Comparative proteolytic processing of rat prosomatostatin by the convertases PC1, PC2, furin, PACE4 and PC5 in constitutive and regulated secretory pathways

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Abstract Recombinant vaccinia virus vectors were used to coexpress each of the candidate prohormone convertases PC1, PC2, furin, PACE4 and PC5 with rat prosomatostatin (rProSOM) in the constitutive secreting cell line LoVo and in the endocrine corticotroph cell line AtT-20, which exhibits regulated secretion. Mammalian ProSOM is cleaved at a dibasic Arg-Lys↓ site to produce somatostatin-14 (S-14) and at a monobasic Gln-Arg↓ site to yield somatostatin-28 (S-28). The analysis of processed products by gel-permeation high performance liquid chromatography shows that in LoVo cells PC1, furin and PACE4 generate S-14, S-28 and a mixture of S-14 and S-28, respectively, while PC2 is unable to process ProSOM in these constitutive cells. In contrast, PC2 can generate S-14 in AtT-20 cells. The convertase PC5 is unable to process ProSOM in either cell line. These data suggest that PC2, PC1 and PACE4 are candidate S-14 convertases, while PACE4 and furin are candidate S-28 convertases.

Key words: Somatostatin; Convertase; Processing; Coexpression; Vaccinia virus; Constitutive and regulated cell

1. Introduction

Post-translational proteolysis is a common mechanism required for the synthesis of biologically active peptides and proteins in eukaryotes [1,2]. Such processing occurs at either single or pairs of basic amino acids [3]. Genetic evidence suggests that pairs of basic amino acids are necessary for prohormone processing, since mutations in one of the basic residues present in the cleavage site of proinsulin, for example, result in secretion of the precursor rather than the mature protein [4]. However, not all single or pairs of basic residues are signals for intracellular cleavage. There is now ample evidence that the presence of basic amino acids, although necessary, is not sufficient to direct recognition by the physiologically relevant endoproteases, and that other secondary structure determinants favor the processivity of a given site within a precursor [5].

Six processing enzymes, generally referred to as the precursor convertases (PCs), have been identified in mammalian species. These Ca^{2+}-dependent serine proteinases belonging to the subtilisin/kexin family are called PC1/3, PC2, furin, PC4, PACE4 and PC5/6 (for reviews see [2,3,6]). In general, it was found that the catalytic segment of each convertase represents the sequence that is most conserved between the members of the family, with PC2 showing the least sequence similarity. Of all these convertases, only furin is ubiquitously expressed, while PACE4 and PC5 exhibit a widespread tissue distribution in both regulated and constitutive cells [7]. In contrast, PC1 and PC2 seem to be predominantly expressed in endocrine and neural cells, and PC4 is exclusively synthesized within germ cells of the testis [7].

The cleavage preference of the convertases has been studied by their coexpression with a number of pro-proteins and prohormones. From these studies it became apparent that each convertase exhibits aspecificity of cleavage post single or pairs of basic residues (for reviews see [2,8]). In addition, the data showed that the intracellular cleavage kinetics of specific bonds in precursors are determined by a multiplicity of factors, including convertase activation, substrate specificity and intracellular environment [9]. Coexpression studies with vaccinia virus recombinants have proven useful to determine if a given precursor substrate can be cleaved intracellularly by a candidate convertase either in constitutive or regulated cells [10–13].

In mammals a unique gene encodes prosomatostatin which contains a single Arg↓ and an ArgLys↓ cleavage site [14]. Processing occurs at the COOH-terminal segment of the molecule and generates two bioactive forms: somatostatin-14 (S-14) by cleavage post ArgLys↓ and somatostatin-28 (S-28) following a GlnArg↓ cleavage. Cleavage at the ArgLys↓ site yields S-14 and an 8 kDa peptide [15–17], whereas endoproteolysis at the monobasic Arg↓ site generates S-28 and a 7 kDa peptide [18,19].

In a previous work we have shown that PC1 is able to process ProSOM in the constitutive COS-7 cells, whereas PC2 was unable to do so [20]. In a subsequent study it was shown that stable transfectants of mPC2 together with rProSOM in the regulated cells GH4C1 or GH3 produce S-14 (Galanopoulou, A.S., Seidah, N.G. and Patel, Y.C. 1995, submitted). In the present work, we exploited the vaccinia virus expression system to compare the processing of rat ProSOM by each of the convertases PC1, PC2, furin, PACE4 and PC5 in either AtT-20 or LoVo cells, as prototypes of regulated and constitutive cells, respectively. Although LoVo cells endogenously express PACE4 and furin [7], the latter convertase is not functional [21]. In order to identify the enzyme(s) involved in the generation of S-14 and/or S-28, the resultant peptides present in cellular extracts and culture media were identified by a C-terminally directed radioimmunoassay following fractionation by gel-

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permeation high performance liquid chromatography (GP-HPLC). The data demonstrate that S-14 is generated by the action of either PC1 or PC2 in AtT-20 cells and by only PC1 in LoVo cells, whereas PACE4 generates both S-28 and S-14 in both cell types. Moreover, in either cells furin processes ProSOM into S-28, and PC5 is relatively inactive.

2. Materials and methods

The coinfections of either AtT-20 or LoVo cells (50×10^6 cells) with a vaccinia virus recombinant of each convertase VV:mPC1, VV:mPC2, VV:hfurin, VV:hPACE4 and VV:mPC5 with VV:rProSOM were performed overnight at a multiplicity of infection of 1 plaque forming unit/cell, as previously reported [9,11,12]. Following an overnight infection, the cells were washed and then further incubated in a serum free-medium for 4 h, as reported [12]. The cells were then extracted with 0.5 vol. of glacial acetic acid, and sonicated four times for 30 s, and the supernatant following centrifugation was passed through an ODS-silica cartridge (Sep-pak C_{18}; Waters Inc.) which was equilibrated with 0.1% trifluroacetic acid (TFA). Similarly, the media were diluted with 0.5 vol. of glacial acetic acid and then loaded onto the ODS cartridge. The adsorbed peptides were eluted with 80% acetonitrile in 0.1% TFA. Peptides from both the cell extracts and the culture media were fractionated by GP-HPLC using two tandem Protein-Pak 60 columns (Waters Inc.), eluted with 40% acetonitrile, 0.1% TFA. Fractions of 0.5 ml were collected and then analyzed using a C-terminally directed RIA recognizing S-14 [19]. The percentages of the products were calculated following integration of the areas of the S14-like immunoreactivities and dividing the area occupied by either S-14 or S-28 by the total area.

3. Results and discussion

We have previously shown that S-28 and S-14 are generated in the constitutive and regulated pathways, respectively [22]. In an effort to identify the candidate subtilisin/kexin-like enzyme(s) involved in vivo in the generation of these peptides, an RIA directed against S-14 was performed on GP-HPLC fractions obtained from infected cells and conditioned medium. Two kinds of cells were used: the constitutive LoVo cells which are devoid of furin activity [21] but not of PACE4 [7], and the regulated AtT-20 cells which possess both regulated and constitutive secretory pathways.

The control coinfection of Lovo cells with VV:rProSOM and VV:mpro-opiomelanocortin (mPOMC; CTL) resulted in no S-14 production and only a very limited cleavage of ProSOM into S-28 (12%) which was secreted into the medium (Fig. 1A). It is interesting to note that media collected for 4 h contained more ProSOM than the cell extract, in accordance with the constitutive character of these cells. This cleavage may be afforded by the endogenous convertase PACE4 which is detectable in LoVo cells [7]. A similar control experiment performed in AtT-20 cells, although revealing an intracellular 28% processing into S-14 and 10% into S-28, also showed that only ProSOM is released under basal conditions (Fig. 1B). This pattern differs somewhat from the one we recently reported [22], in which the presence of S-28 and S-14 were detected in the culture medium under basal conditions. The differences may probably be related to our analysis in this study of a 4 h basal secretion medium, as compared to a 16 h secretion medium analyzed previously [22]. The intracellular processing pattern observed in AtT-20 (Fig. 1B) probably reflects the presence of endogenous convertases, with PC1 being the major one and PC2/PACE4 being minor [7]. Above these endogenous activities, coinfection of VV:rProSOM with each VV:PC is expected to enhance, in some cases, the extent of processing of ProSOM.

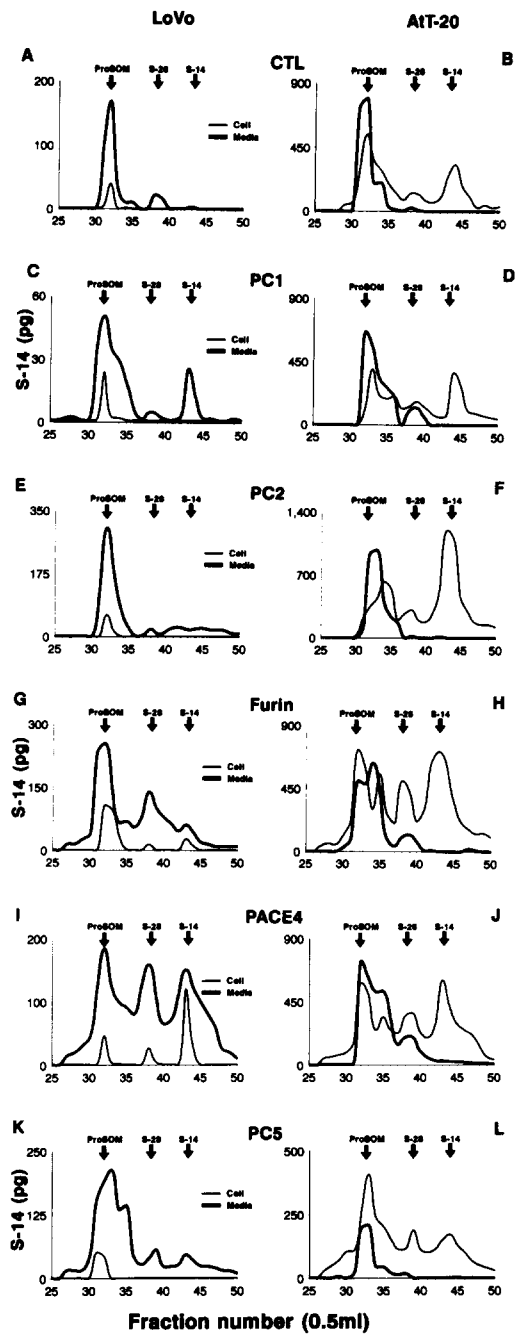

Fig. 1. GP-HPLC analysis of ProSOM processing in LoVo (A,C,E,G,I,K) and AtT20 (B,D,F,H,J,L) cells. The cells were coinfected with VV:rProSOM and either VV:POMC (CTL) or each of VV:mPC1, VV:mPC2, VV:hfurin, VV:hPACE4 and VV:mPC5. The media represent the 4 h collection following overnight growth. The chromatograms presented correspond to the elution pattern of the media and the cell extract equivalent to 12.5×10^6 cells which were applied to two tandem gel-permeation HPLC columns (Protein-Pak 60; Waters). Each fraction (0.5 ml) was lyophylized and the S-14-like immunoreactivity was estimated by a C-terminally directed radioimmunoassay and expressed as pg/total fraction. The arrows indicate the elution positions of pro-somatostatin (ProSOM), somatostatin-28 (S-28) and somatostatin-14 (S-14).

From the results presented in Fig. 1 and the calculations of

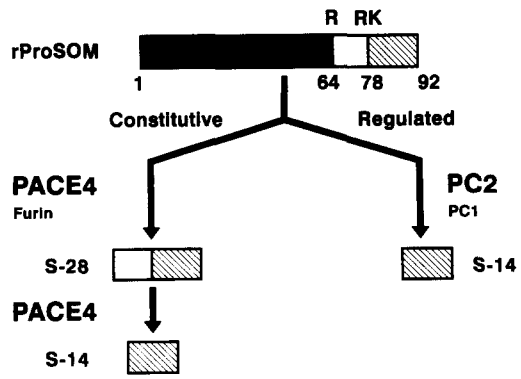

Fig. 2. Proposed processing scheme of the 92 amino acid rat rProSOM emphasizing the convertases involved in the production of either S-28 (single R↓ cleavage) or S-14 (RK↓ cleavage) in the constitutive pathway and S-14 in the regulated pathway. It should be noted that within the constitutive pathway of regulated cells such as AtT20, the production of S-28 occurs, but to some extent the latter is further processed to S-14.

the levels of each product with respect to the total somatostatin-like immunoreactivity (IR-SOM), we were able to deduce the convertases which are likely to be responsible for the cleavage of ProSOM into S-14 and S-28 in either LoVo (Fig. 1A,C,E,G,I,K) or AtT-20 (Fig. 1B,D,F,H,J,L) cells. Accordingly, in LoVo cells S-14 is best produced by PACE4 (38% of media IR-SOM; Fig. 1I) and to a lesser extent by PC1 (19% of media IR-SOM; Fig. 1C). In AtT-20 cells, however, PC2 is the best S-14 convertase (54% of cellular IR-SOM; Fig. 1F), followed by furin (40% of cellular IR-SOM; Fig. 1H) and PACE4 (38% of cellular IR-SOM; Fig. 1J). We note that furin mostly generated S-28 in LoVo cells (Fig. 1G) but significantly stimulated the production of both S-28 and S-14 in AtT-20 cells (Fig. 1H). This may suggest that in AtT-20 cells this Golgi-localized enzyme converts ProSOM into S-28 which is then further processed into S-14 by the PC1 endogenous to AtT-20 but not LoVo cells [7]. As previously observed [20], we note that in constitutive cells, PC2 is unable to process ProSOM into S-14 or S-28 (Fig. 1E). The present data and those reported earlier [20] allow us to conclude that PC1 could produce S-14 in constitutive cells but that PC2 needs a regulated secretory pathway to be active. We also note that PC5 is not able to produce significant amounts of S14 in either cell (Fig. 1K,L). This is not due to low expression levels of inactive PC5 since Northern blot analyses of VV:mPC5 infected cells demonstrated a large increase in the mRNA level of this enzyme (not shown), and a similar coexpression of this convertase with the neuroendocrine protein pro7B2 demonstrated that PC5 can process this precursor to 7B2 [23]. The results presented in Fig. 1 also suggest that both furin and PACE4 are as efficient in processing ProSOM into S-28 in either LoVo (27% of media IR-SOM; Fig. 1G,I) or AtT-20 (19% of cellular IR-SOM; Fig. 1H,J) cells. We note that, especially in the case of AtT-20 cells, a significant shoulder of S-14 immunoreactivity is detected, eluting just after the precursor. The amount of this material, which must contain the C-terminal epitope, is increased by coexpression of the convertases, especially furin and PACE4. This N-terminally truncated intermediate, which has been previously observed [20], probably results from the cleavage of the N-terminal segment of proSOM at Lys^{10} in order to produce antrin. Finally, it is also apparent that neither PC1, PC2 or PC5 can cleave ProSOM into S-28 (Fig. 1C,D,E,F,K,L).

Our results are in good agreement with recent immunocytochemical colocalization studies in rat and mouse pancreatic islets showing that only PC2, but not PC1, colocalizes with somatostatin [24]. The analysis of the different forms of immunoreactive somatostatin in neuronal and non-neuronal tissues revealed that in neuronal and pancreatic tissues S-14 is the major end-product, whereas in mucosal tissues S-28 is the most abundant form [25]. This tissue-specific processing of ProSOM is in agreement with our present findings which demonstrated that S-14 is primarily produced in cells endowed with a regulated secretion mechanism whereas more of the S-28 end-product is observed in constitutive cells. Since PC1 and PC2 are primarily expressed in regulated cells [7,26], we propose (Fig. 2) that PC2, and possibly PC1, could participate in the production of S-14 in regulated cells, but that in pancreatic δ-cells PC2 would be the major S-14 convertase [24].

Our data also suggest that both PACE4 and furin, which are either widely or ubiquitously expressed, respectively [7], are the likely candidate S-28 convertases (Fig. 2). This result is all the more remarkable as the **Arg**LeuGluLeuGln**Arg**↓SerAla cleavage site responsible for the production of S-28 contains a P6 Arg rather than the canonical P4 Arg usually found in furin and PACE4 cleavage sites (see reviews [2,3]). In a previous study [22], we have shown that, in AtT-20 cells, the constitutive secretion of S-28 and S-14 is abolished by a mutation at the monobasic Arg↓ cleavage site preceding S-28. This suggested that the constitutively secreted S-14 results from the cleavage of S-28 by constitutive convertases, which in view of our present results may well be PACE4 and/or furin. The furin data presented in this work are also in accord with a detailed recent study demonstrating a direct role of furin in the production of S-28 in COS-7 cells [27,28]. However, at present we cannot exclude the possibility that other processing enzymes not yet characterized at the structural level [29], could also be responsible for the cleavage at this monobasic site or at the monobasic Lys^{10} site previously identified in the pro-segment of ProSOM, the cleavage of which results in the production of antrin [20].

In conclusion, our data identified the best candidates among the known subtilisin/kexin-like convertases which could be responsible for the processing of ProSOM into either S-14 or S-28 in mammals. Our results are in good agreement with those observed for angler fish ProSOM-I processing, where PC2 was shown to effectively cleave this precursor into S-14 [29], emphasizing the fact that the S-14 convertase(s) were conserved during evolution.

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