The integrity of the RRGDL sequence of the proprotein convertase PC1 is critical for its zymogen and C-terminal processing and for its cellular trafficking

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In order to define the functional importance of the conserved RRGDL motif in the P-domain of the mammalian proprotein convertases (PCs) we generated and cellularly expressed three mutant PC1 vaccinia-virus (VV) recombinants: ARGDL-PC1, RAGDL-PC1 and RRGEL-PC1. Functionally, these mutants caused a decreased level of processing of pro-opiomelanocortin (POMC) into β -lipotropic pituitary hormone (β -LPH), especially in the constitutively secreting BSC40 cells. Pulse-chase analyses demonstrated that, in part, this effect was due to both an increased degradation of the mutant PC1s within the endoplasmic reticulum and to a diminished level of zymogen processing in the same compartment. In addition, within cells containing secretory granules such as PC12 and GH4C1 cells, such mutations prevented the C-terminal auto-processing of PC1 into the fully mature 66 kDa form stored in the secretory granules of regulated cells. Since the 66 kDa PC1 is the most active form of the

enzyme, it is proposed that the RRGDL sequence is critical for the generation of maximal intracellular PC1 activity. In regulated cells, co-expression of POMC with PC1 or its mutants together with the general PC inhibitor α_1 -antitrypsin Portland (α_1 -PDX), which acts primarily within the constitutive secretory pathway, demonstrated that the latter completely inhibited the formation of β -LPH by PC1 mutants, whereas it only partially inhibited the ability of wild-type PC1 to process POMC. This suggests that RRGDL mutations prevent PC1 from entering secretory granules and hence the formation of the 66 kDa PC1, and result in the mis-sorting of PC1 mutants towards the constitutive secretory pathway. This conclusion was further supported by immunocytochemical data demonstrating that RRGDL mutants exhibit an intracellular localization pattern different from that of the granule-associated wild-type PC1, but similar to that of the Golgi-localized convertase PC5-B.

INTRODUCTION

Limited proteolysis of prohormones and proprotein precursors allows the regulation of the production of their active polypeptide components. In the majority of cases, this activation step involves specific cleavage(s) post single or pairs of basic residues. Recently, seven mammalian serine proteinases related to yeast kexin and bacterial subtilisins were identified and shown to be responsible for such cleavages. These proprotein convertases (PCs) were called furin (also called PACE), PC1 (PC3), PC2, PACE4, PC4, PC5 (PC6) and PC7 (LPC, SPC7 or PC8) (for reviews and updates see [1–5]). While furin, PACE4, PC5 and PC7 exhibit a widespread tissue distribution, PC4 is found only in testicular germ cells. The expression of PC1 and PC2 is mostly restricted to endocrine and neural cells. All of these PCs exhibit an N-terminal signal peptide, followed by a prosegment, a catalytic domain, a P-domain and an enzyme-specific C-terminal segment [4]. The prosegment is thought to act as a molecular chaperone guiding the folding of the PCs in the endoplasmic reticulum and, once cleaved by an autocatalytic mechanism, it acts as an inhibitor until it is disposed of during cellular transit to the *trans*-Golgi network (TGN) [5,6]. The most conserved catalytic domain

of eukaryotic precursor convertases exhibits the closest similarity to bacterial subtilisins and contains the catalytic triad Asp-His-Ser as well as the oxyanion-hole Asn [4]. In contrast with the relatively short bacterial subtilases, the catalytic domain of eukaryotic convertases is extended at the C-terminus by a 140–150-amino-acid P-domain whose function is not well understood. Finally, the P-domain is followed by PC-specific sequences which may control their intracellular trafficking and localization [7–9].

The P-domain has been shown to be critical for the zymogen cleavage and for enzyme secretion of pro-kexin [10], pro-PACE4 [11], pro-PC2 [12], for the substrate cleavage activity of furin [13,14], and for the sorting of PC2 [15]. The N-terminus of the P-domain starts at the end of the catalytic domain, which is where the subtilisin sequence stops. Its C-terminal border has been defined as that amino acid close to an L-X-(L/F)-X-G sequence [4], beyond which further deletions would irreversibly abolish the activity of the PC [10,11,13]. Interestingly, this functional definition coincides nicely with the endpoint of similarity between the PCs [4,10].

In addition to the above consensus sequence at the end of the P-domain, ten other amino acids are absolutely conserved among

Abbreviations used: PC, proprotein convertase; ER, endoplasmic reticulum; TGN, *trans*-Golgi network; m, mouse; α_1 -PDX, α_1 -antitrypsin Portland; POMC, pro-opiomelanocortin; β -LPH, β -lipotropic pituitary hormone; VV, vaccinia virus; pfu, plaque-forming unit; BFA, brefeldin A.

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mFurin	Tyr	Asn	Arg	Arg	Gly	Asp	Leu	Ala	Ile
dFur1	Ser	Gln	Arg	Arg	Gly	Asp	Ile	Gln	Leu
mPC1	Tyr	Ser	Arg	Arg	Gly	Asp	Leu	His	Val
afPC1	Tyr	Thr	Arg	Arg	Gly	Asp	Leu	His	Ile
hydPC1-A	His	Arg	Arg	Arg	Gly	Asp	Val	Ser	Ile
mPC2	Ala	Thr	Arg	Arg	Gly	Asp	Leu	Asn	Ile
xenPC2	Ser	Thr	Arg	Arg	Gly	Asp	Leu	Asn	Ile
mPC4-a	Tyr	Ser	Arg	Arg	Gly	Asp	Leu	Glu	Ile
mPACE4	His	Pro	Arg	Arg	Gly	Asp	Leu	Gln	Ile
mPC5	His	Pro	Arg	Arg	Gly	Asp	Leu	Ala	Ile
ceBlis-D	Tyr	Leu	Lys	Arg	Gly	Asp	Leu	Lys	Leu
mPC7	His	Pro	Arg	Arg	Gly	Ser	Leu	Glu	Leu
aFurin	Ser	Ser	Arg	Arg	Gly	Glu	Ile	Arg	Ile
dFur2	Phe	Phe	Pro	Arg	Gly	Asn	Leu	Arg	Ile
yKexin	Thr	Glu	Ile	Arg	Gly	Thr	Thr	Thr	Val

Fibronectin	Val	Thr	Gly	Arg	Gly	Asp	Ser	Pro	Ala
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Figure 1 Conservation of the RRGDL motif in mammalian PCs and their orthologues.

This Figure aligns the sequences surrounding the conserved RRGDL motif in the P-domain of the mouse proprotein convertases mPC1, mPC2, mPACE4, mPC4, mPC5, mFurin in comparison with mPC7 and with the yeast (y) enzyme yKexin and with the kexin-like enzymes: the *Drosophila melanogaster* dFurin1 and dFurin2, *C. elegans* Blisterase-4 isoform D (ceBlis-D), the anglerfish PC1 (afPC1), the *Aplysia californica* aFurin, the *Hydra* hydPC1-A, as well as the *Xenopus laevis* xenPC2. These PC-related sequences were compared with the RGD motif in human fibronectin. All the sequences were obtained from the EMBL data bank.

all PC orthologues ([4]; N. G. Seidah, unpublished work). Among these, the RRGDL sequence is conserved in six (except for PC7) of the seven mammalian PCs, and its RG dipeptide is always present in all PC orthologues (Figure 1). In view of the RGD sequence found in PCs and in fibronectin, it was originally suggested that this motif may be important for the interaction of the PCs with cell-adhesion integrins [16]. The conservation of this motif suggests a structural or functional role which may be common to mammalian convertases. Binding of fibronectin to its integrin receptor was shown to be dependent on the RGD sequence and to be abrogated when it was replaced by RGE [17]. Interestingly, three RGD copies exist in *Hydra* PC1. The PCs which do not contain an RGD sequence include mammalian (human, rat and mouse) PC7, *Drosophila* (fruitfly) furin 2, *Limnaea stagnalis* (pond snail) PC2, *Aplysia californica* (sea hare) PC1 and PC2 and yeast XPR6 (reviewed in [4]).

In the present study we addressed the question of the functional relevance of the RRGDL motif within the model enzyme mouse PC1 and its importance for POMC cleavage, precursor processing (prosegment removal and C-terminal cleavage), stability of the enzyme and its intracellular trafficking. For this purpose we constructed PC1 mutants in which the RRGDL sequence was modified and compared their properties with those of wild-type PC1 using the VV expression system in both constitutively secreting and regulated cells. The data revealed that the RRGDL motif is critical for the stability of PC1 and for its

zymogen activation in the endoplasmic reticulum (ER), as well as for the elaboration of maximal PC1 activity releasing β -lipotropic pituitary hormone (β -LPH) from POMC. Finally, biosynthetic analysis rationalized our observations and demonstrated that processing of PC1 to the C-terminally truncated 66 kDa form, which usually occurs in regulated cells [18,19], is surprisingly prevented in all mutants. Co-expression of POMC with PC1 or its RGDEL mutant and with the PC inhibitor α_1 -antitrypsin Portland (α_1 -PDX) [20], which primarily inhibits processing within the constitutive secretory pathway [21], suggested that the granular formation of β -LPH from POMC by PC1 does not occur with the PC1 mutant. These data suggest that mutation of the RGD sequence prevents PC1 from entering secretory granules, resulting in its cellular release via the constitutive secretory pathway.

MATERIALS AND METHODS

Cell culture and VV infections

Four different cell lines were used for infections. The constitutively secreting BSC40 cells (kidney epithelial cells, African green monkey), the regulated rat sommatolactotroph GH4C1 and GH3 cells and the more granulated pheochromocytoma PC12 cells (a gift from Dr. S. Tooze, Imperial Cancer Research Fund, London, U.K.) were used as described in [19,22]. All VV infections were performed for 30 min–2 h as previously described using 1 plaque-forming unit (pfu)/cell (1.5×10^6 BSC40 cells or 3×10^6 for either GH3, GH4C1 or PC12 cells) of each recombinant virus. The cells were analysed either 5 or 17 h post infection [19,23].

Site-directed mutagenesis and production of RRGDL mutant VV recombinants of PC1

In the mPC1-RRGDL recombinant VV mutants produced, the R¹⁹⁰RGDL⁴⁹⁴ motif was replaced by either ARGDL (VV: mPC1[ARGDL]), RAGDL (VV: mPC1[RAGDL]) or by RRGEL (VV: mPC1[RRGEL]). We used as control an mPC1 mutant in which the Cys²¹² in the catalytic domain was replaced by a Val²¹² (VV:mPC1[Cys/Val]). For mutagenesis of mPC1, its full-length cDNA was subcloned into the *SalI* and *EcoRI* sites of M13mp19 vector (Bio-Rad.). Single-stranded DNA was produced and annealed with mutant oligonucleotides. *In vitro* synthesis of the mutated double-stranded DNA was performed using T7 polymerase of a mutagenesis kit from Bio-Rad. The primers used for the constructions were: 5'GAATATTCTGCGCGGTGACCTT^{3'} for mPC1[ARGDL], 5'GAATATTCGCGCGCAGGTGACCTT^{3'} for mPC1[RAGDL], 5'CGTAGAGGAGAGCTCCATGTC^{3'} for mPC1[RRGEL] and 5'CATGTACCAGAGTTGCAGGTGA^{3'} for mPC1[Cys/Val]. The sequenced mutant double-stranded cDNAs were then cloned into the *SacII* and *SalI* sites of a modified PMJA602 transfer vector and recombinant VVs were generated by infection/transfection of Ltk⁻ cells, as described [19,23]. The VV recombinant mouse mPOMC (VV:mPOMC) and human α_1 -PDX (VV: α_1 -PDX) were generously given by G. Thomas (Vollum Institute, Portland, OR, U.S.A.).

Metabolic labelling of cells

Briefly, following either 5 h or 17 h VV infections of cells plated on 10 cm² dishes, the cells were pulse-labelled with either 100 μ Ci of [³⁵S]methionine or 500 μ Ci of Na₂³⁵SO₄, and in the brefeldin A (BFA) experiment this was followed by a chase of 90 min. The cell extracts and media were then immunoprecipitated with antisera against either β -endorphin, N-terminal-mPC1 (recog-

nizing the 84–100 segment of mPC1 [19,24]) or C-terminal-mPC1 (recognizing the 629–726 segment of mPC1 [19,24]). In experiments with BFA, the cells were first preincubated for 60 min without or with of 5 μ g/ml BFA (Epicentre) and then pulse-labelled for 10 min with [35 S]methionine and chased for 90 min in the absence or presence of BFA. The immunoprecipitates were separated by SDS/PAGE on either 15 % (β -endorphin immunoprecipitations) or on 8 % acrylamide gels. Quantification of each band was carried out using Digital Imaging System IS-1000 (Alpha Innotech Corp., San Leandro, CA, U.S.A.). All experiments were repeated two to four times.

Immunofluorescence studies

Localization of PC1, 7B2 and PC5-B in PC12 cells, infected with the respective VV recombinants, was determined by indirect immunofluorescence. Typically, cells grown on polylysine (Sigma)-coated chamber slides (Nunc) for 72 h, were infected for 2 h with either VV:mPC1, VV:mPC1-RRGEL, VV:m7B2 [25,26] or VV:mPC5-B [7], at a multiplicity of 10 pfu/cell at 37 °C. At 5 h post-infection, the cells were rinsed once in PBS and fixed in 4 % formaldehyde/0.1 % picric acid in 0.1 M phosphate buffer, pH 7.2, for 1 h at room temperature. The cells were then washed three times with PBS containing 0.01 % Triton X-100 (PBS/T) at 4 °C and subsequently allowed to react overnight with the primary antibodies diluted in 10 % normal goat serum and 0.3 % Triton X-100 in PBS. The N-terminal-PC1 [19], MAP-peptide PC5 [7] and 7B2_{23–39} [25] antisera were used at a dilution of 1:250. After rinsing with PBS/T, cells were incubated for 30 min at 37 °C with rhodamine-labelled goat anti-rabbit IgG diluted 1:15 in 10 % normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.). Samples were examined using a Zeiss microscope with a standard epifluorescence attachment, equipped with a Plan-Neofluor 40 $\times$ /0.75 objective.

RESULTS

Functional importance of the RRGDL sequence

In an effort to define the functional relevance of the conserved RRGDL motif, we prepared VV recombinants of PC1 in which this sequence was replaced by either ARGDL, RAGDL or by RRGEL. Functional analysis of the consequences of these PC1 mutations is shown in Figure 2, where the production of β -LPH from POMC by PC1 and its mutants was analysed in the medium of constitutively secreting BSC40 cells (upper panel) and of regulated GH4C1 cells (lower panel). Positive controls consisted either of the wild-type PC1 (RRGDL) or of a PC1 in which the Cys in the H*GTRC²¹² sequence [24], four residues C-terminal to the His* of the active site, has been replaced by Val. The choice of the Cys/Val mutation is based on the presence of Val in most subtilisins and Cys in kexin-like convertases [16]. In both cell types, the ratio β -LPH/POMC in the two controls is comparable, suggesting that the Cys²¹²-to-Val mutation does not appreciably affect the activity of PC1. In contrast, the P-domain mutations resulted in a net decrease in β -LPH production, especially in BSC40 cells, but did not yield new products, suggesting that these mutations do not alter the cleavage selectivity of PC1. In BSC40 cells, the upper of the two observed β -LPH bands has been previously reported to be due to a late post-translational modification within the β -endorphin segment [21,23,27]. Interestingly, the mutants RRGEL and RAGDL caused the most dramatic decrease in the level of β -LPH generated by PC1. The observed decreased level of POMC processing by the RRGDL mutants of PC1 could either be due to a lower production, an

Figure 2 Activity of mPC1-RRGDL mutants in BSC40 and GH4C1 cells

BSC40 and GH4C1 cells were infected with VV:mPOMC together with either VV:WT, VV:mPC1-RRGDL, VV:mPC1-ARGDL, VV:mPC1-RAGDL, VV:mPC1-RRGEL or with VV:mPC1-Cys/Val, at a multiplicity of infection of 1 pfu/cell of each VV:recombinant. At 15 h post-infection, the cells were pulse-labelled with [35 S]methionine for 2 h. Media were immunoprecipitated with a β -endorphin-specific antibody [23]. The immunoprecipitates were separated by SDS/PAGE (15 % acrylamide with 1.3 % cross-linker) and the dried gel autoradiographed. The migration positions of unprocessed POMC and of β -LPH are indicated by arrows.

increased degradation [19] or to a decreased level of activated enzyme forms [18,19].

Expression of RRGDL-PC1 mutants and their susceptibility to ER degradation

In order to assess the level of early production of mutated proteins, GH4C1 cells were pulse-labelled for 10 min and the cellular extracts were immunoprecipitated with either C-terminally or N-terminally directed antibodies (Figure 3A). Using either antibody, this study revealed that, in all PC1 mutants, the enzyme is produced, albeit at a lower level as compared with the wild-type convertase. In addition, the ER processing of proPC1 (87 kDa) to PC1 (83 kDa) [19,28] is significantly decreased. Accordingly, in all other experiments, although we used both antibodies, we only present the results obtained with the N-terminal antiserum, as it recognizes all PC1 forms [18,19].

The levels of PC1 were also measured in the presence or absence of the fungal metabolite BFA. This agent causes the disassembly of the Golgi complex and fusion of the *cis*-, *medial*- and *trans*-Golgi (but not the TGN) with the ER [29]. Figure 3(B) shows the biosynthesis of PC1 and its mutants following a 10 min pulse-labelling and a 90 min chase period. Under these pulse-chase conditions, most of the wild-type and Cys/Val mutant proPC1 (87 kDa) is converted into PC1 (83/84 kDa). As

Figure 3 Post-translational processing of proPC1 and its mutants

GH4C1 cells were infected with either VV:WT, VV:mPC1-RRGDL, VV:mPC1-ARGDL, VV:mPC1-RAGDL, VV:mPC1-RRGEL or VV:mPC1-Cys/Val. At 15 h post-infection, the cells were either pulse-labelled with [³⁵S]methionine for 10 min (**A**) or labelled for 10 min followed by a chase of 90 min in the presence or absence of brefeldin A ($\pm$ BFA) (**B**). In (**A**), cellular extracts were immunoprecipitated with either a C-terminal or a N-terminal mPC1 antibody at a dilution of 1:125. In (**B**), cellular extracts and media were immunoprecipitated with the N-terminal mPC1 antibody. The immunoprecipitates were separated by SDS/8%-PAGE and the dried gel autoradiographed. The molecular masses of the marker proteins (Amersham) are shown, as well as the positions of the 87 kDa proPC1 and 83/84 kDa and 66 kDa PC1.

previously observed [19], BFA treatment abolishes PC1 secretion and affects the trimming of its glycosyl moieties, resulting in a reduction of its apparent molecular mass by about 2 kDa. However, in the cases of the P-domain mutants, a significant decrease in the level of radiolabelled PC1 is evident, and such a diminution is not affected by the presence of BFA. Thus the P-domain mutants are partially degraded early along the secretory pathway, possibly within the ER itself, and their zymogen processing is seemingly reduced. Although the secretion level of the PC1-RRGDL mutants is very low, it is detectable when films are exposed for longer times (results not shown; also see the medium of RAGDL-PC1). Furthermore, we also observe that the secreted C-terminally truncated 66 kDa form [18,19] can only be detected for the wild-type PC1 and its Cys/Val mutant.

The RRGDL motif and the processing of the prosegment and the C-terminus of PC1

We next evaluated the extent of processing of proPC1 to PC1 and of the 84 kDa PC1 into its 66 kDa form in three cell types. Thus we compared the biosynthetic products obtained in the constitutively secreting BSC40 cells with those in the regulated GH4C1 and PC12 cells. As shown in Figure 4, in all cell types following either 2 h or 3 h pulse-labelling, the RRGDL mutations

Figure 4 Biosynthesis of mPC1-RRGDL mutants in BSC40, GH4C1 and PC12 cells

BSC40, GH4C1 or PC12 cells were infected with either VV:WT, VV:mPC1-RRGDL, VV:mPC1-ARGDL, VV:mPC1-RAGDL, VV:mPC1-RRGEL or with VV:mPC1-Cys/Val, at 3 (BSC40 and PC12 cells) or 6 (GH4C1 cells) pfu/cell. At 5 (BSC40 and PC12 cells) or 15 h (GH4C1 cells) post-infection, cells were pulse-labelled with [³⁵S]methionine for 2 h (BSC40 and PC12 cells) or 3 h (GH4C1 cells). Cellular extracts and media were immunoprecipitated with an N-terminal mPC1 antibody. The immunoprecipitates were separated by SDS/8%-PAGE and the dried gel autoradiographed. The molecular masses of the marker proteins (Amersham) are shown, as well as the positions of the 87 kDa proPC1 and 83/84 kDa and 66 kDa PC1.

significantly decreased the extent of proPC1 (87 kDa) to PC1 (83/84 kDa) conversion, in agreement with the results of Figure 3(A). We estimate that, as compared with controls, the RRGDL mutations caused a decrease in the extent of mature PC1 production, evaluated as the ratio PC1/(proPC1 + PC1), by at least 4-fold in BSC40 cells, and by about 1.4-fold in GH4C1 and PC12 cells.

As previously reported [18,19,30,31], we observed that either wild-type PC1 (84 kDa) or its Cys/Val mutant are further

Figure 5 Analysis of Tyr-sulphated PC1 products

BSC40 and GH4C1 cells were infected with either VV:WT, VV:mPC1-RRGDL, VV:mPC1-ARGDL, VV:mPC1-RAGDL, VV:mPC1-RRGEL or with VV:mPC1-Cys/Val, at a multiplicity of infection of 3 pfu/cell. At 15 h post-infection, cells were pulse-labelled with $\text{Na}_2^{35}\text{SO}_4$ for 2 h. Cellular extracts and media were immunoprecipitated with an N-terminal mPC1 antibody. The immunoprecipitates were separated by SDS/8%-PAGE and the dried gel autoradiographed. The molecular masses of the marker proteins (Amersham) are shown, as well as the position of the 84 kDa Tyr-sulphated PC1. Notice the absence of the 66 kDa form in GH4C1 cells, since sulphation likely occurs at Tyr⁶⁹⁴ of PC1 [19].

processed into a C-terminally truncated 66 kDa form (resulting from cleavage at the AsnAspArgArg↓GlyVal⁵⁹³ sequence [24,30]) in GH4C1 and PC12 cells, which contain secretory granules, but not in BSC40 cells, which lack a regulated secretory pathway. Furthermore, in GH4C1 and PC12 cells, a 71 kDa intermediate (indicated by * in Figure 4) can also be detected, which was shown [30] to result from a cleavage of the 84 kDa PC1 at the GluGlyArgArg↓AspGlu⁶³⁰ sequence [24]. In contrast, for all P-domain mutants, only the 83/84 kDa form of PC1 is the final product in GH4C1 and PC12 cells and media, and no 66 or 71 kDa forms are detectable (Figure 4). Similar data were obtained at either 5 h or 17 h post-infection periods, suggesting that the observed inability of the RRGDL mutants to be further processed to the 71/66 kDa forms in regulated cells is not affected by the level of PC1 production, which is much higher at 17 h than at 5 h post-infection. Therefore, since C-terminal processing into the final 66 kDa form occurs following exit of the 84 kDa PC1 from the TGN, presumably within immature secretory granules [19,30,31], it is possible that the RRGDL mutants do not enter the regulated secretory pathway and that the observed basal secretion of the 84 kDa PC1 mutants occurs either from the constitutive [32] or constitutive-like pathways [33].

Secretion of PC1 and its RRGDL mutants

We have previously reported that PC1 is Tyr-sulphated and that only the 84 kDa form can be labelled with $\text{Na}_2^{35}\text{SO}_4$ [19]. Accordingly, we exploited the fact that sulphation occurs in the TGN [34], in order to define the rate of basal secretion of PC1 and its mutants following their exit from the TGN. As shown in Figure 5 (upper panel), when BSC40 cells overexpressing PC1 or

its mutants were pulse-labelled for 2 h with $\text{Na}_2^{35}\text{SO}_4$, the overall level of sulphated 84 kDa PC1 is diminished in the RRGDL mutants as compared with controls, especially for the RRGEL mutant. This suggests that RRGDL mutations lower the amount of PC1 that reach the TGN in 2 h and which subsequently is secreted. This result predicts that the RRGEL mutant would produce the least amount of active PC1 capable of processing precursors in the TGN. This is indeed what was observed in Figure 2, where the order of decrease of POMC processing into β -LPH follows the observed order of sulphated PC1 production. In contrast, when the same experiment was performed in GH4C1 cells (Figure 5, lower panel), we did not observe an appreciable reduction in overall sulphated-PC1 production. Thus the less prominent effect of the RRGDL mutations on POMC processing in GH4C1 cells (Figure 2) is not due to a decreased level of PC1 reaching the TGN, but could be rationalized by the lack of the more active 66 kDa PC1 [30] in the RRGDL mutants (Figure 4).

The RRGDL mutants of PC1 are mis-sorted to the constitutive secretory pathway

In order to further investigate the trafficking of PC1 and its RRGDL mutants, we first tried to stimulate their release from either GH4C1 or PC12 cells using 50 mM KCl [7]. However, either at 5 h or 17 h after VV infection, we could not clearly demonstrate any stimulation of secretion, even for the wild-type PC1 (results not shown). This may be due to the effects of viral infections on the sensitivity of cells to cellular depolarization by KCl [7]. Therefore, in order to compare the cellular secretory pathway of PC1 and its mutants, we selected the RRGEL-PC1 mutant, which exhibits the most prominent effects (Figures 2–5) and exploited the recently reported capacity of the antitrypsin variant α_1 -PDX [20] to inhibit PCs primarily within the constitutive secretory pathway, without significantly reducing overall processing within granules [21]. Thus the inhibitory potential of α_1 -PDX on wild-type PC1 and its RRGEL mutant was estimated upon their co-expression with POMC in the regulated GH3 cells, which contain more granules than GH4C1 cells, and in PC12 cells. As shown in Figure 6, following 5 h [^{35}S]methionine labelling of GH3 cells, α_1 -PDX was shown to decrease the level of β -LPH produced by wild-type PC1 by about 40% with a concomitant increase in unprocessed POMC. In contrast, this inhibitor completely abolishes the β -LPH production by the RRGEL-PC1 mutant. Identical results were also obtained in PC12 cells (results not shown). This result supports the notion that, unlike wild-type PC1, which exits the cells by both the constitutive and regulated pathways, the RRGEL mutant does not enter the regulated secretory pathway and is therefore susceptible to complete inhibition by α_1 -PDX within the constitutive secretory pathway of GH3 cells. Thus a single point mutation of the RRGDL sequence causes the mis-sorting of PC1 to the constitutive or constitutive-like secretory pathways.

Immunocytochemical analysis of PC1 and its RRGEL mutant in PC12 cells

Immunocytochemistry of PC12 cells expressing either wild-type PC1 or its RRGEL mutant was performed in an attempt to compare their intracellular localization. Thus 5 h post infection of PC12 cells with the recombinant VV:PC1s, we compared by immunofluorescence the expression patterns of PC1 and PC1-RRGEL with that of the secretory-granule marker protein 7B2 [2,25,35], and PC5-B, which does not enter secretory granules and localizes in the ER and TGN [7]. Clearly the PC1 immunoreactivity in PC12 cells overexpressing wild-type PC1 is very

Figure 6 Inhibition of POMC processing by PC1 and its RRGDL mutants by α_1 -PDX

GH3 cells were infected with VV:mPOMC (2 pfu/cell), together with either VV:WT + VV: α_1 -PDX, VV:mPC1-RRGDL + VV:WT, VV:mPC1-RRGDL + VV: α_1 -PDX, VV:mPC1-RRGEL + VV:WT, VV:mPC1-RRGEL + VV: α_1 -PDX or with VV:WT alone. Multiplicities of infection were 2 pfu/cell of PC1 recombinant virus and 3 pfu/cell of VV: α_1 -PDX. At 15 h post-infection, cells were pulse-labelled with [35 S]methionine for 3 h. Cellular extracts and media were immunoprecipitated with a β -endorphin-specific antibody. The immunoprecipitates were analysed as described for Figure 2.

Figure 7 Immunocytochemical analysis of the localization of PC1 and its RRGEL mutant in PC12 cells.

PC12 cells were plated out on to plastic slides and, 2 days later, infected at a multiplicity of infection of 10 pfu/cell with either (A) VV:mPC1-RRGDL, (B) VV:m7B2, (C) VV:mPC1-RRGEL or (D) VV:mPC5-B. At 5 h post-infection, cells were fixed, permeabilized and stained using primary antibodies directed against the N-terminal segment of mPC1, 7B2 [22], or mPC5 [7]. Immunoreaction was revealed using a rhodamine-labelled secondary antibody raised in goat.

similar to that of overexpressed 7B2, including its concentration in paranuclear positions, likely representing the Golgi apparatus [7] (Figures 7A and 7B). In contrast, in addition to a paranuclear immunostaining (Figure 7C), the RRGEL-PC1 exhibits a remarkably dense perinuclear immunostaining when compared with the perinuclear staining of wild-type PC1, arguing in favour of an increased ER localization [7] of the mutant PC1. Furthermore, this labelling is similar to that of PC5-B (Figure 7D). These data agree with the notion that wild-type PC1 is sorted to compartments similar to those containing the granule-associated 7B2, whereas the RRGEL mutant's immunoreactivity resembles that of PC5-B, which was shown to be sorted to the TGN and not into the regulated secretory pathway [7].

DISCUSSION

The aim of the present work was to investigate the functional relevance of the conserved RRGDL pentapeptide (Figure 1) found in the P-domain of six out of the seven known mammalian PCs [3–5,16]. Site-directed mutagenesis was used to modify this motif within the model mPC1 sequence, and the corresponding VV recombinants were thereupon derived. Analysis of the expression and intracellular activity of wild-type PC1 and its RRGDL mutants in both constitutively secreting and in regulated cells allowed us to compare the molecular forms of PC1 as well as the ability of this enzyme or its mutants to process POMC into β -LPH. The results presented in the present paper demonstrated that the RRGDL sequence is implicated in multiple steps of PC1 biosynthesis.

First, RRGDL mutations significantly decreased the level of prosegment processing within the ER (Figures 3 and 4). This may mean that this motif is important for the folding of pro-PC1 and/or its subsequent autocatalytic cleavage [28,36]. Zymogen cleavage of the PCs has been reported to be very sensitive to mutations or deletions within either the prosegment [12,15,37], the catalytic domain [14,37] or the P-domain [11,12,15,37,38]. It is generally proposed that such alterations affect the folding of the zymogen in such a way as to prevent or significantly decrease the rate of its autocatalytic processing within the ER. The consequence of this is usually a retention in the ER and partial degradation in the ER/cytosol, as evidenced by the fact that removal of the prosegment is a prerequisite for the transport, from the ER to the TGN, and activity of kexin [10], furin [8,37], PC1 [9,19], PACE4 [11] and PC5 [7]. In part this may be due to the presence of ER-resident proteins which interact with the prodomain [4]. In this context it is noteworthy that, in Chinese-hamster CHO-K1 cells [38] and in human LoVo cells [39], the endogenous furin is localized in the ER as pro-furin in view of a point mutation in either the catalytic domain in which Cys³⁰³ is replaced by Tyr [38] or in the P-domain where a conserved Trp⁵⁴⁷ is replaced by Arg [39].

Our data revealed that the P-domain RRGDL sequence is important for the efficient zymogen cleavage of pro-PC1 to PC1, with the RRGEL mutation being the most critical one in this process (Figures 3 and 4). Thus it is possible that PC7, which is the only PC containing an RRGSL sequence [3], is unique among the mammalian convertases and that some of the amino acids within its P-domain which are critical for its folding in the ER may be different from those of the other PCs. The mechanistic importance of the RRGDL sequence is nevertheless intriguing. Thus the prosegment and the P-domain, possibly in a collaborative fashion, are needed for correct folding and/or zymogen cleavage. However, the available evidence suggest that the prodomain is dispensable once the enzyme reaches the TGN, whereas no such data are available for the P-domain. In fact, no

active PC form lacking the P-domain or part of it has yet been identified or produced. Constructions deleting parts of the P-domain invariably led to inactive zymogens retained in the ER [9–13,15,36,38]. In the present work the RRGDL mutations, although critical for zymogen cleavage, are only partially deleterious to this process, as some of the proenzyme is able to be processed and reach the TGN, where it can exert its activity towards substrates such as POMC and be secreted (Figures 2–5). However, the introduced mutations did not affect the cleavage selectivity of PC1 in producing β -LPH, suggesting that the RRGDL sequence does not play a role in the catalytic cleavage preference of PC1. This is significant, as we still do not know whether the P-domain (which is not found in the less specific bacterial subtilisins) is actually part of the catalytic domain, possibly required for the strict cleavage specificity of the PCs, or is a *bona fide* independent domain needed for their folding and/or intracellular trafficking. Finally, our data revealed that mutations in the RRGDL sequence led to a significant increase in the ER/cytosol degradation of proPC1/PC1 (Figure 3B). Tunicamycin prevention of N-glycosylation of PC1 and PC2 also resulted in a massive ER/cytosol degradation [19]. Therefore we believe that the RRGDL sequence is important, but not sufficiently critical, for the correct folding of PC1 in the ER. In contrast, for PC2, which is normally associated with its binding protein 7B2 [26,40] and which undergoes zymogen cleavage in the TGN/immature granules [9,19], the RRGEL mutation did not significantly affect its proPC2 to PC2 processing [41].

Secondly, the integrity of the RRGDL sequence of PC1 seems to be less critical for efficient zymogen processing in regulated cells as compared with constitutively secreting cells (Figure 4). This may mean that regulated cells express intracellular binding proteins which are able to compensate for the lack of an integral RRGDL sequence in PC1. Experiments aimed at defining such proteins revealed that secretogranin II, which is enriched in regulated cells [42] and which can act as a 'quality-control' protein preventing the 'artefactual' post-translational modification of β -LPH and nerve growth factor in constitutive cells [27], did not increase the level of active PC1 in the RRGEL mutant (results not shown). The putative binding protein(s) is thus not known, but the integrins represent attractive candidates, especially as it was recently shown, by phage display, that the integrin $\alpha 5 \beta 1$ preferentially recognizes an RRGDL motif as compared with $\alpha v \beta 3$ [43]. Alternatively, the higher ER residence time of proteins in BSC40 cells as compared with GH4C1 cells [21] may also contribute to the more extensive degradation of mutant PC1s in the former cells.

Thirdly, the most striking effect of the RRGDL mutations examined is the complete absence of the C-terminally processed 71 and 66 kDa forms of PC1. Previously these forms were shown to occur by an autocatalytic mechanism operating only in regulated cells in a post-TGN compartment, possibly within immature secretory granules [18,19,28,36,44]. In a similar fashion it was also shown that processing of PC5-A into an equivalent 65 kDa form also takes place in a post-TGN compartment and that mutants which prevent the entry of PC5-A into the regulated pathway abrogated the production of the 65 kDa form [7]. Using the general PC inhibitor α_1 -PDX [20,21,45], we demonstrated that processing of POMC into β -LPH is completely abrogated for the RRGEL mutant, whereas it is only partially inhibitory to the wild-type PC1-activity (Figure 6). Since α_1 -PDX operates primarily within the constitutive secretory pathway [21], we interpret these results to mean that the RRGEL mutant does not enter secretory granules, in accord with its inability to generate the 66 kDa PC1 form. In agreement, our immunocytochemistry data obtained in PC12 cells (Figure 7) suggest that only wild-type

PC1 resembles the secretory-granule-associated 7B2 [35] in its intracellular localization, whereas the RRGEL mutant shows a pattern similar to the TGN-localized PC5-B [7].

It is thus concluded that the integrity of the RRGDL motif is critical for the sorting of PC1 into dense-core secretory granules, possibly through the binding of a receptor-like protein. Co-expression of PCs with a subgroup of integrins, including $\alpha 5 \beta 1$, may shed some light on the possible ability of the latter to interact with the convertases. The critical need of the RRGDL for the autocatalytic conversion of the 84 kDa PC1 into its 66 kDa form suggests that this motif plays a significant role in the regulation of the intracellular specific activity of PC1, as the 66 kDa form is much more active than the longer forms [30,31]. It remains to be seen how this selection is performed following exit of the 84 kDa PC1 from the TGN. Finally, future work should define whether the results presented here for PC1 are applicable to other members of the PC family which undergo zymogen cleavage in the ER, such as furin, PACE4, PC4 and PC5, and whether the integrity of the RRGDL sequence is also critical for the formation of the C-terminally truncated, granule-associated, 65 kDa PC5 [7] and 76 kDa furin [46]. In addition, it will be informative to define whether the variant RRGSL motif found in PC7 also plays a critical role for this convertase [3].

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