Processing of proSAAS in neuroendocrine cell lines

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ProSAAS, a recently discovered granin-like protein, potently inhibits prohormone convertase (PC)1, and might also perform additional functions. In the present study, the processing of proSAAS was compared in two neuroendocrine cell lines over-expressing this protein: the AtT-20 mouse pituitary corticotrophic line and the PC12 rat adrenal phaeochromocytoma line. The processing of proSAAS was examined by pulse–chase analysis using [^{3}H]leucine, by MS, and by chromatography and radioimmunoassay. Various smaller forms of proSAAS were detected, including peptides designated as little SAAS, PEN and big LEN. Because the PC-12 cells used in the present study do not express either PC1 or PC2, the finding that these cells efficiently cleave proSAAS indicates that these cleavages do not require either enzyme. Two of the peptides identified in AtT-20 media represent novel C-terminally truncated forms of PEN. In both cell lines, the secretion of the small proSAAS-derived peptides is stimulated by secretagogues. However, long-term treatment of wild-type AtT-20 cells with two different secretagogues (8-bromo-cAMP and a phorbol ester) does not affect levels of proSAAS mRNA; this treatment significantly increases PC1 mRNA by approx. 60–80 %. The lack of co-regulation of proSAAS and PC1 mRNA implies that enzyme activity can be induced without an accompanying increase in the inhibitor. In addition, the finding that the peptides are secreted via the regulated pathway is consistent with the proposal that they may function as neuropeptides.

Key words: granin, neuropeptide, peptide processing, prohormone convertase, 7B2.

INTRODUCTION

Neuroendocrine peptides are typically produced from larger precursors by selective cleavage. Often, these cleavage sites contain basic amino acids that are recognized by one of the prohormone convertases (PCs), such as PC1 or PC2 [1–3]. These two enzymes are broadly distributed in the neuroendocrine system, and are co-localized to secretory vesicles, along with neuroendocrine peptides [4–6]. In addition, other PCs may also contribute to peptide processing in the Golgi and/or late secretory pathway. One of the PC5 isoforms has been found in the regulated secretory pathway [7]. Other members of the PC family have been detected primarily in the *trans*-Golgi network, and are thought to play a larger role in the processing of proteins than of neuroendocrine peptides. These *trans*-Golgi-enriched enzymes include furin, paired basic amino acid converting enzyme 4 (PACE4) and PC7 [8–12].

Following the action of the endopeptidases, a carboxypeptidase is generally required to remove C-terminal basic residues from the processing intermediates. Like PC1 and PC2, carboxypeptidase E (CPE) is broadly distributed in the neuroendocrine system, where it is present in the late secretory pathway [13,14]. In contrast, carboxypeptidase D (CPD) is primarily enriched in the *trans*-Golgi network along with furin and related enzymes [15,16]. In some cases, further post-translational modifications are required (after the carboxypeptidase step) before the peptide becomes biologically active; these additional modifications include acetylation, sulphation, phosphorylation or C-terminal amidation [17].

The importance of CPE in peptide processing is evident from the large effect on peptide processing in Cpe^{fat}/Cpe^{fat} mice that have a point mutation ($Ser^{202} \rightarrow Pro$) within the coding region of the *CPE* gene [18]. This mutation leads to a complete loss of CPE activity [19]. As a result, the production of numerous peptide hormones and neurotransmitters is affected in these mice [18,20–24]. Using a novel procedure to purify peptides that accumulate in the Cpe^{fat}/Cpe^{fat} mice, a number of peptides were identified [24,25]. These peptides include known neuroendocrine peptides, as well as several novel mouse peptides. Some of these mouse peptides correspond to previously identified bovine peptides of unknown function [26]. Isolation of the cDNA revealed that five of these peptides arose from a single precursor protein by cleavage at basic amino-acid-containing sites; this precursor was termed 'proSAAS' because one of the peptides (named 'SAAS') contained the sequence Ser-Ala-Ala-Ser within a larger sequence.

Although there is no sequence similarity among proSAAS and other proteins, there are some features in common between proSAAS and 7B2, another member of the granin family [27]. Both proteins are approx. 26 kDa and contain a number of cleavage sites for the PCs. In addition, both have broad neuroendocrine tissue distributions [24,27,28]. One of the functions of 7B2 is to inhibit PC2 [29,30]. Similar studies showed that proSAAS is a potent and selective inhibitor of PC1 [24,31]. However, this inhibition is due only to a small region near the C-terminus of proSAAS [31,32]. Thus the other regions of proSAAS that have been conserved during evolution might serve other functions. In support of this possibility is the observation that proSAAS is present in neuroendocrine cells that lack PC1 [28]. Furthermore, proSAAS is processed extensively in the brain into smaller peptides that are no longer able to inhibit PC1 [33].

Previously, the processing of proSAAS was examined in AtT-20 cells using Western blotting, pulse–chase experiments and MS analyses [24]. However, each of these techniques gave distinct

Abbreviations used: CPE, carboxypeptidase E; DMEM, Dulbecco's modified Eagle's medium; ir, immunoreactive; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; MS/MS, tandem MS; PACE4, paired basic amino acid converting enzyme 4; PC, prohormone convertase; RIA, radioimmunoassay; RP, reversed phase; TFA, trifluoroacetic acid.

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results. The Western blot analysis showed only large-molecular-mass forms, the pulse–chase experiments with [^{3}H]leucine showed mainly intermediate forms, and MS revealed primarily low-molecular-mass forms [24]. Thus one of the aims of the present study was to use a quantitative analysis to address the question of the extent of proSAAS processing in AtT-20 cells. In addition, similar studies were performed after expression of proSAAS in the PC-12 cell line (a cell line that lacks PC1 and PC2 activity). The finding that proSAAS is extensively processed in this cell line indicates that neither PC1 nor PC2 is required for the processing of proSAAS. The secretion of the proSAAS-derived peptides via the regulated pathway, which is an important criterion for peptide neurotransmitters and hormones, was also examined. Finally, the question as to whether PC1 mRNA could be regulated independently of proSAAS mRNA in the AtT-20 cell line was addressed. The finding that these two mRNAs are not co-regulated suggests that cells can alter the amount of active PC1 without an accompanying increase in the inhibitor, proSAAS.

MATERIALS AND METHODS

Cell culture and transfection

The AtT-20 mouse pituitary corticotrophic cell line was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) bovine calf serum and penicillin/streptomycin (10 units/ml). AtT-20 cell lines stably overexpressing rat proSAAS were generated as described previously [24]. The PC-12 rat phaeochromocytoma cell line was maintained in DMEM supplemented with 5 % (v/v) horse serum, 10 % (v/v) bovine calf serum and penicillin/streptomycin (10 units/ml). To overexpress rat proSAAS in PC-12 cells, full-length rat proSAAS cDNA was subcloned into the *Eco*RI/*Not*I sites of the pcDNA3 vector (Invitrogen). Approx. 10^6 PC-12 cells were transfected with 10 μg of plasmid DNA combined with 25 μl of LIPOFECTAMINE™ reagent (Life Technologies). Stable cell lines were selected using 1.0 mg/ml Geneticin (also known as G418). The overexpressed proSAAS protein was identified by radioimmunoassay (RIA), as described below. Two clones expressing high levels of proSAAS were chosen for this study.

Biosynthetic studies

Cells grown in 60 mm dishes to $\approx$ 80–90 % confluence were washed twice with PBS, incubated in leucine-free medium for 1 h, and then labelled with [^{3}H]leucine (50 μCi/ml) for 10 min (the 'pulse'), followed by incubation in DMEM medium containing unlabelled leucine for 2 h (the 'chase'). The cells and media following pulse or chase were subjected to immunoprecipitation using little-SAAS antiserum (#2768) and big-LEN antiserum (#87), as described previously [24]. A protease inhibitory cocktail (Sigma) was added during the immunoprecipitation mixture. The immunoprecipitates were analysed by denaturing PAGE and autoradiography, as described previously [24].

Secretion studies

AtT-20 or PC-12 cells were cultured in 60 mm six-well dishes to $\approx$ 80–90 % confluency, washed twice with PBS, and then incubated with Hepes-buffered Krebs–Ringer bicarbonate in the presence or absence of either 10 μM forskolin or 56 mM KCl (with a proportionately reduced NaCl concentration to maintain the same ionic strength). After 30 min, the medium was collected and analysed by RIA, as described below.

For analysis of secreted peptides by HPLC and matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF)-MS, cells were washed twice with PBS, and then incubated for 3 h in DMEM medium containing 56 mM KCl. The medium was harvested and stored at -80 °C before analysis.

RIA

The quantification of peptides by RIA was performed as described previously [33] using antisera generated against little SAAS (proSAAS 42–59), PEN (proSAAS 221–242) and big LEN (proSAAS 245–260). Antibody dilutions that bound 30 % of the radioactivity were 1:3000 for anti-SAAS (antibody #2766) and anti-LEN (antibody #85), and 1:4500 for anti-PEN (antibody #141). The concentrations of non-radioactive peptides required to reduce tracer binding by 50 % (i.e. IC_{50} values) were 400 fmol/tube for SAAS, 300 fmol/tube for PEN and 200 fmol/tube for LEN.

Peptide extraction

Cells were collected, and peptides were extracted by boiling for 10 min in water containing an inhibitory cocktail [1 mM EDTA/1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride/40 μM bestatin/14 μM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane ('E-64')/22 μM leupeptin/15 μM pepstatin/0.8 μM aprotinin], followed by centrifugation at 13000 ***g*** for 30 min. The spent medium was extracted by boiling in the presence of the inhibitory cocktail for 10 min, dried under vacuum, and stored at -20 °C. Prior to RIA and/or size-exclusion chromatography, samples were resuspended in sodium phosphate buffer, pH 7.5, containing 0.2 % (v/v) Triton X-100.

Size-exclusion chromatography

Size-exclusion chromatography was performed essentially as described previously [33]. Briefly, the sample in a total volume of 50–100 μl was applied to the Superdex Peptide HR 10/30 column (Pharmacia), and separated with 0.1 % (v/v) trifluoroacetic acid (TFA) containing 30 % (v/v) acetonitrile (Fisher). Fractions at 1 min intervals were collected, with a flow rate of 0.5 ml/min. Fractions were dried and resuspended in sodium phosphate buffer, pH 7.5, containing 0.2 % (w/v) Triton X-100, before being subjected to RIA.

Reversed-phase (RP)-HPLC

For RP-HPLC, the material representing the peak of immunoreactive (ir)-peptides was pooled and loaded on to a C_{18} column (250 mm $\times$ 4.6 mm, internal diameter 5 μm; Column Engineering, Inc., Ontario, CA, U.S.A.) maintained in 0.1 % (v/v) TFA solution. The peptides were eluted with a linear gradient of acetonitrile (5–75 %) in 0.1 % TFA over 70 min with a flow rate of 0.5 ml/min; fractions were collected at 1 min intervals. The synthetic peptides (big SAAS, little SAAS, PEN, little LEN and big LEN) were analysed in a separate run to determine their retention times.

MS

Media or cell extracts were acidified by adding 0.1 % (v/v) TFA and then applied to C_8 Sep-Pak (Waters) cartridges. The cartridges were washed with 10 vol. of 0.1 % TFA, the bound peptides were eluted first with 50 % (v/v) acetonitrile in 0.1 % TFA, and then with 70 % acetonitrile in 0.1 % TFA. The two eluates were combined and dried under vacuum. The peptides

were reconstituted in 40 μl of water, and a 1 μl aliquot was analysed by MALDI–TOF-MS.

MALDI–TOF spectra were obtained in positive linear mode on a Perceptive Biosystems Voyager-DE STR mass spectrometer (Framingham, MA, U.S.A.). Approx. 100 laser shots were summed per spectrum. α-Cyano-4-hydroxycinnamate saturated in 30 % (v/v) acetonitrile and 0.1 % (v/v) TFA in water was used as a matrix. External calibration was performed with des-Arg^1-bradykinin ($[M-H]^+$ 904.4681) and neurotensin ($[M-H]^+$ 1672.9170).

Nano-electrospray ionization MS was performed on a Q-TOF instrument (Micromass, Manchester, U.K.) equipped with a Z-spray nano-electrospray ion source. Samples in 50 % (v/v) acetonitrile/1 % (v/v) acetic acid were sprayed from gold-coated, medium-length borosilicate capillaries (Protana, Odense, Denmark). The appropriate ion was selected by the quadrapole mass filter, and then induced to fragmentation by collision with argon. The collision energy was varied from 15 to 45 V to optimize fragmentation. The spectra were analysed using Mass Lynx MaxEnt 3 software (Micromass).

RNA isolation and Northern blot analysis

To examine the regulation of proSAAS mRNA, AtT-20 cells were cultured in 60 mm plates to $\approx$ 80–90 % confluence, washed twice with PBS, and then incubated with 1 mM 8-bromo-cAMP, 10 nM PMA or control medium for 6 h. Total cellular RNA was extracted as described previously [34]. The RNA integrity was confirmed by gel electrophoresis followed by ethidium bromide staining, and then quantified by measuring absorbance at 260 nm. Of the total RNA, 10 μg was separated on a formaldehyde–agarose gel and transferred on to a nitrocellulose membrane. The amount of 18 S rRNA in each lane of the Northern blot was quantified by ethidium bromide staining followed by densitization, using the Java Image analysis program (Jandel Scientific). PC1 or proSAAS mRNA was detected using ^{32}P-radiolabelled proSAAS or PC1 riboprobes, generated as described previously [24,35]. After hybridization for 16 h at 65 °C, membranes were washed with 0.1 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 0.1 % (w/v) SDS and 5 mM EDTA at 68 °C for 1–4 h. Filters were dried and exposed to Kodak XAR-5 film. The images were quantified by the Java Image analysis program (Jandel Scientific).

RNA from PC12 cells was also obtained as described in [34], and analysed on Northern blots that were probed with PC1 and PC2 cDNA probes, as described previously [24,35]. RNA from AtT-20 cells and rat brain were included on these blots as positive controls.

RESULTS

Biosynthesis of proSAAS in PC-12 cells

Previously, proSAAS has been found to be processed and secreted in AtT-20 cells and in *Cpe^{fat}*/*Cpe^{fat}* mice [24], although this study did not provide quantitative information on the levels of the various peptides. A summary of the previously detected proSAAS-derived peptides is shown in the top panel of Figure 1; except for KEP and little LEN, all of these peptides were detected in media from AtT-20 cells overexpressing proSAAS.

In the present study, the biosynthetic fate of proSAAS in PC-12 cells was compared with that in AtT-20 cells by pulse–chase analysis using a labelling time of 10 min, instead of the 20 min used in a previous study of AtT-20 cells [24]. After labelling with [^{3}H]leucine, cells were either analysed immediately or subjected to a 2 h chase before analysis. ProSAAS-derived peptides in cells and medium were analysed by immunoprecipitation using antiserum raised against little SAAS or big LEN, representing the N-terminal and C-terminal regions of proSAAS respectively (Figure 2). A 26 kDa protein that represents proSAAS is detected in both PC-12 and AtT-20 cells following the 10 min pulse (Figure 2). The intensity of this band is higher in the proSAAS-overexpressing PC-12 cells compared with the wild-type PC-12 cells, which presumably express proSAAS as gauged from the presence of the mRNA in this cell line [24]. The proSAAS is no longer detected with either of the antisera following a 2 h chase (Figure 2, upper and lower panels). A truncated form of approx. 22 kDa is detected in the AtT-20 cells and in the media from both cell lines using the antiserum raised against the N-terminus (Figure 2). In addition, a shorter approx. 3.4 kDa form is detected with this antiserum in the medium from AtT-20 cells (Figure 2). The complete absence of proSAAS following the 2 h chase indicates that proSAAS can be processed efficiently in both AtT-20 and PC-12 cells. To confirm that the PC-12 cells used in the study did not express either PC1 or PC2, RNA from wild-type PC-12 cells and the PC-12 cell lines overexpressing proSAAS was examined by Northern blot analysis. No signal for either PC1 or PC2 mRNA could be detected (results not shown), consistent with a previous report that this cell line does not express either enzyme [36].

To examine the lower-molecular-mass forms of proSAAS secreted from PC-12 cells, MS analysis of conditioned medium was performed (Figure 3). Several peptides corresponding to the predicted mass of proSAAS-derived peptides are detected in medium from PC-12 cells overexpressing proSAAS (Figure 3, lower panel). These peptides include big LEN, little SAAS, PEN, big SAAS, PEN–LEN, proSAAS 246–260, proSAAS 40–59 and proSAAS 186–218. Some of these peptides have been detected previously in proSAAS-overexpressing AtT-20 cells [24] and/or in the mouse brain [33]. Peptides with masses close to proSAAS 40–59 (2038.95) and PEN (2302.75) are detected in wild-type PC-12 cells (Figure 3, upper panel); these ions might represent endogenous proSAAS-derived peptides in this cell line.

Processing of proSAAS in AtT-20 cells and medium, and in PC-12 cells

In order to examine the extent of proSAAS processing in AtT-20 and PC-12 cells stably expressing high levels of proSAAS, peptide extracts were subjected to gel-filtration chromatography, followed by RIA with antisera raised against three different domains of proSAAS. ir-SAAS is eluted as a single peak of approx. 2 kDa with AtT-20 cell extracts and medium, and as two overlapping peaks of approx. 3 and approx. 2 kDa with PC-12 cell extracts (Figure 4). This suggests that, whereas SAAS is largely processed to a single form in AtT-20 cells, two forms of SAAS are present in PC-12 cells (Figure 4). ir-PEN is eluted as a peak of approx. 2–3 kDa both with AtT-20 cell extracts and medium and with PC-12 cell extracts. ir-LEN is eluted as a small peak of approx. 10 kDa and a larger peak of approx. 2 kDa with AtT-20 cell extracts, and as two distinct peaks of approx. 4 kDa and approx. 2 kDa with AtT-20 medium and PC-12 cell extracts (Figure 4). Taken together, these results indicate that proSAAS is efficiently processed in both cell lines, albeit to different extents.

RP-HPLC analyses of ir-proSAAS-derived peptides in AtT-20 and PC-12 cells

To characterize the peptides further, gel-filtration fractions representing the 1.5–3 kDa material were pooled and subjected to fractionation on an RP-HPLC column. The majority of ir-

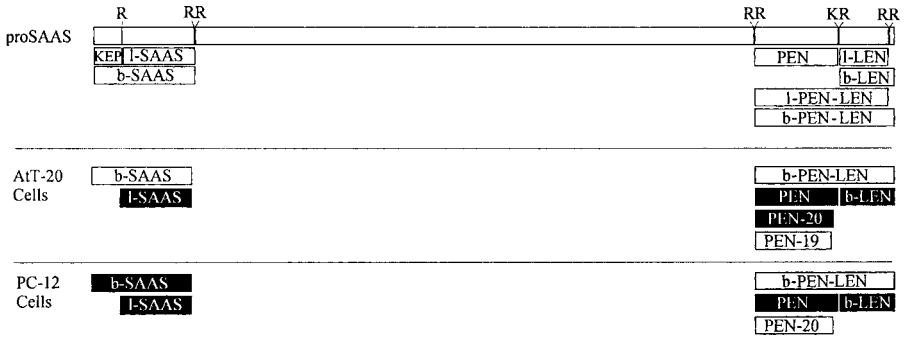

Figure 1 Schematic diagram of proSAAS

ProSAAS-derived peptides previously found in AtT-20 cells or in the *Cpe*^{fat}/*Cpe*^{fat} mouse brain [24] are indicated in the top section of the Figure. ProSAAS-derived peptides detected in the present study in AtT-20 cells or in PC-12 cells are indicated in the middle and bottom sections respectively. Filled-in boxes represent the major peptides; open boxes represent less abundant ones. All of the indicated peptides were detected by MS as well as by RIA following HPLC, as described in the Results section, and peptides PEN-20 and PEN-19 were also sequenced by MS/MS analysis, as shown in Figure 6. The amino acid positions of the various peptides within rat proSAAS are: big (b-)SAAS, 34–59; little (l-)SAAS, 42–59; KEP, 34–40; b-LEN, 245–260; l-LEN, 245–254; PEN, 221–242; b-PEN-LEN, 221–260; l-PEN-LEN, 221–254. R, arginine; K, lysine.

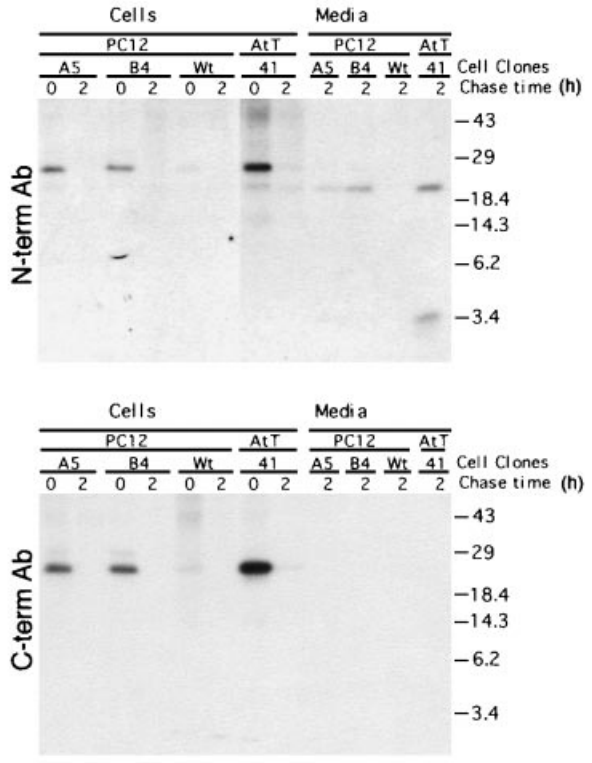

Figure 2 Pulse–chase analysis of proSAAS in wild-type PC-12 cells (Wt), two proSAAS-overexpressing PC12 cell lines (A5 and B4) and a proSAAS-overexpressing AtT-20 cell line (41)

The cells were labelled for 10 min with [^{3}H]leucine, 'chased' for 0 or 2 h, and the cell extracts and medium were subjected to immunoprecipitation, as described in the Materials and methods section using SAAS antiserum #2768 (N-term Ab; upper gels) or LEN antiserum #87 (C-term Ab; lower gels). The positions of pre-stained molecular-mass markers (in kDa) are indicated to the right of the gels. Similar results were obtained in two separate experiments.

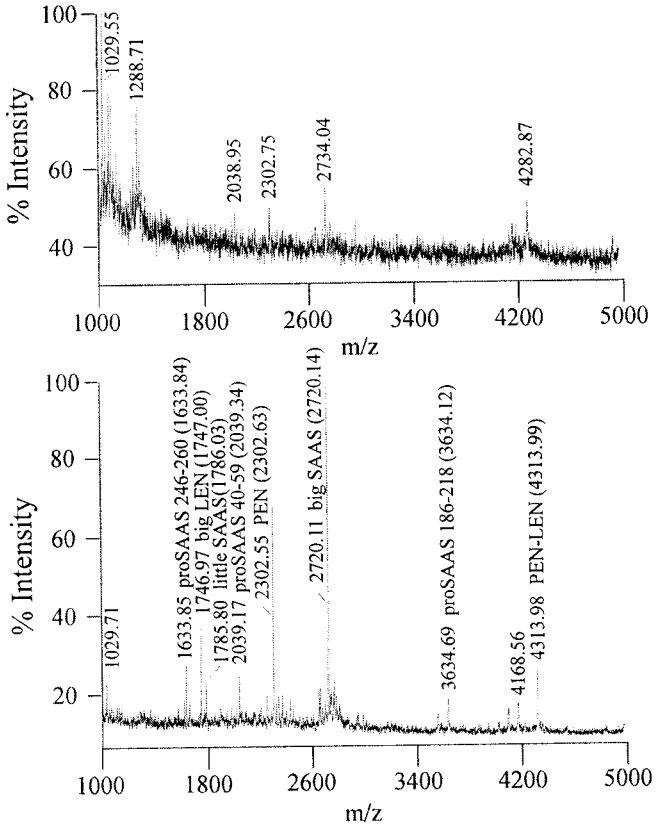

Figure 3 MS analysis of medium from wild-type PC-12 cells (upper panel) or the B4 proSAAS-overexpressing PC12 cell line (lower panel)

The culture medium was analysed using MALDI–TOF analysis, as described in the Materials and methods section. The theoretical masses are indicated in parentheses for various proSAAS-derived peptides that are less than 0.015% different from the observed mass. Two additional proSAAS-overexpressing PC-12 cell lines were examined with similar results.

SAAS in AtT-20 cell extracts and medium is eluted at the position of little SAAS (Figure 5). In contrast, the ir-SAAS in PC-12 cell extracts is eluted as two peaks corresponding to big SAAS and little SAAS (Figure 5, lower panel). Analysis of ir-PEN by HPLC shows four distinct forms in AtT-20 cell extracts and medium; two minor forms that are eluted between 40% and 45% (v/v) acetonitrile, and two major forms that are eluted

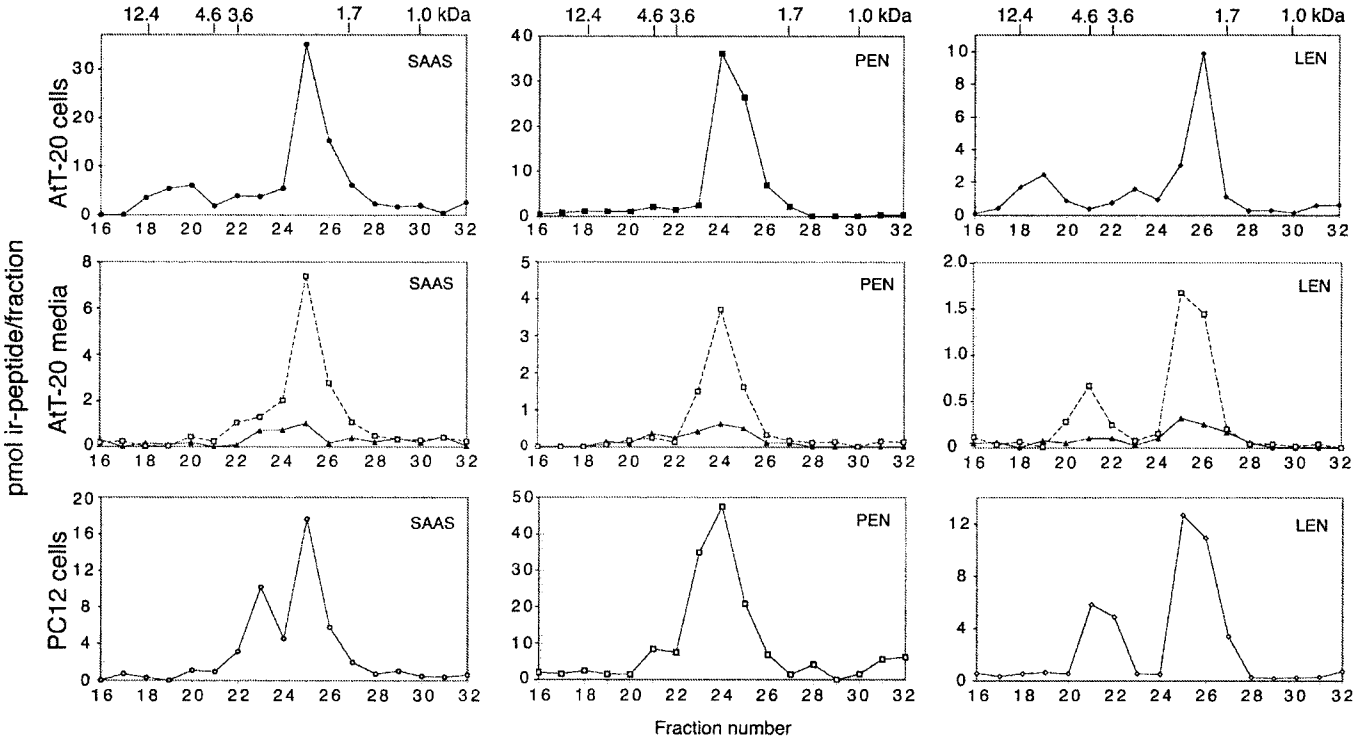

Figure 4 Fractionation of cell extracts and media from proSAAS-overexpressing cell lines by gel-filtration chromatography on a Superdex Peptide 10/30 column

Top row: AtT-20 cell extracts. Middle row: AtT-20 media collected for 30 min in the presence (broken line) or absence (continuous line) of forskolin. Bottom row: PC-12 cell extracts. Aliquots were analysed by RIA for SAAS (left column), PEN (middle column) and LEN (right column). Molecular-mass calibration standards are: cytochrome *c*, 12.4 kDa; adrenocorticotrophic hormone, 4.6 kDa; β-endorphin, 3.6 kDa; α-melanocyte-stimulating hormone, 1.7 kDa; dynorphin A-8, 1.0 kDa.

between 50 % and 52 % acetonitrile. The peak at 52 % acetonitrile has the same retention time as PEN (Figure 5). The other ir-PEN peaks were analysed further by MS (described in detail below). Of the four peaks of ir-PEN found in AtT-20 cell extracts, only two are readily detected in PC-12 cell extracts, and their relative ratios vary between the two cell lines (Figure 5). With respect to ir-LEN, a single peak that has the retention time of big LEN was seen in AtT-20 cell extracts and medium (Figure 5). Additional ir-LEN peaks are present in PC-12 cell extracts; one of these might correspond to LEN lacking the N-terminal residue (proSAAS 246–260), which was detected in the original MS analysis of the medium (Figure 3).

A pool of the fractions representing approx. 3–4 kDa ir-LEN and ir-PEN from the gel-filtration analysis was subjected to RP-HPLC. This resulted in the elution of a single peak that contained both ir-LEN and PEN immunoreactivities (results not shown). Because the anti-LEN serum recognizes big PEN–LEN, but does not cross-react with little PEN–LEN [33], these results suggest that the approx. 3–4 kDa peak observed upon gel-filtration of PC-12 cell extracts (Figure 4) corresponds to big PEN–LEN.

MS of peptides isolated from AtT-20 cells and medium

To confirm the identity of proSAAS-derived peptides in AtT-20 medium and cell extracts, HPLC fractions that contained the peaks of immunoreactivity were subjected to MALDI–TOF-MS analysis. Fraction no. 37 contains a peptide with an average $[M-H]^+$ mass of 1787.2 (cells) and 1786.53 (medium); this corresponds to little SAAS (theoretical mass of 1786.03). A peak with an average $[M-H]^+$ mass of 2720.53 was identified in HPLC fraction no. 35; this corresponds to big SAAS (theoretical mass of 2720.14). The ir-LEN in HPLC fraction no. 31 was identified as big LEN, since the observed average $[M-H]^+$ mass of 1747.07 (medium) and 1747.03 (cells) matches the theoretical mass of 1747.00 for this peptide. Fraction no. 47 contains a peptide with an average $[M-H]^+$ mass of 2303.58 (medium) and 2302.70 (cells); this corresponds to the full-length 22-residue form of PEN (theoretical mass of 2302.63).

MALDI–TOF analysis of the other ir-PEN peptides in fractions 35, 40 and 45 (representing peaks at 40 %, 45 % and 50 % acetonitrile respectively) did not reveal any peptides with a mass corresponding to that of the full-length PEN. Instead, fraction 40 contained a peptide with an average $[M-H]^+$ mass of 1934.84; this corresponds to the predicted mass of a 19-residue form of PEN that lacks the three C-terminal residues of full-length PEN (theoretical mass of 1935.15). To confirm this assignment, the sample was subjected to tandem MS (MS/MS) analysis with an electrospray ionization Q-TOF mass spectrometer (Figure 6A). The majority of the observed MS/MS fragments match up with expected b- and y-series ions of this 19-residue peptide (Figure 6A). We have designated this novel peptide 'PEN-19', to distinguish it from the full-length 22-residue form of PEN. Fraction no. 45 contains a peptide with an average $[M-H]^+$ mass of 2047.86; this corresponds to a 20-residue form of PEN lacking the two C-terminal residues of full-length PEN (theoretical mass of 2047.31). This assignment was also confirmed by MS/MS analysis (Figure 6B), and this peptide has been designated 'PEN-20'. The identity of the ir-PEN peptide in

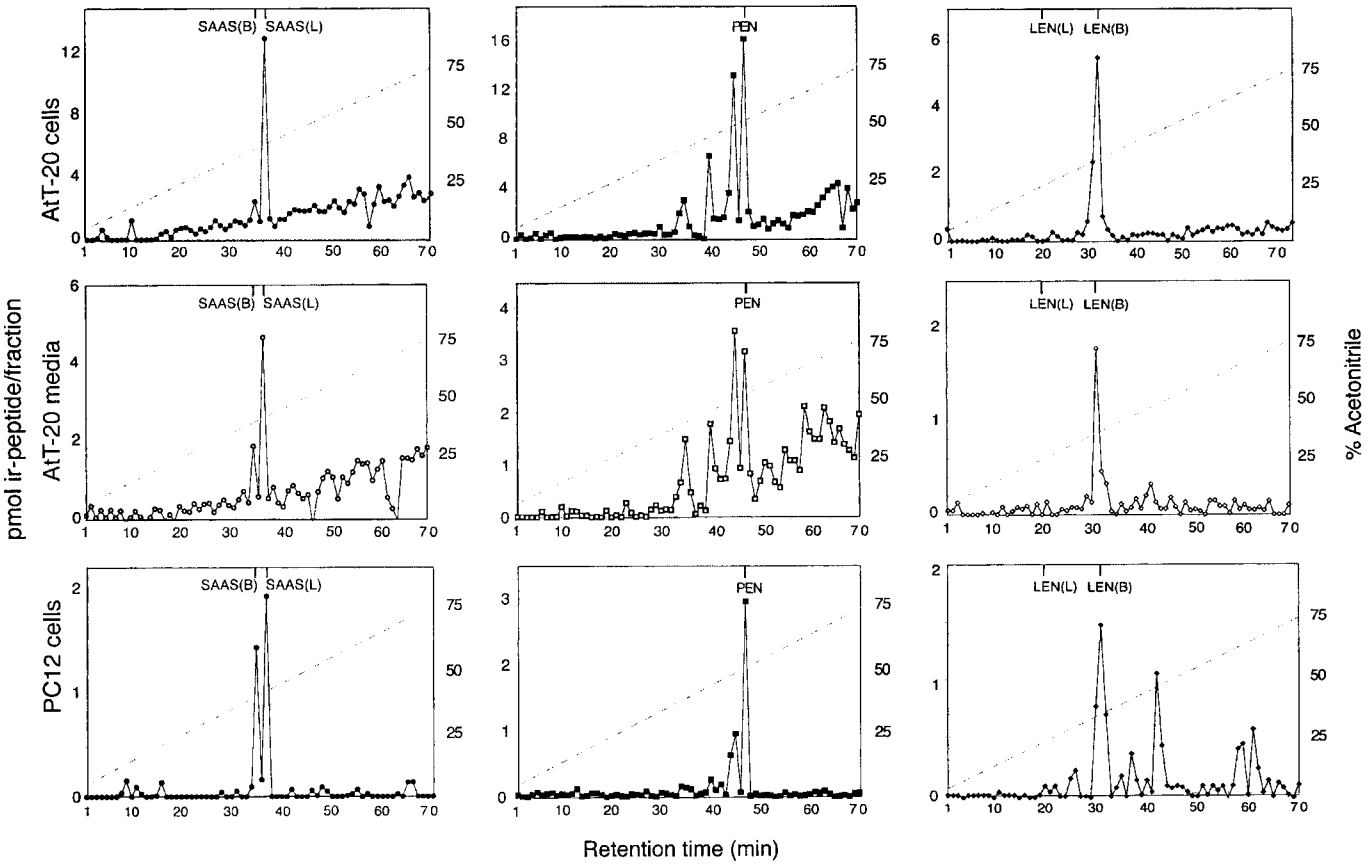

Figure 5 HPLC analysis of the 1.5–3 kDa peak of ir-proSAAS-derived peptides following gel-filtration analysis of proSAAS-overexpressing cell lines

Shown are AtT-20 cell extracts (top row), forskolin-stimulated AtT-20 medium (middle row) and PC-12 cell extracts (bottom row). Gel-filtration fractions representing the peaks of ir-SAAS, ir-PEN and ir-LEN were pooled and subjected to HPLC on a C_{18} column with a linear gradient of acetonitrile from 5–75% (broken line). The fractions were analysed for ir-SAAS (left column), ir-PEN (middle column) and ir-LEN (right column). The lines on the top margin denote the elution positions of synthetic peptides (B, big form; L, little form).

fraction 35 could not be determined, because of the relatively low abundance of this peptide.

Stimulated release of proSAAS-processing products from AtT-20 and PC-12 cells

In order to determine whether proSAAS-derived peptides could be released via the regulated secretory pathway, cell lines stably expressing rat proSAAS were treated with different secretagogues, and the medium was assayed for ir-SAAS, ir-PEN and ir-LEN by RIA. A 30 min treatment of AtT-20 cells with forskolin results in a large increase over basal secretion in all three peptides (Figure 4, middle panels). Similarly, the secretion of all three peptides from two different proSAAS-expressing lines of PC-12 cells is stimulated 3–10-fold by 56 mM KCl (Figure 7).

Regulation of proSAAS and PC1 mRNA expression

To examine whether the expression of proSAAS is co-regulated with that of PC1, wild-type AtT-20 cells were incubated for 6 h with 8-bromo-cAMP or phorbol ester (PMA). These treatments have been shown previously to increase PC1 mRNA levels in other cell lines [37,38]. Northern blot analysis of AtT-20 cells revealed that the mRNA level of PC1 was increased by approx. 60–80% with 8-bromo-cAMP, and by 40–60% with PMA (Figure 8). This increase is statistically significant ($P < 0.01$ for 8-bromo-cAMP and $P < 0.05$ for PMA). In contrast, the mRNA level of proSAAS was not affected significantly by either 8-bromo-cAMP or PMA.

DISCUSSION

Neuroendocrine cell lines have proven to be a useful model to identify the enzymes involved in the maturation of neuropeptide and hormone precursors. In the present study, we have investigated the processing of proSAAS in cell lines containing (AtT-20) and lacking (PC-12) the endopeptidases PC1 and PC2. Although proSAAS was processed in both cell lines, there were differences in the extent of the processing. In AtT-20 cells, the proSAAS was mainly converted into little SAAS, big LEN, PEN and PEN-20 (Figure 1). In contrast, big SAAS was not processed as completely in PC-12 cells, with approx. 40% of the total ir-SAAS peptides represented by big SAAS. Similarly, the relative level of PEN-20 is much lower in PC-12 cells than in AtT-20 cells. Neither PEN-19 nor the unidentified form of ir-PEN that was eluted in HPLC fraction no. 34 was detected in the PC-12 cell line. Both cell lines contained PEN–LEN, but at lower levels than big LEN. On the basis of different processing profiles of proSAAS in the AtT-20 and PC-12 cell lines, it is likely that PC1 and/or PC2 contribute to the processing of proSAAS to the shorter peptides that are more abundant in the AtT-20 cells. However, the finding that many of these peptides are produced

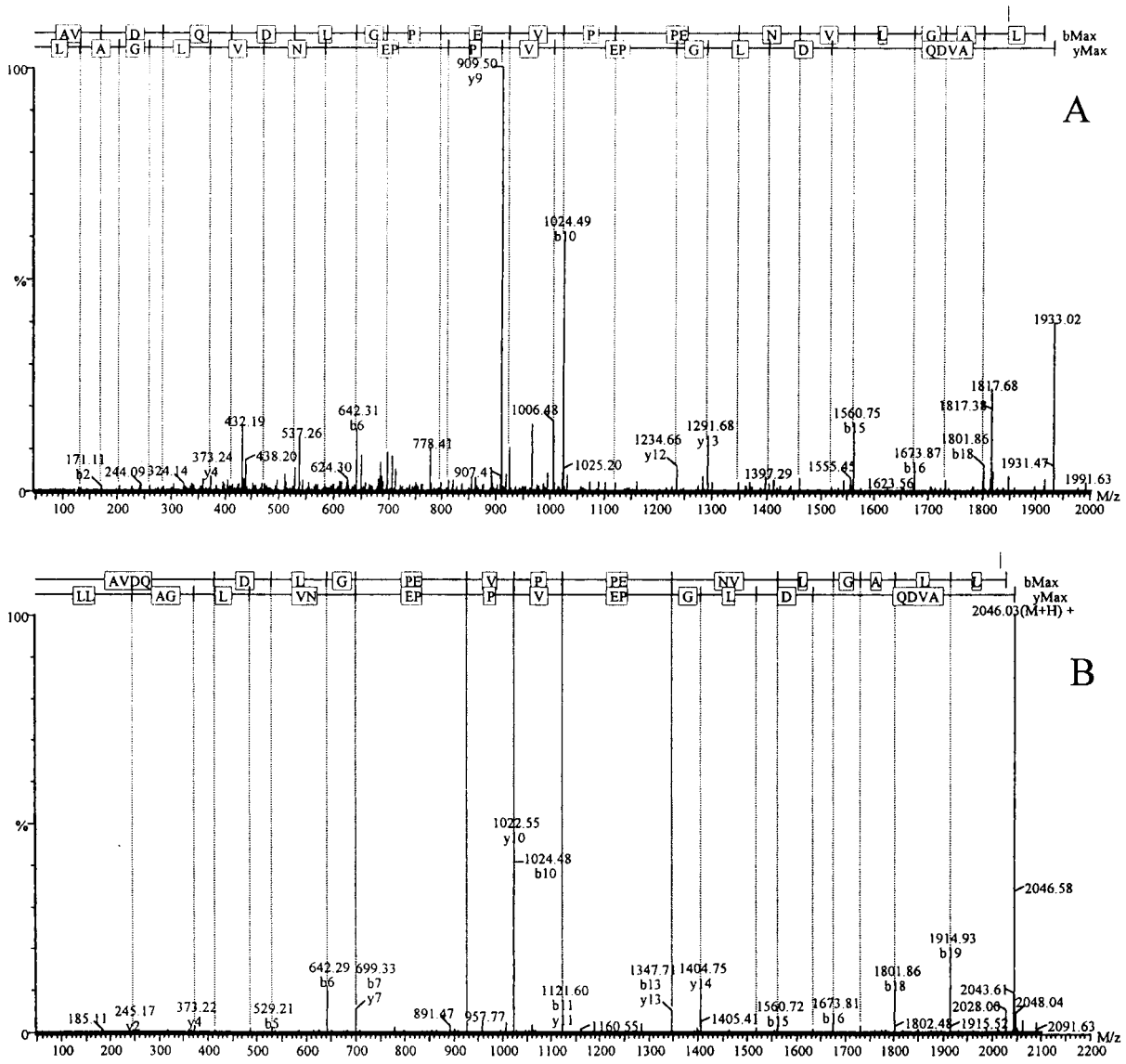

Figure 6 Nano-electrospray ionization Q-TOF MS/MS analysis of the ion with a [$M-H^+$] mass of 1934.84 in HPLC fraction no. 40 (A), and the ion with a [$M-H^+$] mass of 2047.86 in HPLC fraction no. 45 (B) of the AtT-20 medium

The spectra were acquired for 5–10 min with approx. 0.2–0.3 μl of sample. The indicated amino acid sequences were determined from the mass difference between the fragment ions.

in PC-12 cells indicates that neither PC1 nor PC2 is essential for these cleavages. Most of the cleavage sites required to generate the peptides found in PC-12 cells fit the consensus site for furin [9]. Recently, purified furin was found to process bacterially expressed proSAAS *in vitro* [39]. Although a detailed analysis of the specific forms of proSAAS was not performed, the general sizes of the products of furin cleavage correspond to big SAAS, PEN–LEN and big LEN [39]. In addition to furin, it is possible that other members of the PC family (such as PC5, PC7 and PACE4) may participate in proSAAS processing.

A major finding of the present study is the identification of two novel PEN-derived peptides. Previously, studies on the forms of proSAAS peptides in the mouse brain detected ir-PEN peptides that were eluted from RP-HPLC with similar retention times, and presumably represent these two novel forms [33]. However, due to the large number of peptides present in mouse brain, it was not possible to identify these novel forms of PEN in the previous study. Because the HPLC-purified samples from proSAAS-expressing AtT-20 medium contained only a few peptides upon analysis by MS, it was possible to perform MALDI–TOF and Q-TOF MS/MS analyses and to identify two of the peptides. Both correspond to C-terminally truncated forms of PEN, one (PEN-20) lacking two residues, the other (PEN-19) lacking three. When analysing peptides in medium, one has to consider the possibility that the cleavages occur following secretion, either by a cellular enzyme or a residual enzyme from the serum (even though the cells were incubated in serum-free medium for the collection period, small amounts of serum proteases might still be bound to the cells). This is unlikely to account for the finding of similar peptides in the AtT-20 cell extracts (Figure 5) and mouse brain extracts [33]. Furthermore, because the cells and mouse brains were extracted rapidly by boiling for 10 min in a protease inhibitor cocktail, it is also unlikely that they represent post-extraction cleavages.

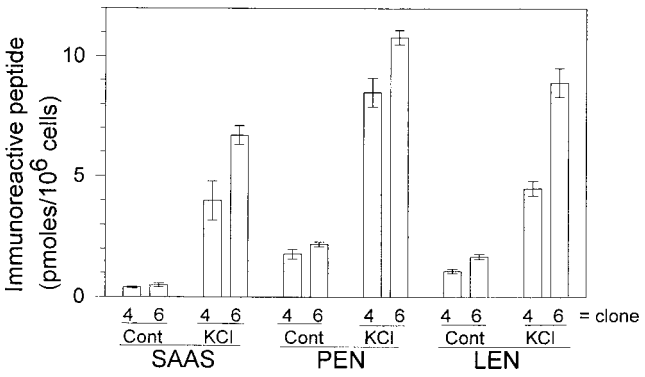

Figure 7 Secretion of peptides from proSAAS-expressing PC-12 cells

Two independent cell lines (clone B4 and clone B6) were exposed for 30 min to either standard Hepes-buffered Krebs–Ringer bicarbonate containing the normal amount of NaCl and KCl (Cont), or to a similar buffer in which the KCl concentration was 56 mM and the concentration of NaCl was decreased to maintain the same ionic strength. The medium was collected and analysed by RIA, as described in the Materials and methods section. Error bars show the S.E.M. for triplicate determinations.

It is not clear which enzymes are involved in the formation of PEN-19 and PEN-20. The generation of PEN-20 requires cleavage between leucine and arginine, and the generation of PEN-19 requires processing between leucine and leucine, within the Leu-Leu-Arg-Val^{242} sequence (numbering system of human/rat proSAAS). Although peptide processing frequently occurs at basic residues, a large number of peptides also require cleavage at non-basic residues [25,26,40–46]. Alternatively, it is possible that PEN-19 and PEN-20 arise by carboxypeptidase trimming of full-length PEN. When proSAAS was incubated with thrombin, substantial cleavage was found to occur at Arg^{241} of proSAAS, even though this is not predicted to be a consensus site for thrombin. Thus this region of the protein is presumably folded into a protease-accessible domain. Cleavage at Arg^{241}, followed by CPE action, would generate PEN-20. However, the formation of PEN-19 would require either a carboxypeptidase to remove leucine from PEN-20, or an endopeptidase to cleave full-length PEN (or PEN–LEN) directly between the Leu-Leu sequence; neither type of activity has been reported previously to be present in the secretory pathway. The relative proportions of PEN, PEN-20 and PEN-19 vary between the two cell lines investigated, suggesting that the enzymes that generate these different forms are expressed at different levels in AtT-20 and PC-12 cells.

Another finding of the present study is that the peptides derived from proSAAS are secreted from AtT-20 and PC-12 cells via the regulated pathway. However, unlike some of the other components of the regulated secretory pathway, proSAAS mRNA levels are not altered by treatments that stimulate secretion. For example, 8-bromo-cAMP and phorbol esters increase PC1 mRNA levels, but do not affect proSAAS mRNA levels (Figure 8). The lack of co-regulation of proSAAS and PC1 mRNA implies that PC1 enzyme activity can be modulated independently of proSAAS. Examination of the promoter regions of the *proSAAS* and *PC1* genes shows no obvious similarities. Although both genes lack TATA and CAAT boxes, the *PC1* gene contains two cAMP-response elements that mediate the effect of 8-bromo-cAMP on mRNA levels [47]. In contrast, neither the mouse nor the human *proSAAS* genes contain sequences resembling cAMP-response elements within several hundred nucleotides of the promoter region.

Taken together, the results of the present study have implications for the role of proSAAS as an endogenous inhibitor of PC1. Because PC1 is potently inhibited by proSAAS and processing intermediates such as PEN–LEN, it was not previously clear how active PC1 could exist in cell lines that contain equal amounts of the two proteins. Although PC1 has been shown previously to cleave PEN–LEN into smaller peptides, presumably PEN and LEN, this cleavage is extremely slow [32]; overnight incubation of PEN–LEN with a 10-fold excess of PC1 resulted in cleavage of only a fraction of the PEN–LEN peptide (Y. Qian, F.-Y. Che and L. D. Fricker, unpublished work). The finding that enzymes other than PC1 can process proSAAS into PEN and big LEN provides a mechanism for the activation of PC1. In this model, PC1 is inactive in the endoplasmic reticulum and Golgi apparatus, due to both the sub-optimal environment (neutral pH and relatively low Ca^{2+} levels) and the presence of proSAAS. Subsequently, when proSAAS is cleaved into PEN and LEN, the inhibition of PC1 by proSAAS is eliminated.

In addition to their function as endogenous inhibitors of PC1, it is possible that various proSAAS-derived peptides perform additional functions, perhaps as neurotransmitters. To test this hypothesis further, it is essential to know the exact molecular forms of the proSAAS-derived peptides that exist *in vivo*. In this respect, the identification of two novel C-terminally truncated

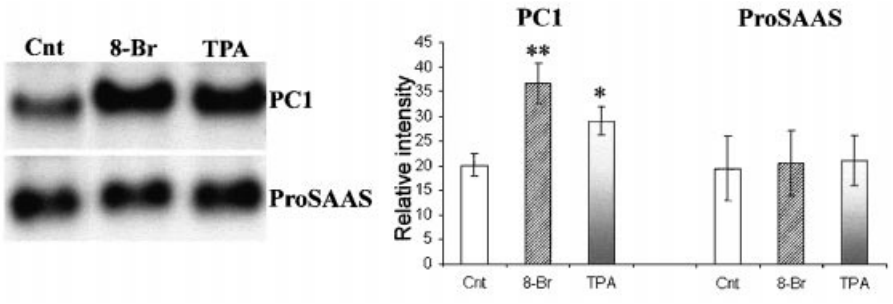

Figure 8 Northern blot analysis of proSAAS mRNA from AtT-20 cells

Wild-type AtT-20 cells were grown in 150 mm dishes to 80–90% confluency, then incubated without (Cnt) or with 8-bromo-cAMP (8-Br) or PMA (TPA) for 6 h. The mRNA was isolated, and 10 μg was separated on a formaldehyde/agarose gel, as described in the Materials and methods section. The 18 S rRNA was detected by ethidium bromide staining and proSAAS mRNA and PC1 mRNA were detected using ^{32}P-labelled riboprobes. Left panel: Representative autoradiogram of Northern blot analysis of PC1 and proSAAS mRNA. Right panel: images were quantified using the Java Image analysis program, and the relative amount of PC1 or proSAAS mRNA was divided by the relative amount of 18 S rRNA. Error bars show the S.E.M. ($n = 4$). Statistical analysis was performed using Student's t test (SigmaPlot, Jandel Scientific). $^*P < 0.05$ compared with the control; $^{**}P < 0.01$ compared with control.

forms of PEN is important for further studies investigating the bioactivities of each of the proSAAS-derived peptides.

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REFERENCES

1 Seidah, N. G. and Chretien, M. (1998) Proprotein convertase I. In Handbook of Proteolytic Enzymes (Barrett, A. J., Rawlings, N. D. and Woessner, J. F., eds.), pp. 349–353, Academic Press, San Diego
2 Seidah, N. G. and Chretien, M. (1998) Prohormone convertase 2. In Handbook of Proteolytic Enzymes (Barrett, A. J., Rawlings, N. D. and Woessner, J. F., eds.), pp. 345–357, Academic Press, San Diego
3 Zhou, A., Webb, G., Zhu, X. and Steiner, D. F. (1999) Proteolytic processing in the secretory pathway. J. Biol. Chem. **274**, 20745–20748
4 Smeekens, S. P. and Steiner, D. F. (1990) Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease Kex2. J. Biol. Chem. **265**, 2997–3000
5 Seidah, N. G., Marcinkiewicz, M., Benjannet, S., Gaspar, L., Beaubien, G., Mattei, M. G., Lazure, C., Mbikay, M. and Chretien, M. (1991) Cloning and primary sequence of a mouse candidate prohormone convertase PC1 homologous to PC2, furin, and Kex2: distinct chromosomal localization and messenger RNA distribution in brain and pituitary compared to PC2. Mol. Endocrinol. **5**, 111–122
6 Bennett, D. L., Bailyes, E. M., Nielsen, E., Guest, P. C., Rutherford, N. G., Arden, S. D. and Hutton, J. C. (1992) Identification of the type 2 proinsulin processing endopeptidase as PC2, a member of the eukaryotic subtilisin family. J. Biol. Chem. **267**, 15229–15236
7 De Bie, I., Marcinkiewicz, M., Malide, D., Lazure, C., Nakayama, K., Bendayan, M. and Seidah, N. G. (1996) The isoforms of proprotein convertase PC5 are sorted to different subcellular compartments. J. Cell Biol. **135**, 1261–1275
8 Molloy, S. S., Thomas, L., VanSlyke, J. K., Stenberg, P. E. and Thomas, G. (1994) Intracellular trafficking and activation of the furin proprotein convertase: localization to the TGN and recycling from the cell surface. EMBO J. **13**, 18–23
9 Nakayama, K. (1997) Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins. Biochem. J. **327**, 625–635
10 Kiefer, M. C., Tucker, J. E., Joh, R., Landsberg, K. E., Saltman, D. and Barr, P. J. (1991) Identification of a second human subtilisin-like protease gene in the fes/fps region of chromosome 15. DNA Cell Biol. **10**, 757–769
11 Mains, R. E., Berard, C. A., Denault, J., Zhou, A., Johnson, R. C. and Leduc, R. (1997) PACE4: a subtilisin-like endoprotease with unique properties. Biochem. J. **321**, 587–593
12 Seidah, N. G., Hamelin, J., Mamarbachi, M., Dong, W., Tadros, H., Mbikay, M., Chretien, M. and Day, R. (1996) cDNA structure, tissue distribution, and chromosomal localization of rat PC7, a novel mammalian proprotein convertase closest to yeast kexin-like proteinases. Proc. Natl. Acad. Sci. U.S.A. **93**, 3388–3393
13 Fricker, L. D. and Snyder, S. H. (1982) Enkephalin convertase: purification and characterization of a specific enkephalin-synthesizing carboxypeptidase localized to adrenal chromaffin granules. Proc. Natl. Acad. Sci. U.S.A. **79**, 3886–3890
14 Fricker, L. D. (1998) Carboxypeptidase E/H. In Handbook of Proteolytic Enzymes (Barrett, A. J., Rawlings, N. D. and Woessner, J. F., eds.), pp. 1341–1344, Academic Press, San Diego
15 Varlamov, O. and Fricker, L. D. (1998) Intracellular trafficking of metallocarboxypeptidase D in AtT-20 cells: localization to the *trans*-Golgi network and recycling from the cell surface. J. Cell Sci. **111**, 877–885
16 Fricker, L. D. (1998) Metallocarboxypeptidase D. In Handbook of Proteolytic Enzymes (Barrett, A. J., Rawlings, N. D. and Woessner, J. F., eds.), pp. 1349–1351, Academic Press, San Diego
17 Bennett, H. P. J. (1991) Glycosylation, phosphorylation, and sulfation of peptide hormones and their precursors. In Peptide Biosynthesis and Processing (Fricker, L. D., ed.), pp. 111–140, CRC Press, Boca Raton
18 Naggert, J. K., Fricker, L. D., Varlamov, O., Nishina, P. M., Rouille, Y., Steiner, D. F., Carroll, R. J., Paigen, B. J. and Leiter, E. H. (1995) Hyperproinsulinemia in obese *fat/fat* mice associated with a point mutation in the carboxypeptidase E gene and reduced carboxypeptidase E activity in the pancreatic islets. Nat. Genet. **10**, 135–142
19 Varlamov, O., Leiter, E. H. and Fricker, L. D. (1996) Induced and spontaneous mutations at Ser^{202} of carboxypeptidase E: effect on enzyme expression, activity, and intracellular routing. J. Biol. Chem. **271**, 13981–13986
20 Fricker, L. D., Berman, Y. L., Leiter, E. H. and Devi, L. A. (1996) Carboxypeptidase E activity is deficient in mice with the *fat* mutation: effect on peptide processing. J. Biol. Chem. **271**, 30619–30624
21 Rovere, C., Viale, A., Nahon, J. and Kitabgi, P. (1996) Impaired processing of brain proneurotensin and promelanin-concentrating hormone in obese *fat/fat* mice. Endocrinology **137**, 2954–2958
22 Udupi, V., Gomez, P., Song, L., Varlamov, O., Reed, J. T., Leiter, E. H., Fricker, L. D. and Greeley, G. H. J. (1997) Effect of carboxypeptidase E deficiency on progastrin processing and gastrin mRNA expression in mice with the *fat* mutation. Endocrinology **138**, 1959–1963
23 Berman, Y., Mzhavia, N., Polonskaia, A. and Devi, L. A. (2001) Impaired prohormone convertases in Cpe^{fat}/Cpe^{fat} mice. J. Biol. Chem. **276**, 1466–1473
24 Fricker, L. D., McKinzie, A. A., Sun, J., Curran, E., Qian, Y., Yan, L., Patterson, S. D., Courchesne, P. L., Richards, B., Levin, N. et al. (2000) Identification and characterization of proSAAS, a granin-like neuroendocrine peptide precursor that inhibits prohormone processing. J. Neurosci. **20**, 639–648
25 Che, F., Yan, L., Li, H., Mzhavia, N., Devi, L. and Fricker, L. D. (2001) Identification of novel peptides from brain and pituitary of Cpe^{fat}/Cpe^{fat} mice. Proc. Natl. Acad. Sci. U.S.A. **98**, 9971–9976
26 Sigafoos, J., Chestnut, W. G., Merrill, B. M., Taylor, L. C. E., Diliberto, E. J. and Viveros, O. H. (1993) Novel peptides from adrenomedullary chromaffin vesicles. J. Anat. **183**, 253–264
27 Hsi, K. L., Seidah, N. G., DeSerres, G. and Chretien, M. (1982) Isolation and NH_2-terminal sequence of a novel porcine anterior pituitary polypeptide: homology to proinsulin, secretin and Rous sarcoma virus transforming protein TVFV60. FEBS Lett. **147**, 261–266
28 Feng, Y., Reznik, S. E. and Fricker, L. D. (2001) Distribution of proSAAS-derived peptides in rat neuroendocrine tissues. Neuroscience (Oxford) **105**, 469–478
29 Martens, G. J., Braks, J. A., Eib, D. W., Zhou, Y. and Lindberg, I. (1994) The neuroendocrine polypeptide 7B2 is an endogenous inhibitor of prohormone convertase PC2. Proc. Natl. Acad. Sci. U.S.A. **91**, 5784–5787
30 van Horssen, A. M., van den Hurk, W. H., Bailyes, E. M., Hutton, J. C., Martens, G. J. and Lindberg, I. (1995) Identification of the region within the neuroendocrine polypeptide 7B2 responsible for the inhibition of prohormone convertase PC2. J. Biol. Chem. **270**, 14292–14296
31 Qian, Y., Devi, L. A., Mzhavia, N., Munzer, S., Seidah, N. G. and Fricker, L. D. (2000) The C-terminal region of proSAAS is a potent inhibitor of prohormone convertase 1. J. Biol. Chem. **275**, 23596–23601
32 Cameron, A., Fortenberry, Y. and Lindberg, I. (2000) The SAAS granin exhibits structural and functional homology to 7B2 and contains a highly potent hexapeptide inhibitor of PC1. FEBS Lett. **473**, 135–138
33 Mzhavia, N., Berman, Y., Che, F., Fricker, L. D. and Devi, L. A. (2001) ProSAAS processing in mouse brain and pituitary. J. Biol. Chem. **276**, 6207–6213
34 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
35 Gee, P., Rhodes, C. H., Fricker, L. D. and Angeletti, R. H. (1993) Expression of neuropeptide processing enzymes and neurosecretory proteins in ependyma and choroid plexus epithelium. Brain Res. **617**, 238–248
36 Bloomquist, B. T., Eipper, B. A. and Mains, R. E. (1991) Prohormone-converting enzymes: regulation and evaluation of function using antisense RNA. Mol. Endocrinol. **5**, 2014–2024
37 Mania-Farnell, B. L., Botros, I., Day, R. and Davis, T. P. (1996) Differential modulation of prohormone convertase mRNA by second messenger activators in two cholecystokinin-producing cell lines. Peptides. **17**, 47–54
38 Udupi, V., Townsend, C. M., Gomez, G., Zhang, T. and Greeley, G. H. J. (1995) Downregulation of prohormone convertase-1 by a phorbol ester. Biochem. Biophys. Res. Commun. **217**, 495–500
39 Sayah, M., Fortenberry, Y., Cameron, A. and Lindberg, I. (2001) Tissue distribution and processing of proSAAS by proprotein convertases. J. Neurochem. **76**, 1833–1841
40 Feistner, G. J., Hojrup, P., Evans, C. J., Barofsky, D. F., Faull, K. F. and Roepstorff, P. (1989) Mass spectrometric charting of bovine posterior/intermediate pituitary peptides. Proc. Natl. Acad. Sci. U.S.A. **86**, 6013–6017
41 Flanagan, T., Taylor, L., Poulter, L., Viveros, O. H. and Diliberto, E. J. (1990) A novel 1745-dalton pyroglutamyl peptide derived from chromogranin B is in the bovine adrenomedullary chromaffin vesicle. Cell. Mol. Neurobiol. **10**, 507–523
42 Nielsen, E., Welinder, B. S. and Madsen, O. D. (1991) Chromogranin-B, a putative precursor of eight novel rat glucagonoma peptides through processing at mono-, di-, or tribasic residues. Endocrinology **129**, 3147–3156
43 Vilim, F. S., Aarnisalo, A. A., Nieminen, M., Lintunen, M., Karlstedt, K., Kontinen, V. K., Kalso, E., States, B., Panula, P. and Ziff, E. (1999) Gene for pain modulatory neuropeptide NPFF: induction in spinal cord by noxious stimuli. Mol. Pharmacol. **55**, 804–811

44 Strub, J., Garcia-Sablone, P., Lonning, K., Taupenot, L., Hubert, P., Van Dorsselaer, A., Aunis, D. and Metz-Boutigue, M. (1995) Processing of chromogranin B in bovine adrenal medulla. Eur. J. Biochem. **229**, 356–368

45 Ahn, K. (1998) Endothelin-converting enzyme 1. In Handbook of Proteolytic Enzymes (Barrett, A. J., Rawlings, N. D. and Woessner, J. F., eds.), pp. 1085–1089, Academic Press, San Diego

46 Ahn, K. (1998) Endothelin-converting enzyme 2. In Handbook of Proteolytic Enzymes (Barrett, A. J., Rawlings, N. D. and Woessner, J. F., eds.), pp. 1090–1091, Academic Press, San Diego

47 Jansen, E., Ayoubi, T. A. Y., Meulemans, S. M. P. and van de Ven, W. J. M. (1995) Neuroendocrine-specific expression of the human prohormone convertase 1 gene. J. Biol. Chem. **270**, 15391–15397

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