Direct role of furin in mammalian prosomatostatin processing

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We have previously reported that rat prosomatostatin (rPSS) undergoes conversion at Arg¹ and Lys¹ monobasic sites to SS-28 and PSS-(1–10) respectively in COS-7 cells, and have proposed furin or a related enzyme of the constitutive secretory pathway as the endoprotease responsible. Here we have tested directly the ability of furin to cleave rPSS at the two monobasic sites as well as at the RXRK dibasic site of SS-14 conversion (a furin motif, except for Lys substituting for Arg at P1). Recombinant vaccinia virus (VV) vectors were used to co-express rPSS with graded doses of furin in COS-7 cells and LoVo colon carcinoma cells deficient in furin. PSS and cleavage products in cell extracts and media were characterized by HPLC analysis and C-terminal [SS-14-like immunoreactivity (SS-14 LI)] and N-terminal [PSS-(1–10) LI] directed radioimmunoassays. There was a dose-dependent increase in SS-28 production from rPSS by furin in COS-7 cells from 29 % (control) to 58 % (high-dose furin) associated with a

progressive decrease in unprocessed PSS from > 60 % to ~ 20 % of total SS-14 LI. Significant SS-14 production occurred only at high levels of furin infection. Control LoVo cells infected with VV:rPSS exhibited production of ~ 21 % SS-28, ~ 15 % PSS-(1–10) and 3.5 % SS-14. Infection of LoVo cells with VV:hfurin (hfurin = human furin) enhanced SS-28 production to 30–34 %. SS-14 synthesis also increased to 25–40 %, probably by conversion from SS-28. Overexpression of furin in COS-7 or LoVo cells failed to increase PSS-(1–10) production. These results show that furin is a candidate SS-28 convertase. Arginine is the preferred residue at the P1 site of furin cleavage. Furin does not process rPSS to PSS-(1–10), suggesting the existence of another monobasic convertase with a preference for Lys rather than Arg at P1. Such an enzyme could also explain the presence of endogenous SS-28-, PSS-(1–10)- and SS-14-producing activities in LoVo cells.

INTRODUCTION

Mammalian prosomatostatin (PSS) consists of a 10 kDa precursor which is cleaved endoproteolytically to generate several mature products (reviewed in [1]) (Figure 1). Processing occurs principally at the C-terminal segment of the molecule to produce the two bioactive peptides, somatostatin-14 (SS-14) and somatostatin-28 (SS-28), together with SS-28-(1–12), PSS-(1–76) (8 kDa; N-terminal PSS peptide) and PSS-(1–64) (7 kDa; N-terminal PSS) [1–7]. In addition, processing at the N-terminal segment of PSS generates PSS-(1–10) (1 kDa) [7,8]. These products are generated from differential processing at three sites, dibasic Arg-Lys¹ and monobasic Arg¹ sites in the C-terminal segment of PSS, and a second monobasic Lys site in the N-terminal segment of PSS, which results in tissue-specific amounts of the various mature peptides [2,3,6–8]. For instance, SS-14 is the predominant or exclusive product in pancreatic islets, retina, brain and peripheral nervous tissue, whereas SS-28 is the sole product in intestinal mucosal cells [2,3,9].

On the basis of the wide variability in the patterns of endogenous and heterologous PSS processing to SS-14 and SS-28, as well as the presence in the precursor of both monobasic and dibasic cleavage sites, it has been postulated that separate enzymes with dibasic and monobasic cleavage specificities may be required for PSS maturation [1,2,10,11]. Recently a family of subtilisin-related proprotein convertases homologous to the yeast kex2 proteinase has been identified, having at least six current

members [12–15]. These include furin [paired basic amino acid converting enzyme (PACE)], prohormone convertase (PC) 1/PC3, PC2, PACE4, PC4 and PC5/PC6. Among them, furin is ubiquitously expressed and is one of the two enzymes in the family (the other being an alternatively spliced form of PC5/6 called PC5/6-B) that is membrane-bound [14,15]. Furin is a resident of the trans-Golgi network and mediates processing of constitutively secreted proproteins and membrane glycoproteins, typically at an RXK/RR site [16,17]. PC1/PC3 and PC2 cleave at dibasic residues and are selectively expressed in endocrine and neuroendocrine cells [12–15]. PC4 is exclusively synthesized within germ cells of the testis [18,19], whereas PACE4 and PC5/PC6 are broadly expressed in many but not all tissues; their role in prohormone processing remains to be clarified [13–15,20,21].

We have previously shown that heterologous processing of rat (r)PSS at the two monobasic cleavage sites correlates with the endogenous expression of furin mRNA [22]. For instance, constitutive cells such as COS-7 and PC12 cells, which express furin but not PC1 or PC2, showed significant conversion of rPSS to SS-28 and PSS-(1–10). This suggests that furin or furin-like enzymes may subserve a role as candidate monobasic converting enzymes. Studies of the cleavage specificity of furin have revealed that the minimal required sequence is RXXR [16,17,23–25]. On the basis of structure–function studies of various prorenin mutants, Watanabe et al. [25] have proposed the following rules for cleavage by furin: Arg at P1 is essential and additionally at

Abbreviations used: SS, somatostatin; (r)PSS, (rat) prosomatostatin; HFBA, heptafluorobutyric acid; DMEM, Dulbecco's modified Eagle's medium; PACE, paired basic amino acid converting enzyme; PC, prohormone convertase; TFA, trifluoroacetic acid; VV, vaccinia virus; hfurin, human furin; m.o.i., multiplicity of infection; p.f.u., plaque-forming units; SS-14 LI, SS-14-like immunoreactivity; D-PBS-M, Dulbecco's PBS with Mg²⁺.

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

least two out of three basic residues at P2, P4 and P6 are required. The SS-28-generating cleavage site RXXXXR possesses only two basic residues at P1 and P6, and satisfies two of the three suggested criteria. The PSS-(1-10)-generating cleavage site RXXXXK features a P6 Arg but deviates further in exhibiting a Lys rather than an Arg at the P1 site. Interestingly, the dibasic processing site in rPSS (RXRK) for generating SS-14 is a perfect furin motif except for the substitution of an Arg for a Lys at P1. SS-14, however, is only a minor product of heterologous rPSS processing by endogenous furin in constitutive cells [22].

In the present study we have evaluated directly the role of furin in the processing of rPSS at its three cleavage sites and determined whether Arg at P1 is a strict requirement or whether it is interchangeable with a Lys. We have (i) investigated the heterologous processing of rPSS in constitutive COS-7 monkey kidney cells overexpressing furin by using a vaccinia virus recombinant furin; (ii) correlated the dose-dependence of furin expression with processing at each of the three PSS cleavage sites; and (iii) characterized rPSS processing in LoVo human colon adenocarcinoma cells that are deficient in furin activity [26]. We show that furin can mediate monobasic cleavage of rPSS to SS-28 and qualifies as an SS-28 convertase. Furin is not responsible for N-terminal processing of PSS to PSS-(1-10), suggesting the existence of another monobasic convertase with a preference for Lys or with a broader specificity than furin encompassing both Arg and Lys residues at the P1 site.

EXPERIMENTAL

Chemicals and reagents

Synthetic peptides were obtained as follows: SS-14 (Ayerst Laboratories, Montreal, Quebec, Canada), SS-28 (Hukabel Scientific, Montreal, Quebec, Canada), Tyr-SS-14 (Bachem Fine Chemicals, Torrance, CA, U.S.A.), PSS-(1-10) (Dr. R. Benoit, McGill University), [Tyr¹⁰]PSS-(1-10) (Biomega, Montreal, Quebec, Canada). Acetonitrile and trifluoroacetic acid (TFA) were purchased from Fisher Scientific (Montreal, Quebec, Canada). Pepstatin A, PMSF and heptafluorobutyric acid (HFBA) were obtained from Sigma. Dulbecco's modified Eagle's medium (DMEM), Dulbecco's PBS with Mg²⁺ (D-PBS-M), F12 medium and fetal bovine serum were purchased from Gibco. All other reagents were of analytical grade and were obtained from various commercial sources.

Cell culture

COS-7 monkey kidney epithelial cells were obtained from the National Research Council Biotechnology Research Laboratories (Montreal, Quebec, Canada) courtesy of Dr. D. Banville, and were cultured in DMEM supplemented with 5% fetal bovine serum. LoVo human colon adenocarcinoma cells were obtained from ATCC and grown in F12 medium containing 20% fetal bovine serum.

Expression vectors

Plasmid pKS1 expressing rat prePSS cDNA was kindly provided by Dr. K. Sevarino, Yale University. Plasmid pMJ601:rPSS expressing the rat prePSS cDNA under a late vaccinia virus (VV) promoter was constructed by a two-step process. An *Xba*I-*Eco*RI fragment from pKS5 containing prePSS cDNA was subcloned into pBluescript KS and from this a *Not*I-*Apal* fragment was further subcloned into pRc CMV (pES). Plasmid pMJ601:rPSS was then constructed by insertion of the rat prePSS cDNA from pES into the *Hind*III site of pMJ601. Construction of the VV:rPSS recombinant virus was performed by infecting Ltk⁻ cells with VV:wt (where wt = wild type) and then transfecting them with pMJ601:rPSS (5 µg) and VV:wt DNA (1 µg), using the lipofectin reagent (Gibco BRL) in order to integrate the rPSS cDNA into the VV genome. A crude extract of the virus was collected the following day and was further purified with subsequent passages over BSC 40 monkey kidney epithelial cells. Recombinant virus expressing human furin (VV:hfurin) was obtained as previously described [27].

VV infections

COS-7 cells were plated in 100 mm-diam. plastic Petri dishes and grown as monolayers. After 1-2 days, two plates were trypsinized in order to count the number of cells per plate [(0.7-1.0) × 10⁶ cells/plate]. The remaining cultures were infected with either VV:wt or VV:hfurin. Cells were washed twice with D-PBS-M and virus (diluted in D-PBS-M; 1.5 ml/plate) was applied at a multiplicity of infection (m.o.i.) of 2.4 or 12 plaque-forming units (p.f.u.)/cell for VV:wt-infected cells and 0.8, 2.4 or 12 p.f.u./cell for the VV:hfurin infection group. After a 1 h incubation at 37 °C in 5% CO₂ with frequent rocking of the plates, virus-containing solutions were replaced with normal growth media; 1 h later, cells were transfected with pMJ601:rPSS by calcium phosphate precipitation followed by a 3 min glycerol shock at 7 h post-infection. At 22 h after infection, media were replaced by secretion medium containing DMEM with 1% BSA and enzyme inhibitors (PMSF and pepstatin A; 20 µg/ml each). After a further 4 h, media were collected and centrifuged at 350 g for 5 min to remove floating cells. The supernatant was acidified by addition of 50 µl/ml 1 M acetic acid and stored at -20 °C until further use. Cell extracts were collected in 1 M acetic acid containing the same inhibitors at 4 °C, extracted by sonication and centrifuged at 5000 g for 30 min at 4 °C. The supernatant was stored at -20 °C pending analysis. Samples were assayed for total SS-14-like immunoreactivity (SS-14 LI) and prepared for HPLC analysis as described below.

In the case of LoVo cells, two separate co-infections were performed. To study rPSS processing in wild-type cells, one or two 10 mm plates of LoVo cells were infected with VV:rPSS (2 p.f.u./cell) and VV:wt (1 p.f.u./cell). The effect of furin was studied by co-infection by VV:rPSS and VV:hfurin, also at 2 and 1 p.f.u./cell respectively. Cell extracts and 4 h secretion

media were collected after 19 h and processed as described above.

HPLC

Acidified secretion media and cell extracts were diluted 1:7 in 0.1% TFA and concentrated using Waters Sep-Pak C₁₈ cartridges. The adsorbed peptides were eluted with 80% acetonitrile/0.1% TFA. The eluate was analysed by reverse-phase HPLC on a Waters HPLC apparatus consisting of two solvent delivery pumps (M45 and M6000), an M680 automated gradient controller and a C₁₈ μ Bondapak column [3,22]. The column was eluted at room temperature at 1 ml/min with a 12–55% acetonitrile gradient in 0.2% HFBA and 0.01 M sodium acetate over 150 min. Fractions were collected into 12 mm $\times$ 75 mm borosilicate glass tubes, spiked with 10 μ l of 10% BSA, and stored at -20°C until further use. Aliquots from each fraction were rotary evaporated on a Speed Vac rotary concentrator (Savant) and assayed for SS immunoreactivity with region-specific SS radioimmunoassays.

SS radioimmunoassays

Radioimmunoassay for SS-14 LI

SS-14 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 as previously described [2,3,23]. This assay detects SS-14 and molecular forms extended at the N-terminus of SS-14, including SS-28 and proSS. The minimum detectable dose was 1 pg of SS-14.

Radioimmunoassay of PSS-(1–10) LI

PSS-(1–10) LI was measured using antibody R203, [^{125}I]Tyr¹⁰PSS-(1–10) ligand and PSS-(1–10) standards as previously reported [7,22]. This assay detects PSS-(1–10) and its C-terminally extended forms including PSS and 8 kDa and 7 kDa PSS forms. The minimum detectable dose was 2 pg of PSS-(1–10).

Northern blot analysis

Total RNA was extracted from infected COS-7 and LoVo cells by the acid guanidine thiocyanate/phenol/chloroform method. A [α - ^{32}P]UTP-labelled rat furin cRNA probe was generated using the Promega RNA transcription kit. pSP72 containing 1244 bases of the catalytic domain of rat furin was linearized with *Hind*III and transcribed with SP6 RNA polymerase. Samples of 20 μ g of total RNA was electrophoresed in 1.5% agarose/formaldehyde gels, transferred on Nytran nylon membranes (Schleicher and Schüll) using the Vacugene Vacuum Blotting System (LKB-Pharmacia), and hybridized with the furin cRNA probe at 65°C for 24 h. Membranes were washed under high-stringency conditions. Autoradiograms were prepared by exposing the membranes to Kodak XAR-5 film at -80°C for 1–2 days using intensifying screens.

RESULTS

C-terminal processing of PSS in COS-7 cells

Figure 2 depicts Northern blots of furin mRNA in COS-7 cells infected with VV:wt (2.4 p.f.u./cell) or different doses of VV:hfurin. Control cells infected with VV:wt exhibited a single 4.5 kb transcript corresponding to that of endogenous furin.

Figure 2 Expression of furin mRNA in COS-7 cells infected with VV:wt or VV:hfurin and transfected with pMJ601:rPSS

Total RNA (20 μ g) was analysed by Northern blots using a furin cRNA probe. A single 4.5 kb transcript corresponding to endogenous furin mRNA was detected in control cells (lane 1; VV:wt + pMJ601:rPSS). VV:hfurin-infected cells (lanes 2–4) displayed two transcripts of 3 and 4.5 kb. There is a dose-dependent increase in furin mRNA expression when the m.o.i. is increased from 0.8 to 2.4 to 12 p.f.u./cell.

VV:hfurin-infected cells displayed two transcripts of 3 kb and 4.5 kb, due to the use of a 3' truncated form of furin in our VV:hfurin construct and to the choice of different polyadenylation sites [27]. The abundance of furin mRNA was greater in VV:hfurin-injected cells and there was a dose-dependent increase in both forms with increasing levels of infection.

HPLC profiles of SS-14 LI in cell extracts and secretion media from COS-7 cells infected with VV:wt plus pMJ:rPSS at 2.4 p.f.u./cell are depicted in Figures 3(a) and 3(b). Five dominant peaks eluting with retention times of 55, 65, 75, 99 and 110 min were obtained. The first two peaks, with retention times of 55 and 65 min, coeluted with synthetic SS-14 and SS-28, and the final peak at retention time 110 min represented full-length unprocessed PSS. The two intermediate peaks at retention times 75 and 99 min have been characterized previously by gel permeation chromatography and region-specific antibodies, and shown to represent N-terminally truncated forms of PSS [22]. Accordingly, in all calculations of total unprocessed PSS, the three peaks eluting at 75, 99 and 110 min were combined. COS cells infected with VV:wt plus pMJ601:rPSS processed PSS mainly to SS-28 ($29.3 \pm 8.0\%$ of total SS-14 LI in cell extracts; $31.0 \pm 3.1\%$ of total SS-14 LI in media). SS-14 represented $10.5 \pm 2.3\%$ of total SS-14 LI in cell extracts and $1.2 \pm 1.8\%$ in media. Overall, C-terminal processing of PSS to SS-14 and SS-28 was inefficient, based on the amount of unprocessed PSS ($58.5 \pm 0.7\%$ in cell extracts and $69.4 \pm 9.8\%$ in media). Infection of COS-7 cells with a higher level of VV:wt + pMJ:rPSS (12 p.f.u./cell) resulted in increased PSS expression as expected, without any difference in the pattern of processing (results not shown), suggesting that the level of PSS expression and the overexpression of viral proteases encoded in the VV genome have no effect on PSS processing.

To determine the relative ability of furin to cleave PSS to SS-28 and SS-14, COS-7 cells were infected with VV:hfurin at an m.o.i. of 0.8, 2.4 or 12 p.f.u./cell followed by transfection with pMJ601:rPSS. HPLC profiles of SS-14 LI in cell extracts and media from the 0.8 p.f.u./cell infection are compared with those from the control VV:wt infection in Figures 3(c) and 3(d). The dose-related effects of furin infection on the percentage conversion to SS-14 and SS-28 together with percentage of unprocessed PSS obtained from HPLC chromatograms are summarized in Figure 4. There was a dose-dependent increase in conversion to SS-28 in cell extracts, from 29% in control cells to 42% at 0.8 p.f.u./cell and to 58% at 2.4 p.f.u./cell. At very high levels of furin expression (12 p.f.u./cell) the percentage of intracellular SS-28 dropped. Secreted SS-28 showed a dose-dependent increase from 31% of total SS-14 LI in control

Figure 3 Effect of furin vaccinia infection on C-terminal rPSS processing in COS-7 cells

COS-7 cells were infected with either VV:wt (2.4 or 12 p.f.u./cell) (a, b) or VV:hFurin (c, d) and then transfected with pMJ601:rPSS. Cell extracts (a, c) and 4 h secretion media (b, d) were collected and analysed by HPLC. A C_{18} reverse-phase column was eluted with 12–55% acetonitrile/0.2% HFBA at 21 °C over 120 min. The 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 (b) HPLC profiles of SS-14 LI in cell extracts (a) and media (b) of control cells (VV:wt at 2.4 p.f.u./cell + pMJ601:rPSS). A similar profile was obtained when cells were infected with 12 p.f.u./cell of VV:wt (results not shown). (c) and (d) Pattern of rPSS processing in cells infected with VV:hFurin at 0.8 p.f.u./cell + pMJ601:rPSS. The results shown are representative of two experiments.

Figure 4 Dose-dependence of C-terminal rPSS processing in COS-7 cells on furin infection

The percentages of rPSS processing products SS-14, SS-28 and of unprocessed PSS were determined by HPLC and radioimmunoassay analyses of cell extracts (a) and 4 h secretion media (b). Experimental conditions were identical to those described for Figure 3. Results are representative of two experiments.

infections to 41 % at 0.8 p.f.u./cell, to 38 % at 2.4 p.f.u./cell and to 49 % at 12 p.f.u./cell. There was no change in conversion to SS-14 in cell extracts or media at the low level of furin infection (11 % in cell extracts and 11.2 % in media at 0.8 p.f.u./cell). Higher levels of furin were required to enhance SS-14 production (20 % at 2.4 p.f.u./cell and 25 % at 12 p.f.u./cell intracellularly; 9.7 % at 2.4 p.f.u./cell and 26.4 % at 12 p.f.u./cell in media). The percentage of unprocessed PSS showed a progressive decrease from > 60 % of total SS-14 LI in control infections to lows of 21.5 % in cell extracts (at 2.4 p.f.u./cell) and 24 % in media (at 12 p.f.u./cell). These results clearly indicate a dose-dependent increase in SS-28 production up to a maximum of 58 % with increasing doses of furin infection, associated with a progressive fall in the level of unprocessed PSS. Furin also processed PSS to SS-14, but at higher doses than those required for conversion to SS-28. Furthermore, at the very high levels of furin expression (12 p.f.u./cell) SS-14 conversion occurred at the expense of that of SS-28, suggesting that SS-28 may have become a substrate for SS-14 production.

N-terminal processing of rPSS in COS-7 cells

To determine whether, in addition to cleavage at the PSS monobasic site to generate SS-28, furin also processes at the second monobasic site in the N-terminal segment to generate PSS-(1–10), HPLC fractions from the dose-response study of VV:hFurin infection were analysed for PSS-(1–10) LI forms

Figure 5 Effect of furin vaccinia infection on N-terminal processing of rPSS in COS-7 cells

Cells were infected with VV:wt at 12 p.f.u./cell (control; **a**) or with VV:hfurin at 2.4 p.f.u./cell (**b**) or 12 p.f.u./cell (**c**) and then transfected with pMJ601:rPSS. Cell extracts were fractionated by HPLC and analysed for N-terminal PSS-(1–10) LI by radioimmunoassay. The elution positions of synthetic PSS-(1–10) and of the 1.5, 2.5, 7 and 8 kDa N-terminal PSS forms (explained in Figure 1) are indicated by arrows. Furin overexpression failed to affect the percentage of rPSS to PSS-(1–10). Results are representative of two experiments.

(Figure 5). Control cells infected with VV:wt + pMJ601:rPSS showed a small 1 kDa peak coeluting with PSS-(1–10), accounting for ~10% of total immunoreactivity. Several other HPLC peaks were obtained and were shown to correspond to PSS-(1–10) LI peptides of 1.5 kDa, 2.5 kDa, 3.5 kDa, 7 kDa [PSS-(1–64)] and 10.4 kDa (PSS), as previously described. The relative amounts of the different peaks were comparable at low (2.4 p.f.u./cell) and high (12 p.f.u./cell) levels of VV:wt infection. Infection of COS-7 cells with VV:hfurin produced no significant increase in PSS-(1–10) production, even at the highest levels of furin expression, indicating that an Arg residue at P1 is essential for furin-mediated cleavage and cannot be substituted by Lys. The finding of a significant 7 kDa peak correlated with the increasing amounts of furin-mediated conversion to SS-28. On the other hand, a small 8 kDa peak was seen with infection of VV:hfurin at 2.4 p.f.u./cell which was barely detectable at higher levels of furin overexpression (12 p.f.u./cell), despite the concomitant rise in SS-14 production. These results provide additional evidence that the synthesis of SS-14 observed with high levels of furin expression occurs as a result of cleavage of SS-28 rather than directly from PSS, since such processing would generate the 8 kDa peptide.

Processing of PSS in LoVo cells

LoVo cells express an inactive form of furin resulting from a point mutation [26]. Given the ubiquitous distribution of furin, the availability of a cell lacking this enzyme presented an ideal system for further authenticating a functional role for furin as an SS-28 convertase. rPSS processing was studied in control LoVo cells co-infected with VV:wt + VV:rPSS (representing rPSS processing in the absence of furin) and compared with processing in cells co-infected with VV:rPSS + VV:hfurin (cells overexpressing furin with rPSS). Appropriate expression of furin following VV:hfurin + VV:rPSS co-infection was confirmed by Northern blot analysis of furin mRNA (Figure 6). Wild-type LoVo cells expressed a single 4.5 kb transcript corresponding to the mutant furin. As in the case of COS-7 cells, two furin transcripts of 3 kb and 4.5 kb were detected in LoVo cells infected with VV:hfurin. Infected LoVo cells displayed high basal release of SS-14 LI corresponding to 82% of cell content per 4 h, and failed to stimulate secretion in response to forskolin. SS-14 LI in these cells was therefore secreted through the constitutive secretory

Figure 6 Northern blots of furin mRNA from control and VV:hfurin-infected LoVo cells

Total RNA (20 µg) was probed with a furin cRNA probe. A single 4.5 kb transcript was detected in control cells (lane 1), representing the mutant furin mRNA in LoVo cells. Co-infection with VV:hfurin (lane 3) resulted in two transcripts of 3 and 4.5 kb expressed at very high levels. For further details see the legend to Figure 7.

pathway (as was also the case in COS-7 cells). Control cells produced predominantly rPSS (fraction 110 along with additional peaks at fraction 71 and 99 corresponding to N-terminally truncated forms of PSS) (Figures 7a and 7b). These three C-terminally unprocessed forms of rPSS accounted for 75% of cellular SS-14 LI and 73% of secreted SS-14 LI. A minor peak of SS-14 was observed at fraction 55, accounting for 3.5% and 7% of cellular and secreted peptide respectively. Somewhat larger amounts of SS-28 were synthesized in control infected LoVo cells, eluting at fraction 65 and accounting for ~21% of total immunoreactivity in both cell extracts and secretion media. Since LoVo cells do not have functional furin, these results suggest the existence of another SS-28-generating endopeptidase. Co-infection of LoVo cells with VV:rPSS and VV:hfurin (Figures 7c and 7d) significantly increased the production of both SS-14 (25% in cell extracts, 40% in media) and SS-28 (30% in cell extracts, 34% in media).

In order to determine whether the inability of furin to mediate monobasic cleavage at Lys sites in COS-7 cells was also a feature of LoVo cells, N-terminal immunoreactivity was analysed in HPLC fractions of cell extracts and media from control (VV:rPSS + VV:wt) and furin- (VV:rPSS + VV:hfurin) infected cells (Figure 8). Control infected LoVo cells synthesized large amounts of PSS-(1–10), detected mostly in cell extracts (~15% of PSS-(1–10) LI) with a very small secreted component. Significant amounts of unprocessed PSS as well as peaks cor-

Figure 7 C-terminal rPSS processing in (a, b) control LoVo cells co-infected with VV:wt + VV:rPSS (1 and 2 p.f.u./cell respectively) or (c, d) LoVo cells co-infected with VV:hfurin + VV:rPSS (1 and 2 p.f.u./cell respectively)

Cell extracts (a, c) and 4 h incubation media (b, d) were collected at 19 h post-infection and analysed by HPLC and radioimmunoassay for SS-14 LI. Column conditions and markers are as described in the legend to Figure 3. Results are representative of five experiments.

responding to 7 kDa, 8 kDa and 2.5 kDa forms were identified. The presence of significant PSS-(1–10) production in a cell lacking furin activity excludes a role for this endoprotease in this monobasic cleavage and suggests the existence of another monobasic Lys¹ converting enzyme. LoVo cells co-infected with rPSS and furin showed no additional increase in PSS-(1–10) formation. These cells displayed increased synthesis of the 7 kDa and 8 kDa forms associated with a decrease in the amount of unprocessed PSS, consistent with furin-mediated conversion of PSS to SS-14 and SS-28.

DISCUSSION

In the present study we have tested the ability of furin to mediate endoproteolytic cleavage of rPSS at each of the three known processing sites in the precursor: the two monobasic sites located at the C- and N-terminal segments of the molecule which generate SS-28 and PSS-(1–10) respectively, and the RXRK site resulting in SS-14 production. Using a furin VV recombinant to induce graded doses of furin expression in COS-7 cells, we showed a dose-dependent ability of this protease to cleave PSS to SS-28 with a maximum efficiency of ~ 60%, providing direct evidence that furin qualifies as an SS-28 convertase. Interestingly, the site of PSS conversion to SS-28 RXXXXR contains an Arg and P1 and only one of the other two required basic amino acid residues at P6, and thus meets only two of the three criteria for furin cleavage specificity as proposed by Watanabe et al. [25]. Furin was unable to effect monobasic cleavage of rPSS to PSS-(1–10) at a Lys site, suggesting that Arg rather than Lys is the preferred amino acid at the P1 site of the furin consensus motif. Ad-

ditionally, these findings imply the existence of another monobasic convertase, with a preference for Lys rather than Arg at P1, to account for PSS-(1–10) generation from PSS. Dibasic processing to PSS to SS-14 has been shown to be mediated by both PC1 and PC2 ([22,28] A. S. Galanopoulou, N. G. Seidah and Y. C. Patel, unpublished work). When expressed at high levels, however, furin was also able to effect cleavage at this site, albeit inefficiently. Since the SS-14 conversion site RXRK also contains a Lys at P1, differential cleavage to produce SS-14 but not PSS-(1–10) can be explained by the presence of two arginines at P2 and P4 at the SS-14 site, producing a more favourable substrate for furin compared with the single Arg at P6 at the PSS-(1–10) processing site.

LoVo cells represent a human adenocarcinoma cell line lacking endogenous furin due to a point mutation in the 'homo B' domain of the coding region of furin, resulting in premature termination and an inactive enzyme [26]. Since furin is a resident enzyme of the trans-Golgi network and thus is ubiquitously expressed in all cells, the availability of a mutant cell lacking this enzyme provided the opportunity to further delineate the role of furin as an SS-28 convertase. Despite the absence of functional furin, LoVo cells were still capable of ~ 20% conversion of rPSS to SS-28, indicating the presence of an enzyme other than furin responsible for SS-28 generation. Several candidate SS-28 convertases have been previously proposed. Beinfeld et al. [29] and Kuks et al. [30] have identified two putative monobasic processing serine proteinases, an SS-28-generating rat intestinal convertase and a frog skin endoprotease which cleaves at a typical RXVRG monobasic motif. Mackin et al. [31] have proposed that the anglerfish SS-28-generating enzyme is a

Figure 8 N-terminal processing of rPSS in LoVo cells

Cell extracts (a, c) and 4 h secretion media (b, d) from LoVo cells infected with either VV:wt + VV:rPSS (a, b) or VV:hfurin + VV:rPSS (c, d) were analysed by HPLC and assayed for N-terminal PSS-(1–10) LI. Column conditions and markers were as described for Figure 5. Results are representative of two experiments.

cathepsin D-like aspartyl proteinase of approx. 39 kDa which is distinct from the kexin/subtilisin family. A yeast aspartyl protease YAP3 has been shown to convert anglerfish PSS II to SS-28 *in vitro* [32,33]. The functional role of YAP3 in mammalian SS-28 generation, however, remains uncertain, since this enzyme fails to cleave PSS to SS-28 when coexpressed with rPSS in COS-7 cells (A. S. Galanopoulou and Y. C. Patel, unpublished work). Among the subtilisin/kexin family, we have previously demonstrated that neither PC1/PC3 nor PC2 can effect monobasic conversion of rPSS to SS-28 [23]. Because of its highly selective tissue distribution, PC4 is unlikely to be a significant monobasic convertase. Likewise, preliminary evidence suggests that PC5 does not process rPSS to either SS-14 or SS-28 (A. S. Galanopoulou and Y. C. Patel, unpublished work). PACE4, however, is expressed in LoVo cells [15] and could be the enzyme responsible for the conversion of transfected rPSS to SS-28 that was observed in these cells. Further direct studies are necessary to confirm the role of PACE4 as an SS-28 converting enzyme and to determine how its cleavage specificity for monobasic sites differs from that of furin. Coexpression of hfurin with rPSS in LoVo cells significantly improved SS-28 production, although the maximum efficiency of ~34% was less than that obtained in COS-7 cells. These differences may be explained by cell-specific variations in the levels of functional enzyme in COS-7 cells (expressing both endogenous and exogenous enzyme) and LoVo cells (expressing only exogenous enzyme), by differences in the subcellular compartmentalization of enzyme and substrate, or by an inhibitory effect of the mutant form of furin on exogenously expressed enzyme. Additionally, since SS-14 and SS-28 are independently processed from rPSS [3,34], the high level of SS-14 production that was found in LoVo cells compared

with COS-7 cells could have decreased the availability of precursor substrate for furin-mediated SS-28 generation.

Despite the absence of active furin, LoVo cells transfected with rPSS displayed significantly greater N-terminal processing to PSS-(1–10) than did similarly transfected COS-7 cells. Furthermore, expression of increasing levels of furin failed to alter the efficiency of rPSS processing to PSS-(1–10) in either cell line. These findings exclude a significant role for furin in N-terminal PSS-(1–10) generation and provide additional evidence for the existence of another endoproteinase responsible for this cleavage. Such an enzyme (perhaps PACE4, which is endogenously expressed at higher levels in LoVo cells compared with COS cells [15]) would possess a broader cleavage specificity than furin for conversion at monobasic sites encompassing both Arg and Lys residues at P1 to account for the PSS-(1–10)- and SS-28 generating activity found in these cells.

In addition to its role in SS-28 production, previous studies have suggested that furin is capable of low levels of conversion of rPSS to SS-14, based on indirect evidence correlating endogenous expression of furin with constitutive SS-14 synthesis in COS-7, PC12 and 3T3 cells [10,22,35]. Although overexpression of furin in COS-7 cells in the present study enhanced SS-14 production, the efficiency of conversion was relatively poor (~20%), excluding a significant physiological role for the enzyme in dibasic processing to SS-14. Interestingly, LoVo cells infected with VV:hfurin produced significantly higher amounts of SS-14 compared with COS-7 cells. These differences may be due to the different intracellular microenvironment in the two cell lines which could influence enzyme activity. Alternatively, SS-28 produced from rPSS by furin may then act as a substrate for another endogenous convertase in the constitutive pathway

[36], such as PACE4. Indeed, preliminary evidence suggests that PACE4 is capable of converting rPSS into both SS-14 and SS-28 (A. S. Galanopoulou and Y. C. Patel, unpublished work).

In summary, we have shown that furin can mediate the monobasic cleavage of rPSS to SS-28 and qualifies as an SS-28 convertase. Arginine is the preferred basic amino acid at the P1 site for cleavage by furin. Furin does not mediate N-terminal processing of rPSS to PSS-(1-10), suggesting the existence of another monobasic convertase with a preference for Lys rather than Arg at P1. Such an enzyme, with a broader cleavage specificity than furin encompassing both Arg and Lys residues at P1 of monobasic conversion sites, could also explain the presence of endogenous SS-28- and PSS-(1-10)-generating activities found in LoVo cells, which lack a functional furin.

PrePSS cDNA (pKS5) was kindly provided by Dr. K. Sevarino. We are grateful to Miss S. Zheng and O. Dembinska for technical assistance, to J.-L. Liu for assistance with the illustrations, and to Mrs. M. Correia for secretarial help. This work was supported by grants MT-6196 and PG-11474 from the Canadian Medical Research Council. A.S.G. was the recipient of studentship awards from the Fonds Pour La Formation de Chercheurs et l'Aide a la Recherche, the Royal Victoria Hospital Research Institute and the Department of Medicine.

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