact with PC1 forms when expressed as a chimera with the PC1 propeptide.

In summary, the data presented above indicate that PC1 propeptide expressed *in trans* significantly reduces C-terminal PC1 cleavage and POMC processing in a regulated cell line, AtT-20 cells. In constitutively secreting cells such as HEK293, the PC1 propeptide inhibits processing of proPC1, and the SAAS CT peptide portion of the chimera decreased C-terminal processing of PC1. We conclude that the PC1 propeptide expressed *in trans* can effectively function as an inhibitor of C-terminal processing of PC1. Further studies are required to understand the molecular mechanism of PC1 activation, its interaction with proSAAS, and ultimately to develop highly potent and specific inhibitors of this enzyme potentially usable as pharmacological agents.

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