Structure-function studies on the biosynthesis and bioactivity of the precursor convertase PC2 and the formation of the PC2/7B2 complex

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Abstract **Site directed mutagenesis of the prohormone convertase PC2 was used to define the effect of certain residues on the zymogen activation of proPC2 and on its binding to the neuroendocrine protein 7B2. These included the oxyanion hole Asp^{309} (D309N), the N-terminal Glu^{25} (E25Q and E25K) of proPC2 and the Asp^{519} (D519E) of the RGD motif within the P-domain of PC2. Heterologous vaccinia virus expression of the wild type and mutant PC2's in endocrine pituitary cells such as AtT20 and GH3 cells demonstrated that the most dramatic effect was observed with the D309N mutant which no longer bound pro7B2 and which exhibited a significant reduction in its capacity to produce β-endorphin from pro-opiomelanocortin (POMC).**

Key words: 7B2; PC2; Mutagenesis; Chaperone; Biosynthesis; Oxyanion hole

1. Introduction

Tissue-specific processing of precursor proteins at single or pairs of basic residues is a general mechanism by which organisms generate a diversity of bioactive entities starting from an inactive pro-protein [1,2]. The six convertases responsible for such specific cleavages have recently been identified and shown to be serine proteinases related to the subtilisin/kexin family and called furin, PC1/3, PC2, PACE4, PC4 and PC5/6 [1–5]. Analysis of the mode of biosynthesis of each convertase revealed that furin [3,6] and PC1 [7–11] are themselves first synthesized as zymogens which undergo autocatalytic loss of their pro-segment within the endoplasmic reticulum (ER) and are then rapidly transported to the *trans* Golgi network (TGN) where they begin to exert their catalytic activities. In contrast to furin and PC1, the zymogen proPC2 slowly exits from the ER and is only processed to PC2 within the TGN [7,8,11–13] and such processing may continue in the secretory granules [11]. Concerning proPC2, it has been suggested that its processing to PC2 occurs either via an intermolecular autocatalytic mechanism [12] or that furin and/or PACE4 may participate in this process [13]. Since the zymogen activation and inter-organelle rate of transport of the convertases represent mechanisms by which the cellular activity of these enzymes can be controlled, it was of interest to identify cellular factors which may participate in such a regulatory step. Recently, it was reported that the neuroendocrine precursor pro7B2 [14,15] can act in vitro as an inhibitor of PC2 [16]. Subsequently, it was shown that intracellularly both pro7B2 [13,17] and 7B2 [13] can bind proPC2 and PC2. Heterologous cellular coexpression of 7B2 with each of the convertases demonstrated that pro7B2/7B2 is a specific PC2-binding protein [13]. In the present work, we exploited the techniques of site directed mutagenesis and vaccinia virus cellular expression to define the functional importance of certain key amino acids within the proPC2 sequence. Accordingly, we examined the effect of these mutations on (i) the rate of intracellular processing of proPC2 to PC2 and the participation of furin and PACE4 in this process, (ii) the ability of PC2 to produce β-endorphin from pro-opiomelanocortin (POMC) and, (iii) the binding of proPC2/PC2 to pro7B2/7B2.

2. Materials and methods

2.1. Mutagenesis and the generation of recombinant vaccinia viruses

Full length mouse PC2 (mPC2) cDNA [18] and m7B2 [19] were transferred to the M13 vector *M13mp19* and the single strand copies were annealed with mutant oligonucleotides, and synthesis of mutated double stranded DNA was performed using a mutagenesis kit from Biorad. The chosen mutants included the N-terminal of proPC2 Glu^{25} to either Gln (E25Q) or Lys (E25K); the oxyanion hole Asp^{309} of PC2 to Asn (D309N); the RGD^{519} sequence to RGE (D519E) and the pro7B2 Lys^{139} to Ala (K139A). The mutant double stranded cDNAs were then cloned into the transfer vector PMJ601 and recombinant vaccinia viruses (VV:) generated as reported [8,20].

2.2. Cellular infections, metabolic labeling and immunoprecipitations

The endocrine cells AtT20 and GH3 were infected with the vaccinia virus recombinants VV:mPC2 and its mutants, VV:m7B2 and its mutant and VV:mPOMC as previously described [7,8]. Following VV infections and 17 h incubations, the cells were pulse labeled with either [^{35}S]Met or $Na_2[^{35}SO_4]$ and in some cases the radioactivity was chased for specified periods of time. Immunoprecipitations under denaturing [7,8] or non-denaturing [13] conditions were essentially used as described. The antibodies used were the C-terminally directed mPC2 fusion protein antibody [7,8], the 7B2 antibody directed against the segment 23–39 [15] and a β-endorphin specific antibody. The immunoprecipitates were separated by SDS-PAGE using gels composed of either [8%T, 2.7%C], [12%T, 1.3%C], or [14%T, 6%C]-tricine and analysis by autoradiography was then performed.

3. Results

3.1. Analysis of the biosynthesis of proPC2 and its mutants in AtT20 cells

In this study we wished to examine the functional importance of certain key amino acids in the control of the zymogen cleavage of proPC2 into PC2 within the TGN. The amino acids chosen for mutation were selected from three distinct regions of the mouse proPC2 molecule including the prosegment Glu^{25}

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to either Gln (E25Q) or Lys (E25K); the catalytic domain oxyanion hole Asp^{309} to Asn (D309N); and within the P-domain RGD^{519} sequence to RGE (D519E). Accordingly, as a model of endocrine cells we chose AtT20 cells which were pulse-labeled for 30 min with $^{35}SO_4$ and then chased for 45 and 120 min (Fig. 1). This radiolabel was chosen, since we previously reported that PC2 is sulfated in the TGN at a specific Tyr residue [8], thus allowing us to assess both the rate of transport from the ER to the TGN and the processing of proPC2. As shown in Fig. 1, the intracellular processing of the wild type proPC2 into the 71 and 68 kDa forms [8] is more than 50% complete at the 30 min pulse period (P30). By contrast, all proPC2 mutants reveal a major proPC2 form at P30. Furthermore, the major processed form of wild type PC2 at each chase period was the 68 kDa form, whereas for the majority of the mutants the 71 kDa form predominates. Since microsequencing of the 68 kDa form of PC2 [7] demonstrated that it represents the form which has lost all the 83 amino acid prosegment [18], it is possible that the 71 kDa form is extended at the N-terminus, as was previously suggested [7,8,12]. Relative to wild type PC2, we note that for all mutants the levels of the secreted 71 and 68 kDa forms are lower and that the 71 kDa form predominates. We also note that different from the wild type enzyme, all PC2-mutants seem to secrete much less PC2 and their intracellular levels dramatically decreased at the chase period of 120 min. This is probably related to the fact that the mutations result primarily in the accumulation of the 71 kDa form which is not well secreted from AtT20 cells and, which may thus be intracellularly degraded with time, by an as yet unknown mechanism.

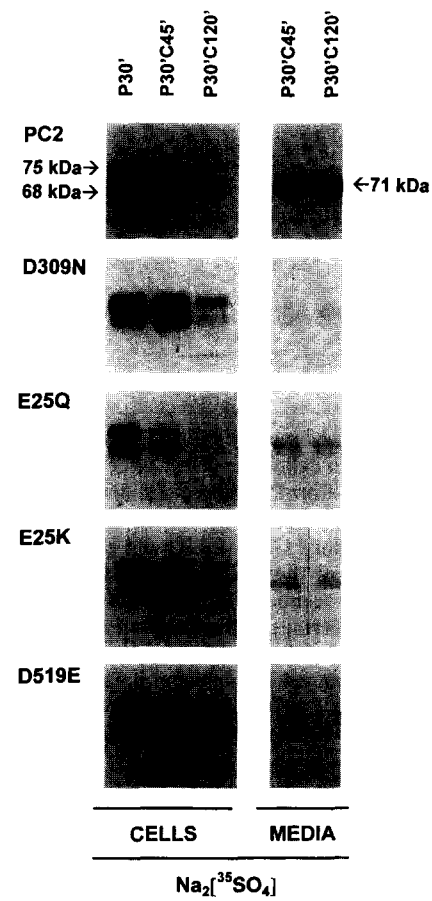

Fig. 1. Autoradiogram of a 8%T (2.7%C) SDS/PAGE slab gel of immunoprecipitated media and extracts of AtT-20 cells infected with either wild type VV:mPC2 or the vaccinia virus recombinants of the PC2-mutants D309N, E25Q, E25K and D519E. The antibody used in this immunoprecipitation is directed against C-terminal 100 amino acid segment of mPC2 [7,8]. The AtT-20 cells were pulsed for 30 min (P30) and chased for 45 min (P30/C45) or 120 min (P30/C120) with $^{35}SO_4$. The positions of pro-PC2 (75 kDa), PC2 (71 kDa) and PC2 (68 kDa) are emphasized.

3.2. *Processing of POMC by PC2 and its mutants in AtT20 and GH3 cells*

In order to assess the functional consequences of the chosen mutations on the intracellular enzymatic activity of PC2, we chose to analyze the known processing of POMC by PC2 into β-endorphin [21] in the regulated AtT20 and GH3 cells which express endogenously PC2 [5,18]. As shown in Fig. 2, relative to wild type PC2, with the exception of the RGD^{519} variant (D519E), all mutants exhibit a significantly reduced capacity to process POMC (AtT20 and GH3 cells) and the intermediate β-LPH (AtT20 cells) into β-endorphin. The production of β-LPH in AtT20 cells is due to their endogenous expression of PC1 [18,21]. Therefore, these data are in general agreement with those deduced from Fig. 1, and together suggest that the mutants D309N, E25Q and E25K produce lower amounts of bioactive PC2. Although the RGD mutation D519E slows down the processing of proPC2 to PC2 (Fig. 1), in the overexpression system used this does not seem to exert a dominant effect on POMC processing by this mutant (Fig. 2).

3.3. *Binding of pro7B2/7B2 with proPC2/PC2 and its mutants in AtT20 cells*

We have recently reported that the 186 amino acid neuroendocrine precursor pro7B2 and its 150 amino acid processed product 7B2 can both specifically bind proPC2 and PC2 [13]. Such binding was reported to be partially dependent on the integrity of the $ArgLysArgArg^{156}\downarrow Leu$ cleavage sequence of pro7B2 into 7B2. The ability of 7B2 to bind PC2, implies the presence of another binding site within the structure of 7B2 [13]. Since it was suggested that 7B2 exhibits some sequence similarity to protease inhibitors and that the scissile bond of the reactive site of the inhibitor proteins aligns with the $Lys^{139}Lys^{140}$ sequence of mouse 7B2 [16], we have also prepared a K139A mutant of pro7B2. The results of our analysis on the ability of the proPC2 mutants and/or the wild type proPC2 to bind 7B2 and its mutant in AtT20 cells pulsed for 2 h with [^{35}S]methionine are shown in Fig. 3. This autoradiogram represents an SDS-PAGE analysis of the 7B2/PC2 complexes immunoprecipitated under nondenaturing conditions with a 7B2 antibody. With the exception of the oxyanion hole mutant of PC2, all other mutants and their resulting proPC2/PC2 products bind equally well to 7B2. With this D309N mutant, 7B2 seems to only bind the 68 kDa form of PC2, which was further shown by microsequencing to start at Gly^{109} (*not shown*) following the major zymogen activation site of proPC2 [7]. We also note that the 7B2 mutation K139A does not affect the binding of proPC2/PC2 to 7B2 suggesting that the significance of the reported alignment to subtilase inhibitors [16] will have to be re-examined.

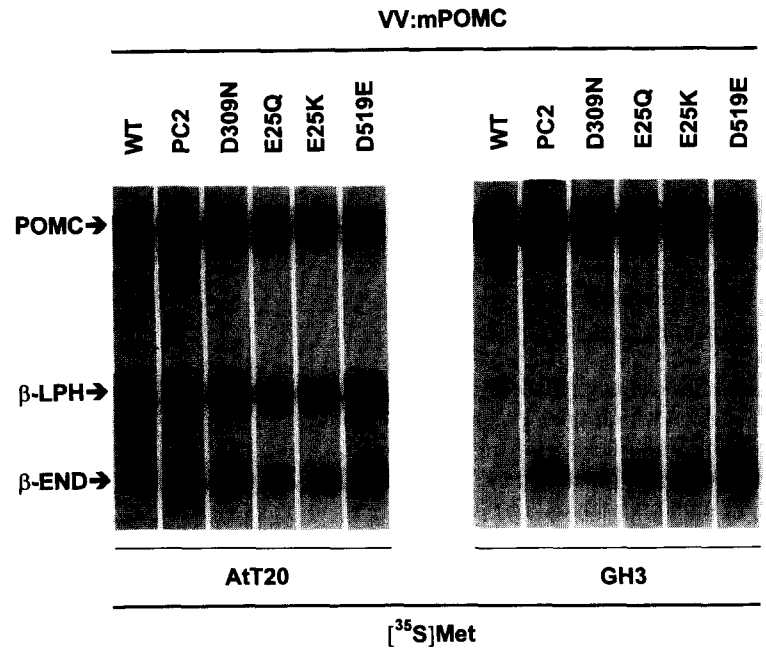

Fig. 2. Autoradiogram of a 14%T (6%C)-tricine SDS/PAGE slab gel of the β-endorphin-immunoprecipitated media of AtT20 and GH3 cells infected with VV:mPOMC together with either wild type vaccinia virus (WT), with wild type VV:mPC2 or its mutants. The cells were pulse-labeled with [^{35}S]methionine for 3 h. The migration positions of POMC, β-LPH and β-endorphin are shown.

3.4. *Processing of proPC2 and its mutants by furin and PACE4 in AtT20 cells*

The analysis of a 3 h pulse with $^{35}SO_4$ of AtT20 cells expressing proPC2 or its mutants in the absence or presence of coexpressed furin, PACE4 or 7B2 is shown in Fig. 4. We first note that furin and to a lesser extent PACE4 increase the level of processing of proPC2 into the 71 kDa form of PC2 both for native and mutants proPC2. We also note that all proPC2 mutants can be partially cleaved by furin, and only for the D519E mutant by PACE4, into a 60 kDa form of PC2 which is not secreted. This suggests that the proPC2 mutants analyzed exhibit conformational changes which expose a cryptic furin-sensitive site. Finally, upon coexpression of pro7B2 with proPC2 or its mutants, we note that the PC2-specific C-terminal antibody used loses much of its capacity to immunoprecipitate proPC2 or the 71 and 68 kDa forms of PC2. This suggests that the binding of 7B2 to PC2 diminishes the accessibility of the C-terminal segment of PC2 to the antibody, even under denaturing conditions. The mutants E25Q, E25K and especially D519E partially restore the ability of the PC2-antibody to immunoprecipitate proPC2 in the presence of 7B2. In addition, in the medium of the PC2 mutant D309N the major PC2 secreted is the 71 kDa form. However, in the presence of coexpressed 7B2, the 68 kDa form is the predominant one, confirming that the presence of 7B2 favors the formation of this form [13] even for the mutant D309N.

4. Discussion

The results presented in this work demonstrate that all the mutations analyzed did not allow an increase in either the rate of zymogen processing of proPC2 into PC2, the transport of proPC2 to the TGN or the ability of this convertase to process POMC into β-endorphin. Interestingly, with the exception of PC2, all subtilisin-like enzymes contain an Asn residue occupying the oxyanion hole formed during catalysis [1–4]. In the case of PC2 this residue is occupied by Asp. Since it was shown that mutagenesis of subtilisin's [22] or kexin's [23] Asn into Asp caused a dramatic decrease in the catalytic efficiency of these enzymes, it was of interest to do the reverse experiment and replace the Asp^{309} of PC2 by Asn^{309}. The results obtained with the D309N mutant clearly demonstrated that this Asp^{309} is very critical for the PC2 activity, as its mutation to Asn lowers the capacity of the mutant PC2 to produce β-endorphin from POMC (Fig. 2). Therefore, the suggestion that this Asp^{309} decreases the catalytic efficiency of PC2 at the expense of increasing its cleavage selectivity [23] does not seem to be true, as this D309N mutant is first of all less effective in producing β-endorphin and, it did not produce other products such as β-lipotropin which is generated by the Asn-containing furin, PC1 and PACE4. Furthermore, this mutation virtually eliminated the capacity of proPC2 to bind 7B2 (Fig. 3) or pro7B2 (not shown). These data suggest that Asp^{309} is important for the binding of pro7B2 to proPC2 which starts in the ER [13].

Our results further defined the critical importance of the Glu^{25} residue at the N-terminus of pro-PC2, as its mutation to either Lys or Gln (residues found at the N-terminus of either proPC1 or profurin, respectively) significantly lowered the ability of proPC2 to be processed to the 68 kDa PC2, and favored the production of the 71 kDa form. These results suggest that this Glu^{25} residue is critical for the choice of the zymogen cleavage site within the pro-segment of proPC2. Such a cleavage has been reported to be an intermolecular reaction occurring autocatalytically [12] or may require the participation of furin and/or PACE4 [13]. The data in Fig. 4, further suggest that furin enhances the production of the 71 kDa but not the 68 kDa form, possibly suggesting that the cleavage producing the 71 kDa is mediated by a furin-like enzyme whereas that

resulting in the 68 kDa form may be autocatalytic [12,13]. In this context, it is worth noting that a very recent report demonstrated that the fusion of the 10 amino acid pro-peptide of the amidation enzyme PAM to the N-terminal Glu^{25} of PC2 increased the exit rate of proPC2 from the ER [24]. From these and our results, it is possible that this reported effect is either due to the properties of the basic peptide sequence PheArgSerProLeuSerValPheLysArg- of proPAM [24], or that this pro-sequence masked the N-terminal α-NH_2 group of Glu^{25}. Since both Glu^{25} mutants (Gln and Lys) rather showed a decreased rate of transport to the TGN, it is also possible that the negative charge of the side chain of Glu^{25} is important for the correct folding of proPC2 within the ER.

Since all the known mammalian convertases exhibit the presence of a conserved **RRGDL** sequence within their P-domain [1,4] it was of interest to investigate the function of this sequence in PC2 by site directed mutagenesis. The data presented in this work revealed that of all the mutants analyzed, the RGD mutant D509E demonstrated the least effect on either the zymogen processing of proPC2 (Fig. 1) or the cleavage of POMC into β-endorphin (Fig. 2). However, this mutation increased the ability of PACE4 to cleave proPC2 into a 60 kDa form, possibly via exposure of a cryptic PACE4-site (Fig. 4). It remains to be established what (if any) is the function of this conserved RGD sequence and whether in PC2 and/or in the other convertases it may be important for the folding of the zymogen possibly via an interaction with integrins within the secretory pathway.

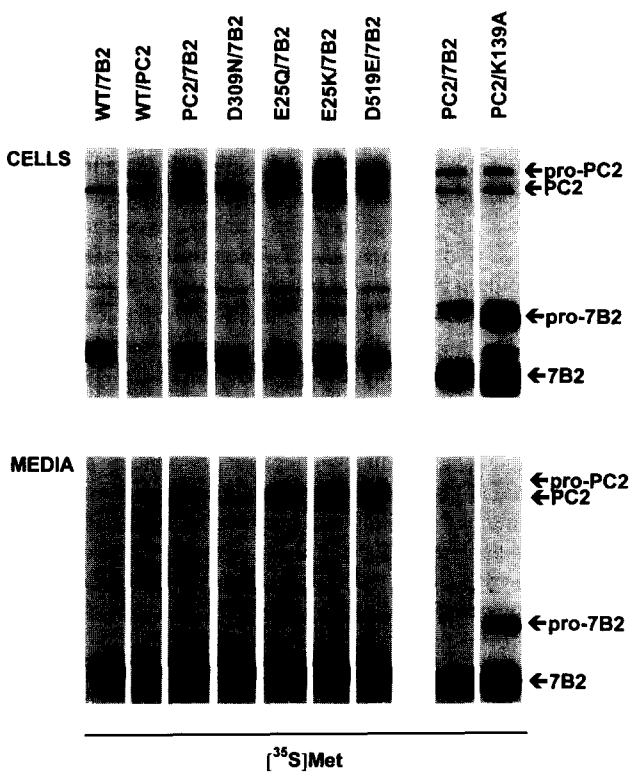

Fig. 3. Autoradiogram of a 12%T (1.3%C) SDS/PAGE slab gel of 7B2-immunoprecipitated (under non-denaturing conditions) media and extracts of AtT-20 cells infected with VV:7B2 and either VV:PC2 or its mutants. The two right lanes describe a similar experiment in which AtT20 cells were coinfected with wild type VV:PC2 and either wild type VV:7B2 or its mutant. The antibody used in these immunoprecipitations is directed against the peptide 23–39 of mouse 7B2 [15]. The AtT20 cells were either pulsed for 2 h with [^{35}S]Met or pulsed for 30 min and chased for 45 min for the K139A mutant. The positions of pro-PC2 (75 kDa), PC2 (68 kDa), pro7B2 (30 kDa) and 7B2 (23 kDa) are emphasized.

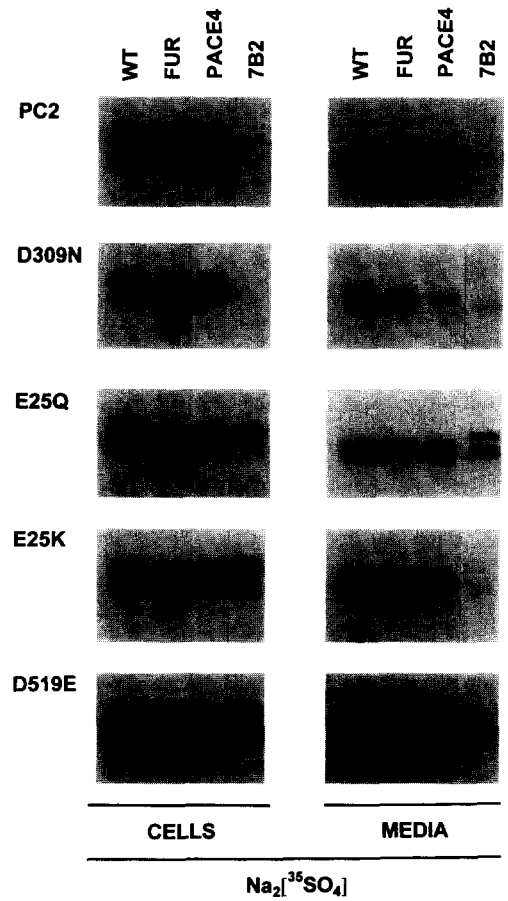

Fig. 4. Autoradiogram of an 8%T (2.7%C) SDS/PAGE slab gel of the PC2-immunoprecipitated media and cell extracts of AtT20 cells infected with either [VV:PC2 + VV:WT], [VV:PC2 + VV:furin], [VV:PC2 + VV:PACE4] or [VV:PC2 + VV:7B2]. Equivalent infections of the PC2-mutants were also performed. The cells were pulse-labeled with $^{35}SO_4$ for 3 h.

In conclusion, the work presented here clearly showed that within the highly conserved primary structure of PC2, single amino acid substitutions can have dramatic effects on the zymogen activation of proPC2, its enzymatic activity and, its ability to interact with its specific binding protein 7B2. Future studies should unravel in more details the structure-function of proPC2/PC2 as well as the sequences involved in its recognition of pro7B2/7B2.

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