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Heterologous processing of rat prosomatostatin to somatostatin-14 by PC2: requirement for secretory cell but not the secretion granule

Aristea S. GALANOPOULOU,* Nabil G. SEIDAH† and Yogesh C. PATEL*‡

*Fraser Laboratories, McGill University Departments of Medicine and Neurology and Neurosurgery, Royal Victoria Hospital and Montreal Neurological Institute, 687 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1, and †Clinical Research Institute of Montreal, Montreal, Quebec, Canada H2W 1R7

The role of PC2 in prosomatostatin (PSS) processing was investigated in GH_3/GH_4C_1 pituitary cells. These cells are sparsely granulated, express different amounts of PC2 and no PC1. We describe heterologous processing of rat PSS (rPSS) co-expressed with PC2 in stably transfected cells, correlate PC2 protein levels under different conditions of transfection with efficiency of PSS processing to somatostatin-14 (SS-14), determine the effect of modulating cell granularity on enzyme expression and PSS processing, and compare the relative potency of PC2 with that of PC1. PSS and cleavage products were monitored by HPLC and radioimmunoassay of SS-like immunoreactivity (SS LI). Radioimmunoassay analysis of N-terminal PC2-like immunoreactivity (PC2 LI) in GH_4C_1:rPSS, GH_4C_1:rPSS+PC2 and GH_3:rPSS transfectants showed a gradient of PC2 protein of 1:2.6:3.4 in cell extracts and 1:4.7:9 in secretion media from these cells respectively. The concentration of PC2 protein correlated with SS-14 conversion efficiency was $36\pm3\%$ in GH_4C_1:rPSS cells, $56\pm7\%$ in GH_4C_1:rPSS-PC2 cells and 100% in GH_3:rPSS cells. Treatment of GH_4C_1:rPSS+PC2 cells with epidermal growth factor, insulin, and β-estradiol to induce granules, significantly increased basal and forskolin-stimulated co-release of SS LI and PC2 LI, but had no influence on SS-14 processing efficiency. Hormone treatment led to a small increase in the ratio of mature PC2 (68 kDa) to proPC2 (75 kDa) forms. PC1 stably transfected in GH_4C_1 cells produced significantly greater SS-14 conversion (62% in cells, 66% in media) compared with PC2 transfectants (53% in cells, 47% in media) These results provide the first proof that PC2 can effect dibasic processing of mammalian PSS, and, along with PC1, qualifies as an authentic SS-14 convertase. The activity of PC2 requires the milieu of the secretory cell but not the secretory granule.

INTRODUCTION

Somatostatin (SS), a multifunctional peptide, is produced in neurons and secretory cells in a variety of tissues, notably brain, pancreatic islets, gut and thyroid, and regulates key cellular processes such as cell secretion, neurotransmission, smooth-muscle contractility and cell proliferation [1,2]. There are two biologically active forms of SS: SS-14 and SS-28 [3,4]. In mammals, these two products are generated by endoproteolytic processing of prosomatostatin (PSS) at two distinct regions at the C-terminal segment of the molecule (Figure 1) [1,5,6]. Cleavage at a dibasic Arg-Lys site produces SS-14, whereas cleavage at a mono-arginyl site releases SS-28. Additionally, there is a second monobasic processing site at the N-terminal segment of the precursor, which generates the decapeptide antrin or $PSS_{[1-10]}$, a molecule without any known biological activity [7,8]. In lower vertebrates, such as fish, there are two distinct SS genes expressed in separate populations of cells which encode for separate SS-14 and SS-28-related products [9–11]. Mammals, on the other hand, are characterized by one SS gene expressed in a single type of SS-producing cell which processes PSS differentially to give rise to tissue-specific amounts of SS-14 and SS-28 [5,6,12,13]. For instance, hypothalamus and cerebral cortex synthesize both SS-14 and SS-28 in an approximate ratio of 4:1, whereas retina, peripheral nerve cells, pancreatic islets and stomach produce SS-14 virtually exclusively [5,6]. In contrast, intestinal mucosal cells synthesize SS-28 as the principal terminal product [5,14].

The subtilisin-related serine convertases comprise an expanding family of enzymes implicated in proprotein processing [15–18]. Six members of this family have been identified and characterized in mammals and named furin [paired basic amino acid converting enzyme (PACE)], PC1 (also called PC3), PC2, PACE4, PC4 and PC5/PC6. Furin is ubiquitously expressed and is one of two enzymes that is membrane bound [17,18]. This enzyme is a resident of the trans-Golgi network (TGN) and

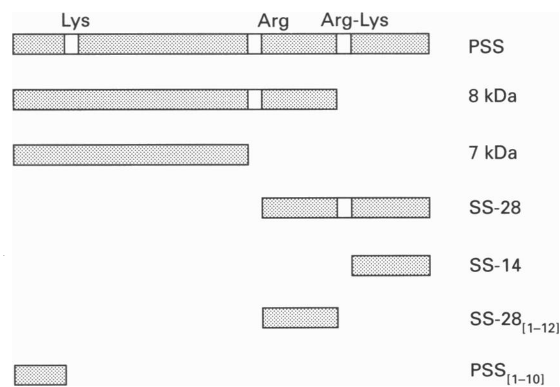

Figure 1 Schematic depiction of mammalian PSS, its dibasic and monobasic processing sites and known cleavage products

Abbreviations used: SS, somatostatin; PSS, prosomatostatin; rPSS, rat prosomatostatin; ER, endoplasmic reticulum; TGN, trans-Golgi network; EGF, epidermal growth factor; TFA, trifluoroacetic acid; HFBA, heptafluorobutyric acid; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PMSF, phenylmethanesulphonyl fluoride; SS LI, somatostatin-14-like immunoreactivity; PC_2 LI, PC_2-like immunoreactivity; PC_1 LI, PC_1-like immunoreactivity; neo^r, neomycin resistance; PACE, paired basic amino acid converting enzyme; D-PBS, Dulbecco's PBS; R.S.D., relative standard deviation.

‡ To whom correspondence should be addressed.

mediates processing of constitutively secreted proteins and membrane glycoproteins, typically at an RXK/RR site [19–21]. By contrast, PC1 and PC2 cleave typically at dibasic residues and are selectively expressed in endocrine and neuroendocrine tissues, including the brain, suggesting a key role of the two proteases in the maturation of neuropeptide and prohormone precursors [15–18]. PC4 has so far been identified only in germ cells in the testis and in the ovary [22,23], whereas PACE4 and PC5/PC6 are broadly but not ubiquitously expressed in both endocrine and non-endocrine cells; their role in prohormone processing remains to be determined [16–18,24,25].

In an attempt to identify the putative convertases involved in PSS processing, we have recently reported that PC1 is capable of generating SS-14 from rat PSS (rPSS) in COS-7 monkey kidney cells and is a candidate protease for dibasic cleavage of the precursor [26]. In direct cotransfection studies, however, we found PC2 to be inactive in PSS conversion in COS-7 cells [26]. Recent studies of the biosynthesis of the convertases have shown that they undergo extensive post-translational modifications themselves, resulting in several molecular forms with differential activity, intracellular storage and secretion [27–33]. In both constitutive and regulated cell lines, the major intracellular form of PC2 is a 75 kDa inactive proPC2 moiety which is slowly transferred to the TGN and then processed to an active 68 kDa form [16–18,28,30,32,33]. PC2 is activated relatively slowly in secretory cells, requiring 1–2 h from the time of synthesis, compared with the more rapid 30 min activation time for PC1 [28,30,33]. Based on these findings, it was probable that the inability of PC2 to effect dibasic processing of PSS in COS-7 cells is due to a slower conversion of proPC2 to PC2, a process which may be relatively more efficient in regulated cells [34].

In the present study we have investigated processing of rPSS in two related rat pituitary mammosomatotroph cells, GH_3 and GH_4C_1. The two cell lines express furin, PC2, low levels of PC5, but not PC1 or PC4 [17,18,24,33,35]. In addition, wild-type GH_3 and GH_4C_1 cells exhibit very few secretory granules which, however, can be induced by hormonal treatment with epidermal growth factor (EGF), β-estradiol and insulin [36]. We describe heterologous processing of rPSS coexpressed with PC1 or PC2 in stably transfected cells, determine the effect of modulating cell granularity on enzyme expression and PSS processing, and correlate PC2 protein levels under different conditions of transfection and regulation with the efficiency of PSS processing to SS-14. We provide the first proof that PC2 can effect dibasic processing of PSS, and, along with PC1, is an authentic SS-14 convertase. Our results demonstrate that the activity of PC2 requires the milieu of the secretory cell but not the secretory granule. Overall, PC1 is a more efficient SS-14-converting enzyme than PC2 when studied under identical conditions in secretory cells.

METHODS

Reagents

Synthetic peptides were obtained as follows: SS-14 (Ayerst Laboratories, Montreal, Quebec, Canada), SS-28 (Hukabel Scientific, Montreal, Quebec, Canada); and [Tyr] SS-14 (Bachem Fine Chemicals, Torrance, CA, U.S.A.). Acetonitrile (CH_3CN) and trifluoroacetic acid (TFA) were purchased from Fisher Scientific (Montreal, Quebec, Canada). Mouse EGF (culture grade) was purchased from UBI (NY, U.S.A.). Beef and pork insulin-Toronto were obtained from Connaught Novo (Ontario, Canada). Pepstatin A, phenylmethanesulphonyl fluoride (PMSF), heptafluorobutyric acid (HFBA), BSA (fraction V), 17-β-estradiol and forskolin were purchased from Sigma. Dulbecco's PBS (D-PBS), F-10 Ham's medium, Dulbecco's modified Eagle medium (DMEM), Optimem-1, neomycin (G418) and horse serum were purchased from Gibco. Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT, U.S.A.). All other reagents were of analytical grade.

Expression vectors

The rat pre-PSS cDNA expression vector pKS5 was provided by Dr. K. Sevarino, Yale University, New Haven, CT, U.S.A. Plasmid pES was constructed by subcloning the rat pre-PSS cDNA into pRc/CMV, a vector that confers neomycin resistance. Mouse pRc/CMV, PC1 and PC2 expression vectors (pmPC1 and pmPC2 respectively) have been described previously [26].

Cell culture, transfections and hormonal induction of granules

GH3 rat pituitary cells were obtained from ATCC; the related GH_4C_1 cells were provided by Dr. Agnes Schonbrunn (University of Texas, Houston, TX, U.S.A.). Both cells were cultured in a 1:1 mixture of DMEM and Ham's F-10 medium supplemented with 15% steroid-free horse serum and 2.5% FBS (culture medium).

GH_3 cells were studied following acute transfection with PSS. Cells were plated in 100 mm diameter plastic Petri dishes and grown as a monolayer. On the 3rd–5th day of culture at $\sim$ 70% confluency, cells were transiently transfected with 20 μg of pKS5 and 20 μg of sheared salmon sperm DNA, by calcium phosphate precipitation, as previously described [26].

GH_4C_1 cells were initially studied following acute transfection with PSS and subsequently as stable transfectants co-expressing PSS with PC1 or PC2. To establish stable transfectants, cells were plated at a density of 4×10^5 cells per well of a 6-well plate (Nuclon, Denmark), incubated with 5 μg of DNA and 30 μg of Lipofectin reagent (Gibco-BRL) in 1.5 ml of Optimem-I medium per well, for 6 h, according to the manufacturer's instructions. GH_4C_1 cells expressing rat PSS alone were established after transfecting the cells with pES vector, which confers neomycin resistance (neo^r). In order to restrict stable expression of rPSS only in cells which also express exogenous PC1 or PC2, GH_4C cells were cotransfected with equal amounts of pKS5, which lacks neo^r, and pmPC1 and pmPC2. Selection (under 300 μg/ml G418), was started on the 3rd day post-transfection and stable cells were grown polyclonally. Acutely transfected GH_3 cells or stably transfected GH_4C_1 cells were studied under normal culture conditions or following hormonal treatment with EGF (10 nM), 17β-estradiol (1 nM) and insulin (300 nM) [36]. In the case of GH_3 cells, treatment with hormones started on the day of transfection, for 3 days; GH_4C_1 cells were cultured to $\sim$ 60% confluency and then treated with the hormonal mixture for 4 days. On the day of experiment, culture medium supplemented with hormones was replaced with DMEM/F-10 medium containing 1% BSA, protease inhibitors (PMSF and Pepstatin A, 20 μg/ml each) and hormones. After 4 h, media were removed, centrifuged at 350 g for 7 min to remove floating cells, acidified with 50 μl of 1 M acetic acid per ml of medium and stored at −20 °C. Cells were scraped on ice in 1 M acetic acid containing 20 μg/ml each of Pepstatin A and PMSF, extracted by sonication, centrifuged at 5000 g for 30 min at 4 °C to remove the particulate fraction and the supernatant was stored at −20 °C. Samples were analysed by HPLC for SS products.

To quantify PC2- or PC1-like immunoreactivity (PC2 LI or PC1 LI), cells were incubated for 4 h in serum-free culture media with 1% BSA, 20 μg/ml each of PMSF and pepstatin A, and aprotinin (10 units/ml). Media were then harvested, centrifuged at 350 g to remove floating cells, acidified with 50 μl/ml 1 M

acetic acid and stored at −20 °C. Cells were lifted in Ca^{2+}/Mg^{2+}-free D-PBS, counted on a haematocytometer and centrifuged at 305 *g* for 5 min. The cell pellet was resuspended in 50 mM Tris/HCl, 150 mM NaCl, 0.05% SDS and 1% Nonidet-T P40 buffer, containing protease inhibitors (20 μg/ml each of Pepstatin A and PMSF, 1 mM EDTA and 10 units/ml aprotinin), sonicated and centrifuged at 5000 *g* for 30 min to remove cellular debris. The supernatant was stored at −20 °C pending analysis.

Northern-blot analysis

Total RNA was extracted from cultured cells by the acid guanidine thiocyanate/phenol/chloroform method. [α-^{32}P]UTP-labelled cRNA probes were generated by use of RNA transcription kits (Promega) according to the manufacturer's instructions. Plasmids containing mPC1 and mPC2 cDNAs were linearized with *Bgl*II and transcribed with SP6 RNA polymerase. Probes of 2520 bases (mPC1) and 2232 bases (mPC2) were generated and purified by chromatography through Sephadex columns. Total RNA (20–40 μg) was fractionated by electrophoresis on 1.5% agarose/formaldehyde gels and transferred to Nytran nylon membranes (Schleicher and Schull) using the VacuGene vacuum blotting system (LKB, Pharmacia). Hybridizations were performed at 65 °C for 24 h in 50% formamide, followed by washing under high-stringency salt conditions. Autoradiograms were prepared by exposing the membranes to Kodak XAR-5 film at −80 °C for 6 days using intensifying screens. Band quantification was done through densitometry in a Quick Scan R & D scanner (Helena Laboratories) and corrected for loading based on comparison with the intensity of the 18S ribosomal band.

HPLC

Pooled, acidified secretion media and cell extracts were diluted 1:7 with 0.1% TFA and concentrated using Waters SepPak C_{18} cartridges. The adsorbed peptides were eluted with 80% CH_3CN/0.1% TFA. The eluate was analysed by HPLC on a C_{18} μBondapak reverse-phase column, using a Waters HPLC apparatus (Waters Millipore) [6,22]. The column was eluted at room temperature at a rate of 1 ml/min with a 12–55% CH_3CN/0.2% HFBA gradient over 150 min. Column eluate was monitored for UV absorbance at 230 nm. Fractions were collected in borosilicate glass tubes (12 × 75 mm; Fisher) using an LKB Ultravac 7000 fraction collector, spiked with 10 μl of 10% BSA and stored at −20 °C until further use. Aliquots from each fraction were evaporated on a Speedvac rotary concentrator (Savant) and assayed for SS immunoreactive peptides by radioimmunoassay.

Radioimmunoassays for somatostatin PC2 and PC1

SS-14-like radioimmunoactivity (SS LI) was measured with rabbit antibody R149 directed against the central segment of SS-14, ^{125}I-Tyr SS-14 radioligand and SS-14 standards [5,6]. This assay detects SS-14 and molecular forms extended at the N-terminus of SS-14, such as SS-28 and pro-SS, with equal affinity. The minimum detectable dose was 1 pg of SS-14.

PC2-LI was measured by radioimmunoassay using rabbit antibody Ab JH1159 (a gift from Drs. R. D. Mains and B. Eipper, Johns Hopkins University, Baltimore, MD, U.S.A.), raised against a tyrosine-containing mPC2 C-terminal peptide (Tyr-mPC2$_{[627-636]}$) which was also used for radio-iodination and PC2 standards. Tyr-mPC2$_{[627-636]}$ was obtained by courtesy of Drs. Iris Lindberg (Louisiana State University, New Orleans, LA, U.S.A.) and C. J. Rhodes (Joslin Diabetes Center, Boston, MA, U.S.A.). Tyr-mPC2$_{[627-636]}$ (2 μg) was radio-iodinated by the chloramine T method [37] to a specific activity of ~ 1400 μCi/μg. Ab JH1159 was diluted 1:50000 to give ~ 30% maximum specific-binding of radioligand. Since PC2 is not known to undergo C-terminal processing, the radioimmunoassay detects all PC2-related peptides [32,33]. Serial dilutions of PC2-containing cell extracts and secretion media exhibited parallelism with standard PC2. All samples from the same experiment were included in a single assay run. Within assay the precision, expressed as coefficient of variation, was $< \pm 5\%$. Between-assay R.S.D. was $< \pm 12\%$ over the linear range of the dose–response curve.

PC1 LI was measured by a previously described radioimmunoassay [26] using a rabbit anti-peptide antibody against synthetic PC1$_{[84-100]}$, ^{125}I-Tyr PC1$_{[84-98]}$ radioligand and PC1$_{[84-100]}$ standards.

Western-blot analysis

Immunoblot analysis of PC2 was carried out in GH_4C_1 stable cotransfectants or COS-7 cells transiently transfected with pmPC2. Protein samples (45 μg) were denatured by boiling in electrophoresis buffer and fractionated by electrophoresis on 10% SDS polyacrylamide gels. The proteins were then transferred onto Immobilon membranes and the blots saturated in 5% de-fatted milk in PBS containing 0.02% sodium azide for 2 h at 37 °C and reacted with rabbit PC2 antibody (1:1000) in 5% milk in PBS at 4 °C under continuous mixing for 24 h. The membranes were washed three times with 5% milk/PBS containing 0.05% Tween 20. PC2-bound antibody was detected by incubating for 4 h at room temperature with ^{125}I-labelled mouse anti-(rabbit IgG). Membranes were then washed in 5% milk/PBS containing 0.05% Tween 20 and exposed to Kodak X-AR-5 film at −80 °C for 2–5 days.

RESULTS

PC1 and PC2 mRNA expression in GH_3 and GH_4C_1 cells

Figure 2 depicts Northern blots of PC1 and PC2 mRNA in GH_3 and GH_4C_1 cells. Neither cell line expressed PC1 mRNA. This is in contrast to AtT-20 cells used as a positive control which, as previously observed, showed two bands of 3 and 5 kb [26]. PC2 mRNA was detected as a 2.8 kb transcript in both GH_3 and

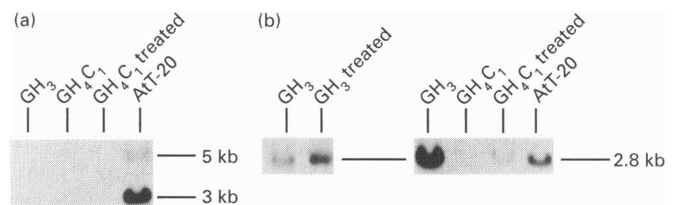

Figure 2 Expression of mRNA for PC1 (a) and PC2 (b) in GH_3, GH_4C_1 and AtT-20 cell lines

Treated cells were studied after incubation with EGF, 17 μg-estradiol and insulin as described in the text. Total RNA (20 μg) was analysed by Northern blot using PC1 or PC2 cRNA probes. PC1 hybridizes to two transcripts of 3.0 and 5.0 kb respectively whereas PC2 hybridizes to a single transcript of 2.8 kb. PC1 mRNA is expressed in AtT-20 cells but not in GH_3 and GH_4C_1 cells. In contrast, PC2 is expressed in all three cell lines and is up-regulated by hormonal treatment. Quantitative analysis of PC2 mRNA in GH_3 cells, treated GH_3 cells, GH_4C_1 cells and treated GH_4C_1 cells gave the proportions 19:35:1:3.

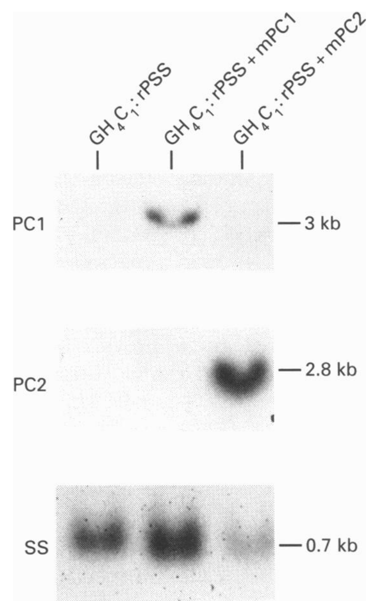

Figure 3 Expression of mRNA for PC1, PC2 and SS in GH_4C_1 stable transfectants

Total RNA from GH_4C_1:rPSS, GH_4C_1:rPSS+mPC1 and GH_4C_1:rPSS+mPC2 cells was extracted and 40 μg samples analysed by Northern blots using PC1, PC2 and SS cRNA probes. All three cell lines express SS mRNA (0.7 kb transcript), whereas PC1 (3 kb transcript) is selectively expressed in the GH_4C_1:rPSS+mPC1 transfectants. PC2 (2.8 kb transcript) is expressed at a low level in GH_4C_1:rPSS cells and at high concentrations in GH_4C_1:rPSS+mPC2 transfectants.

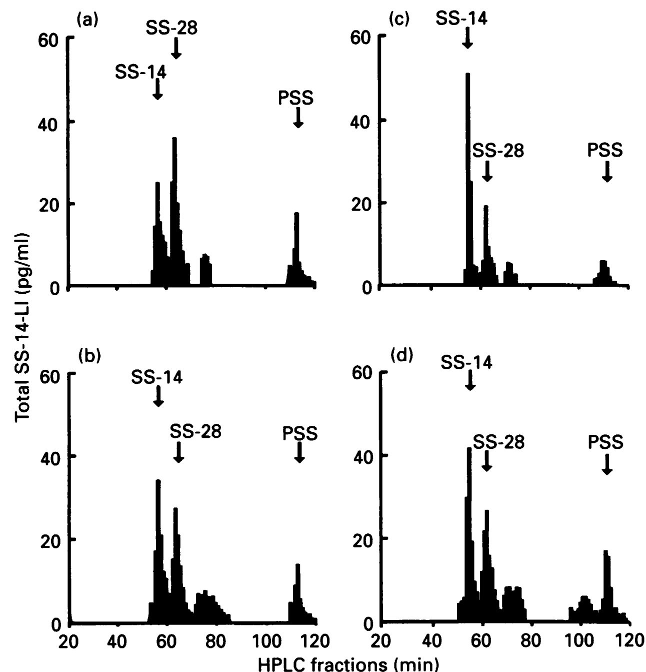

Figure 4 Processing of rPSS in GH_4C_1 cells stably expressing rPSS (a and b) or rPSS+mPC2 (c and d) determined by HPLC

A C_{18} reverse-phase column was eluted with 12–55% CH_3CN/0.2% HFBA at 21 °C over 120 min. Column effluent was analysed by radioimmunoassay for SS-14 LI. The elution positions of standard SS-14, SS-28 and PSS are indicated by arrows. (**a** and **c**) Illustrate HPLC profiles of SS-14 LI forms in cell extracts whereas (**b** and **d**) depict HPLC profiles of secretion media. Similar results were obtained on two separate occasions in the case of GH_4C_1:rPSS cells and on three occasions with GH_4C_1:rPSS+mPC2 cells.

GH_4C_1 cells. Interestingly, although the two cell lines are closely related, they exhibited a striking difference in endogenous PC2 mRNA levels, GH_3 cells expressing 19-fold higher abundance of mRNA than GH_4C_1 cells. Hormonal induction of secretory granules up-regulated PC2 mRNA levels 2- and 3-fold in GH_3 and GH_4C_1 cells respectively (Figure 2). Quantitative analysis of PC2 mRNA in GH_3 cells, treated GH_3 cells, GH_4C_1 cells and treated GH_4C_1 cells gave the ratio: 19:35:1:3.

Development of stable GH4C1 cotransfectants expressing PSS, PC1 or PC2

HPLC analysis of SSLI in GH_3 cells transiently transfected with pKS5 showed complete (99%) processing of PSS to SS-14 in both cell extracts and secretion media (results not shown). In contrast, transiently transfected GH_4C_1 cells processed rPSS to both SS-14 (28% of total SS LI), and SS-28 (34% of SS LI). Processing, however, was relatively inefficient, resulting in 38% unprocessed PSS. This could be due to the very much lower level of expression of endogenous PC2 in GH_4C_1 compared with GH_3 cells, as suggested by PC2 mRNA analyses (Figure 2). Accordingly, stable GH_4C_1 transfectants, coexpressing rPSS and PC2, were established; such cells could also be subjected to granulogenesis with hormone treatment [36]. Stable GH_4C_1 cells co-expressing rPSS and PC1 were also prepared to compare the relative processing activities of PC1 and PC2 in the same cell line (described later). Expression of PC1, PC2 and PSS was confirmed by Northern blots and additionally by protein radioimmunoassay in the case of PC2 and SS. Cells transfected with rPSS, mPC1 and mPC2 featured appropriate mRNA transcripts of 0.7, 3.0 and 2.8 kb respectively (Figure 3). Stable GH_4C_1:rPSS cells expressed 1.6 pg of SS LI/10^6 cells compared with 140 pg/10^6 cells for GH_4C_1:rPSS+mPC1 cells and 53.3 pg SS LI/10^6 cells for GH_4C_1:rPSS+mPC2 cells. These differences can probably be accounted for by the different rPSS constructs used. All three transfectants secreted SS LI constitutively in amounts (expressed as a percentage of total cell content/4 h) ranging from 100% for GH_4C_1:rPSS to 42% for GH_4C_4L:rPSS+mPC1 cells to 78% for GH_4C_1:rPSS+mPC2 cells.

Dibasic processing of rPSS to SS-14 by PC2 in GH_4C_1 cells

In Figure 4 are portrayed HPLC profiles of SS LI in cell extracts and secretion media from GH_4C_1:rPSS (a and b) and GH_4C_1:rPSS+mPC2 cells (c and d). Three main peaks with retention times of 55, 65 and 110 min were observed, corresponding to SS-14, SS-28 and full-length PSS, as described earlier [26]. In addition, two other peaks, eluting at 75 and 97 min, were consistently observed; these peaks have been characterized previously as N-terminally truncated forms of PSS containing an intact C-terminal segment [26] and were included with full-length PSS in all calculations of the percentage of unprocessed PSS. GH_4C_1:rPSS stable transfectants showed high basal secretion of SS-14 LI and processed PSS relatively inefficiently to SS-14 (36% of total SS LI) and SS-28 (39%) with 25% unprocessed PSS (Figure 4). The proportions of SS-14:SS-28:unprocessed PSS in secretion media were 48%:30%:22%. Stable cotransfection of GH_4C_1 cells with rPSS and mPC2 led to increased SS-14 conversion, especially in cell extracts. Overall, SS-14 conversion increased from 36% of total SS-14 LI to 56%, concomitant with a reduction in the level of SS-28 (from 39 to

Table 1 Correlation between PC2 protein expression and SS-14 processing efficiency in GH_4C_1:rPSS, GH_4C_1:rPSS+mPC2 and GH_3:rPSS cells

Results given as mean ± S.E.M. ($n = 3$).

Transfected cell	PC2 LI Cell extracts ($ng/10^6$ cells)	Media (ng secreted/ 4 h per 10^6 cells)	Processing to SS-14 in cell extracts (%)
GH_4C_1:rPSS	0.59 ± 0.06	0.014 ± 0.006	36 ± 3
GH_4C_1:rPSS + mPC2	1.52 ± 0.06	0.08 ± 0.03	56 ± 7
GH_3:rPSS	2.03 ± 0.25	0.16 ± 0.03	100 ± 2

Table 2 Effect of hormone treatment to induce granules on SS-14 LI and PC2 LI in GH_4C_1:rPSS + mPC2 stable cotransfectants

Results given as mean ± S.E.M. ($n = 3$); $^*P \leqslant 0.05$. Similar results were obtained in three separate experiments.

	SS-14 LI ($pg/10^6$ cells)	PC2 LI ($ng/10^6$ cells)
Control	16.7 ± 1.7	1.95 ± 0.06
Hormone treatment	169 ± 12*	2.4 ± 0.23

26%). In secretion media, however, there was no significant difference in the amount of SS-14 between GH_4C_1:rPSS and GH_4C_1:rPSS + mPC2 cells.

Correlation between PC2 protein expression and SS-14 processing efficiency

Table 1 compares SS-14 processing efficiency with PC2 protein measured by radioimmunoassay in ng of total PC2 LI/10^6 cells or 4 h secretion media in GH_4C_1:rPSS stable cells, GH_4C_1:rPSS:PC2 stable cotransfectants and GH_3 cells acutely transfected with rPSS. PC2 LI in GH_4C_1:rPSS and GH_3:rPSS cells reflect endogenous levels of PC2 in GH_4C_1 and GH_3 cells respectively, whereas PC2 LI in GH_4C_1:rPSS + mPC2 cells represents the sum of both endogenous and exogenous PC2. PC2 existed predominantly as an intracellular protein in all three cell types, with only 5–10% of the cell content of PC2 LI secreted per 4 h from any of the three cell types. Results obtained from 4 to 5 different passages showed a consistent gradient of PC2 protein expression of 1:2.6:3.4 in cell extracts and 1:4.7:9 in secretion media from GH_4C_1:rPSS, GH_4C_1:rPSS + mPC2 and GH_3:rPSS cells respectively. There was good correlation between the concentration of PC2 protein and SS-14 conversion efficiency from 36 ± 3% in GH_4C_1:rPSS cells to 56 ± 7% in GH_4C_1:rPSS + mPC2 cells to 100% in GH_3 cells, suggesting that PC2 is capable of mediating dibasic cleavage of PSS to SS-14.

Effect of granule induction on dibasic PSS processing to SS-14 by PC2

Although overexpression of PC2 in GH_4C_1 cells improved dibasic PSS conversion into SS-14, processing efficiency remained below that obtained in GH_3 cells. To determine whether granulation of GH_4C_1 cells could further improve the ability of PC2 to effect dibasic processing of PSS, GH_4C_1:rPSS + mPC2 cotransfectants

Table 3 Effect of granule induction on regulated release of SS-14 LI and PC2 LI from GH_4C_1 stable transfectants

GH_4C_1:rPSS + mPC2 stable transfectants were plated in three groups in triplicate and studied under control conditions, after hormonal treatment (with 10 μM EGF, 1 nM 17β-estradiol and 300 nM insulin to induce granules, or after treatment with 20 μM forskolin. Results given as mean ± S.E.M. ($n = 3$). $^*P < 0.05$ versus control; $^{**}P < 0.05$ versus granule induction alone. Similar results were obtained in three separate experiments.

Treatment	SS-14 LI (pg/4 h per 10^6 cells)	PC2 LI (pg/4 h per 10^6 cells)
Control	90 ± 9	44 ± 13
Granule induction	280 ± 12*	192 ± 17*
Granule induction + forskolin	415 ± 10**	283 ± 13**

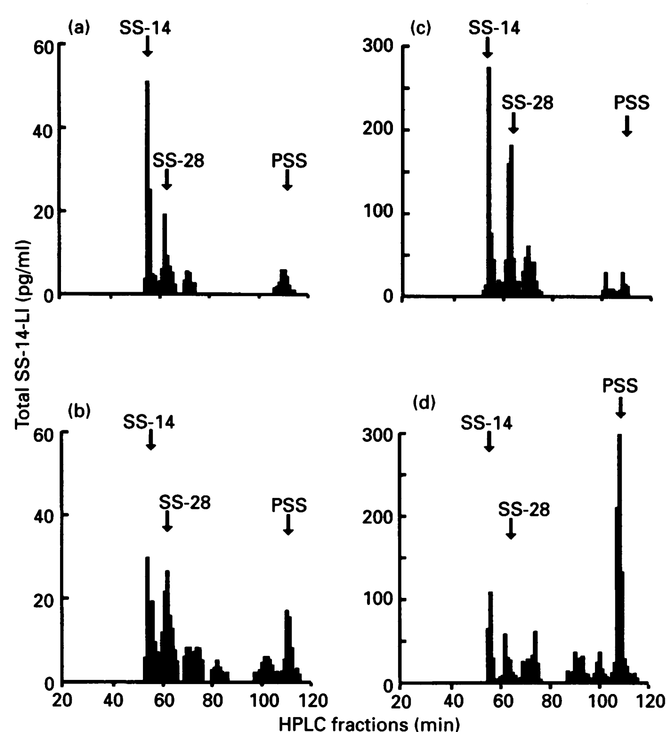

Figure 5 Effect of granule induction on rPSS processing in GH_4C_1:rPSS-mPC2 cells

(**a** and **b**) Depict HPLC profiles of SS-14 LI forms, cell extracts and secretion media from GH_4C_1:rPSS + mPC2 stable transfectants under basal (untreated) conditions, and (**c** and **d**) represent HPLC profiles of rPSS processing in the same cells subjected to hormonal treatment to induce granulation. Column conditions and markers are identical with those described in Figure 4. Profiles are representative of three complete experiments.

were studied under normal culture conditions or following hormonal induction of granules. Hormonal treatment led to a dramatic 10-fold increase in the storage capacity of SS LI in GH_4C_1 cells from 16.7 ± 1.7 to 169 ± 12 pg/10^6 cells (Table 2). In the case of PC2 LI there was a small but not significant (25%) increase in cell content from 1.95 ± 0.06 to 2.4 ± 0.23 ng/10^6 cells. Basal secretion of SS LI increased 3-fold following granule induction, from 90 ± 9 to 280 ± 12 pg/4 h per 10^6 cells with an additional 40% increment of release to 415 ± 10 pg in response to forskolin (Table 3). This was accompanied by a parallel

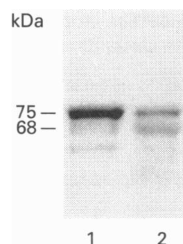

Figure 6 Molecular forms of PC2 in GH_4C_1:rPSS+mPC2 cells subjected to granule induction

Western-blot analysis was performed as described in the Materials and methods section. Cell protein samples (45 μg per lane) were loaded and probed with rabbit PC2 antibody (1:1000). Untreated GH_4C_1:rPSS+mPC2 cells (lane 1) show two main bands of 75 and 68 kDa, corresponding to proPC2 and PC2 respectively, together with a weak band of 56 kDa. Hormone treatment of GH_4C_1:rPSS+mPC2 cells (lane 2) produces a small increase in the ratio of 68 to 75 kDa PC2 forms. Similar results were obtained on three separate occasions.

Table 4 Comparison of the ability of PC1 and PC2 to convert PSS into SS-14 in GH_4C_1 cells

Percentage processing of rPSS to SS-14 and SS-28 in GH_4C_1:rPSS+mPC2 and GH_4C_1:rPSS+mPC2 stable transfectants was determined by HPLC and radioimmunoassay analyses of cell extracts and secretion media. Overall, PC1 is more effective than PC2 in processing rPSS to SS-14, as demonstrated by the higher overall ratio of SS-14 to unprocessed rPSS. Percentage processing to SS-28 is comparable in the two cotransfectants. Results given as mean ± S.E.M. ($n = 3$). *$P < 0.05$ (PC1 versus PC2 transfectants).

Transfectant	SS-14 (Percentage of total SS-14 LI)	SS-28 (Percentage of total SS-14 LI)	Unprocessed rPSS
GH_4C_1:rPSS+PC2			
Cell extracts	53±2.6	27±2.5	20±2
Media	47±4.5	27±3.9	26±3.3
GH_4C_1:rPSS+PC1			
Cell extracts	62±2.1	28±3.2	10±1
Media	66±6*	26±8	8±1*

4-fold increase in basal secretion of PC2 LI (from 44±13 to 192±17 pg/4 h per 10^6 cells) with an additional 50% stimulation (to 283±13 pg/4 h per 10^6 cells) after forskolin challenge. These results demonstrate the existence of a coregulated storage compartment of both SS LI and PC2 LI, which is likely to be the same regulated GH_4C_1 secretory granules.

HPLC analysis of cell extracts and secretion media before and after granule induction showed no change in the pattern of PSS processing (Figure 5). SS-14 accounted for 53% and 47% of total SS LI in treated and untreated cell extracts. In secretion media, SS-14 represented only 25% of total SS LI following hormonal treatment compared with 37% in control cells. There was no significant change in the percentage of unprocessed PSS in control cells (20%) compared with hormone-treated cells (24%). Interestingly, there was a significant increase in the proportion of unprocessed PSS in secretion media from hormone-treated cells (70% of total SS LI) compared with control (26% of SS LI). To characterize the molecular forms of PC2, normally cultured or hormone-treated GH_4C_1:rPSS+mPC2 cells were analysed by immunoblots. Figure 6, lane 1, shows two main bands of PC2LI of 75 and 68 kDa in untreated cells, corresponding to proPC2 and mature PC2 respectively, together with a faint band of 56 kDa [18,28,30,32,33]. Hormone treatment produced a small increase in the ratio of PC2:proPC2 forms (lane 2). COS-7 cells acutely transfected with mPC2 were used as control and revealed a predominant 75 kDa proPC2 band (not shown).

Comparison of relative potency of PC2 with that of PC1 in GH_4C_1 cells

Although PC1 has been shown to be an effective SS-14 convertase in constitutive cells such as COS-7 [26], and although PC2 requires the environment of a secretory cell for processing activity, the question of the relative potency of the two proteases in the same secretory cell remains unresolved. To investigate this further, the pattern of PSS processing in stable GH_4C_1 cells co-expressing rPSS and mPC1 was characterized and compared with that in stable GH_4C_1:rPSS+mPC2 transfectants. The relative proportions of SS-14, SS-28 and unprocessed PSS derived from HPLC analysis of cell extracts and media from the two cells is depicted in Table 4. PC1 transfectants produced significantly greater SS-14 conversion (62% in cells, 66% in media) compared with PC2 transfectants (53% in cells, 47% in media). The relative amounts of SS-28 were comparable in PC1- and PC2-transfected cells. However, there was a significantly lower level of unprocessed PSS in PC1 (10% in cell extracts, 8% in secretion media) than in PC2 transfectants. Analysis of PC1 LI in GH_4C_1:rPSS+mPC2 cotransfectants showed < 1.9 pg of PC1 LI in extracts from 10^6 cells and 5.35±0.36 pg secreted/4 h from 10^6 cells. This level of protein expression is considerably lower than that of PC2 LI in GH_4C_1:rPSS+mPC2 cotransfectants but nonetheless produced greater SS-14 conversion, suggesting that PC1 may be a more efficient SS-14 convertase than PC2.

DISCUSSION

In the present study we have investigated the functional role of PC2 in PSS processing and demonstrated that, as in the case of PC1 studied earlier [26], PC2 is also capable of dibasic cleavage to SS-14, and thus qualifies as a putative SS-14 convertase. Heterologous processing of PSS to SS-14 has been extensively evaluated in non-endocrine as well as endocrine cells with the finding that constitutively secreting cells such as COS-7 and 3T3 are capable of low level SS-14 conversion probably by a furin-like enzyme [26,38], whereas regulated secretory cells such as AtT-20, RINm5f and GH_3 effect virtually complete precursor processing to SS-14 [26,39,40]. The efficiency of processing to SS-14 in these experiments correlates with endogenous expression of either PC1 or PC2, implicating both enzymes in this conversion [26]. For instance AtT-20 and RINm5f cells express predominantly PC1 or PC2 respectively [26], and GH_3 cells express PC2 exclusively (Figure 2), but all three cell lines process PSS nearly completely to SS-14 [26,39,40]. Expression of PC1 mRNA in rat brain by *in situ* hybridization correlates with the regional brain distribution of SS, especially in areas rich in SS neurons, such as the hypothalamic periventricular nucleus [41], lending support for a physiological role of PC1 in PSS processing in these neural cells. On the other hand, rat pancreatic islet D cells which process PSS efficiently to SS-14 [5], express PC2 and not PC1 [42,43], suggesting that PC2 is the physiological SS-14-converting enzyme in the islet. This is further supported by the finding that an enzyme isolated from anglerfish islets as the fish homologue of mammalian PC2 functions as the anglerfish SS-14 convertase [44].

In contrast to our earlier inability to demonstrate SS-14-converting activity of PC2 exogenously expressed in COS-7 cells

[26], this enzyme was fully active in GH_4C_1 cells. This may be related to the poor ability of constitutively secreting cells such as COS-7 and CHO to process proPC2 to the active lower molecular mass forms [26,32] that are produced more efficiently in regulated secretory cells [17,27,33,34]. Recent studies of the biosynthesis of PC2 in GH_4C_1 cells have revealed an elaborate pattern of post-translational processing of this protease [33]. A 75 kDa proPC2 form is synthesized initially in the endoplasmic reticulum (ER) and is slowly transported to the TGN where it undergoes both autocatalytic and furin/PACE-mediated processing to 68 kDa glycosylated and Tyr-sulphated forms of PC2 [33,34]. One of the differences between constitutive and regulated secretory cells is the presence of the neuroendocrine peptide 7B2 [45], which is expressed in GH_3/GH_4C_1 cells but not in CHO or 3T3 cells [18]. Protein 7B2 acts as a molecular chaperone by binding PC2 and regulating its conversion from an inactive proenzyme in the ER–Golgi region into the active enzyme in the TGN and secretory granules [18,34,45,46]. Although GH_3 and GH_4C_1 cells are closely related cell lines, they showed a marked divergence in the endogenous expression of PC2 but not of 7B2 [18]. GH_3 cells expressed very high levels of PC2 mRNA and protein and processed rPSS completely to SS-14. In an earlier study of the processing of anglerfish PSS1 in GH_3 cells, Stoller and Shields [40] also found efficient SS-14 conversion in these cells. GH_4C_1 cells, on the other hand, exhibited 20- and 4-fold lower levels of PC2 mRNA and protein respectively and processed PSS incompletely to both SS-14 and SS-28 (36 and 39% of total SS LI respectively). These findings differ from those of Sevarino et al. [39] who reported an absence of rPSS processing in GH_4C_1 cells associated with constitutive secretion of predominantly unmodified precursor. Although the GH_4C_1 cells in these two studies were obtained from the same source, clonal variations in the expression of endoproteases or in the intracellular milieu may account for these differences. Such variations have indeed been observed in the case of PC2 expression in AtT-20 cells [18,26,35,47,48]. Overexpression of PC2 in stably transfected GH_4C_1 cells increased the level of PC2 protein expression 3-fold and further improved SS-14 conversion from 36 to 56%. A comparison of percentage SS-14 conversion with increasing levels of PC2 protein expression in wild-type GH_4C_1 cells, GH_4C_1 cells overexpressing PC2 and wild-type GH_3 cells showed a linear correlation, providing direct evidence that PC2 is a candidate SS-14 convertase.

GH_3 and GH_4C_1 cells synthesize both prolactin and growth hormone. Wild-type cells have been shown by electron microscopy to lack dense-core secretory granules and to display a reduced capacity for hormone storage and regulated release compared with normal lactotrophs and somatotrophs [36,49]. The cells respond to hormonal treatment with insulin, EGF and estradiol with a 50-fold increase in granule number [36]. The finding of virtually 100% conversion of rPSS into SS-14 in wild-type GH_3 cells clearly suggests that efficient processing of this precursor can occur in a poorly granulated cell and is therefore not dependent on the granule milieu. Additional direct evidence against a requirement for secretion granules in PSS processing was obtained by studies of granule induction in GH_4C_1 cells. Hormonal induction of secretion granules was associated with a 10-fold increase in the cell content of SS LI but led to only a marginal (25%) increase in intracellular concentration of PC2-like protein. Such a marked difference in the storage pattern of SS and PC2 is no doubt due to the fact that a large pool of intracellular PC2 LI exists as inactive proPC2 forms, which are retained in the ER and slowly released for conversion into active PC2 in the Golgi; only a small fraction of mature PC2 is targeted to the regulated pathway for storage and release [33,46]. This is in contrast to PSS and its cleavage products, which are typically targeted to secretory vesicles in regulated cells. Hormonal induction of secretion granules produced an increase in basal as well as forskolin-stimulated secretion of both SS LI and PC2 LI, suggesting the existence of a coregulated storage compartment; conversion of PSS into SS-14, however, was unaltered in either cell extracts or secretion media. It could be argued that although PC2 and SS immunoreactivities were coreleased from a regulated storage pool, enzyme and substrate may be compartmentalized in separate populations of prolactin- or growth-hormone-containing granules, or that granule induction may alter the ratio of inactive proPC2 to active PC2. Secretory granules induced by hormone treatment of GH_4C_1 cells are known to be heterogeneous [36]. However, there are relatively few growth-hormone-containing granules in these cells, whereas the majority ($\sim 75\%$) of secretory vesicles store prolactin. The finding that hormonal treatment significantly increased basal and forskolin-stimulated secretion of both SS-14 LI and PC2 LI suggests coregulation of enzyme and substrate under these conditions. It remains to be seen whether regulated secretion occurs via prolactin granules or a different type of secretory vesicle. Hormonal treatment also produced a small increase in the ratio of mature PC2 (68 kDa) to proPC2 (75 kDa) (Figure 6). Whether this increase occurred in the granule or in another cellular compartment cannot be established from the present data. Importantly, there was no decrease in the relative amount of mature PC2 with hormone treatment, which might account for the absence of any change in PSS processing efficiency as a result of granulogenesis. Overall, our finding of complete PSS processing to SS-14 in wild-type GH_3 cells, and the failure of granule induction in GH_4C_1 cells to improve PSS processing, exclude a significant role of secretion granules in PSS maturation. The notion that PSS processing can be uncoupled from secretion granules is supported by several additional lines of evidence. First, mature products of PSS processing have been localized by immunogold labelling to various Golgi subfractions of neural cells [50]. Secondly, using a low-temperature block to inhibit the exit of secretory vesicles from the TGN in GH_3 cells, Xu and Shields [51] have reported efficient cleavage of anglerfish PSS1 to SS-14 in the TGN before the generation of secretory vesicles. Processing was facilitated by an acidic pH generated by a vacuolar ATPase in the TGN [52]. Thirdly, in rat islet SS-producing 1027 B_2 cells, which express PC1 and PC2 but which are devoid of secretory granules, there is efficient dibasic and monobasic conversion of endogeneous PSS into SS-14 and SS-28 via the constitutive secretory pathway [53]. PSS is not unique in undergoing proteolytic cleavage in the TGN. Recent evidence using electron microscopic immunogold techniques or subcellular fractionation studies suggests that processing of two other precursors, pro-opiomelanocortin in AtT-20 cells [54] or thyrotrophin releasing hormone in transfected AtT-20 cells [55], begins in the TGN before the packaging into secretory granules. In contrast, proteolytic processing of other peptide hormones such as proinsulin [48,56–58], propressophysin [59] and gonadotrophin releasing hormone/gonadotrophin-releasing-hormone-associated peptide [60] appears to occur mainly in secretory granules.

PC1 is synthesized as an 87 kDa proPC1 precursor which is cleaved at the N-terminal segment in the ER to an 84 kDa active form of the enzyme, which is also secreted [27,29,31,33]. This type of processing occurs in both constitutive and regulated secretory cells and explains the SS-14 converting activity of PC1 in transfected COS-7 cells described earlier [26]. Furthermore, since COS-7 cells are constitutively secreting, these findings provide additional confirmation that proteolytic cleavage of PSS

to SS-14 by PC1 also begins in a Golgi or pre-Golgi compartment and does not require secretory granules. In the present study we showed that PC1 is much more potent in converting PSS into SS-14 in GH_4C_1 cells compared with COS-7 cells. Indeed, a comparison of the relative SS-14-converting activity of both PC1 and PC2 in GH_4C_1 cells showed that PC1 is overall more potent than PC2. This means that PC1 and PC2 both qualify as functional SS-14 convertases and that differential tissue-selective processing of PSS to SS-14 must depend on cell-specific expression, post-translational modification and activation as well as affinity of each enzyme for PSS.

In conclusion, the present study provides the first proof that PC2 can effect dibasic processing of PSS, and, along with PC1, qualifies as a putative SS-14 convertase. PC1 is active in both constitutive and regulated secretory cells, whereas PC2 is active only in secretory cells. Overall, PC1 appears to be a more potent SS-14 convertase than PC2. Efficient processing of PSS probably begins in the Golgi compartment. It requires the milieu of the secretory cell but not the secretory granule.

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