Residues unique to the pro-hormone convertase PC2 modulate its autoactivation, binding to 7B2 and enzymatic activity

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Abstract The prohormone convertase PC2 is one of the major subtilisin/kexin-like enzymes responsible for the formation of small bioactive peptides in neural and endocrine cells. This convertase is unique among the members of the subtilisin/kexin-like mammalian serine proteinase family in that it undergoes zymogen processing of its inactive precursor proPC2 late along the secretory pathway and requires the help of a PC2-specific binding protein known as 7B2. We hypothesized that some of these unique properties of PC2 are dictated by the presence of PC2-specific amino acids, which in the six other known mammalian convertases are otherwise conserved but distinct. Accordingly, six sites were identified within the catalytic segment of PC2. Herein we report on the site-directed mutagenesis of Tyr_{194} and of the oxyanion hole Asp_{309} and the consequences of such mutations on the cellular expression and enzyme activity of PC2. The data show that the Y194D mutation markedly increases the ex vivo ability of PC2 to process proopiomelanocortin (POMC) into β-endorphin in cells devoid of 7B2, e.g. BSC40 cells. In these cells, expression of native PC2 does not result in the secretion of measurable in vitro activity against a pentapeptide fluorogenic substrate. In contrast, secreted Y194D-PC2 exhibited significant enzymatic activity, even in the absence of 7B2. Based on co-immunoprecipitations and Western blots, binding assays indicate that Tyr_{194} participates in the interaction of PC2 with 7B2, and that the oxyanion hole Asp_{309} is critical for the binding of proPC2 with pro7B2.

Key words: Precursor convertase 2; 7B2; Prohormone; Convertase; Pair of basic residues; Proopiomelanocortin; Processing

1. Introduction

Biologically inactive protein precursors often undergo limited proteolysis to generate one or more bioactive polypeptide(s). Processing usually occurs at specific single or paired basic residues [1] and is accomplished by one or more of the seven known members of the family of serine proteinases of the subtilisin/kexin type [2–4]. Phylogenetic structural analyses of the catalytic domain of the prohormone convertases (PCs) revealed that PC2 is the most conserved member of the family [4]. All PCs are first synthesized as zymogens (proPCs) which undergo autocatalytic processing of their prosegment within the endoplasmic reticulum (ER), with the exception of proPC2, which is processed to PC2 within the *trans* Golgi network (TGN)/immature secretory granule (ISG) compartments [2,5–7]. Of all the PCs, only PC2 requires a specific binding protein, known as 7B2 [2,8], and such binding improves the efficiency of zymogen activation of proPC2 to PC2 [2,9–12]. The PC2-specific binding protein 7B2 is itself first synthesized as a 186 amino acid (aa) precursor $pro7B2^{186}$ [13], which is cleaved within the TGN by a furin-like enzyme into the 150-aa $7B2^{150}$ [14]. It has been proposed that at least two PC2-binding sites exist in $pro7B2^{186}$. The first one resides in the C-terminal (CT) 31-aa peptide, which is also a potent PC2-specific inhibitor containing a critical Lys-Lys sequence [15,16], whereas the second one [10] may encompass a polyproline-containing segment (aa 88–95) [17]. The oxyanion hole Asp_{309}* [18], which is an Asn* in all other PCs as well as in subtilases [1–4], has been reported to be important in the initial binding of proPC2 to $pro7B2^{186}$ within the ER [11]. However, the other PC2 residues involved in the binding of 7B2 are not known.

Our goal is to delineate in PC2 the critical amino acids which play a key role in its zymogen activation and in its concomitant binding to pro7B2/7B2. Thus, we hypothesized that some of the special properties of PC2 are due to the presence of specific residues within its catalytic segment (aa 109–455) [18] which are unique to this convertase, and which are otherwise different but conserved in all other PCs. Accordingly, alignment of the catalytic segments of all the mammalian PCs, revealed 9 positions where the above criteria were satisfied (not shown). These include $\mathbf{Ser}_{189}$ in PC2 [18] which is an Asn in all other PCs (**Ser**189Asn), **Tyr**194Asp, **Ala**219Asn, **Gln**242Gly, **Asn**273Asp, the oxyanion hole **Asp**309Asn, **Met**325Ile, **Ser**380Thr, and **Val**445Leu. Since the last three replacements are conservative, only the first six substitutions were considered. In this report, we isolated vaccinia virus recombinants (VV:) of mouse PC2-mutants generated by site-directed mutagenesis of Tyr_{194} and Asp_{309}. Following cellular expression of VV:recombinants, in the presence or absence of VV:7B2 or VV:POMC, we analysed the consequential effects of the selected PC2 mutants on proPC2 processing to PC2, their binding to $pro7B2^{186}$/$7B2^{150}$, their ability to generate β-endorphin from its precursor, POMC, and their in vitro activity.

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Abbreviations: VV, vaccinia virus; PC, precursor convertase; POMC, proopiomelanocortin; ER, endoplasmic reticulum; TGN, *trans* Golgi network; ISG, immature secretory granules; aa, amino acid

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2. Materials and methods

2.1. Mutagenesis and the generation of recombinant vaccinia viruses

Mutagenesis of Tyr_{194} of mPC2 [18] into either Tyr194Asp, Tyr194-Leu or Tyr194Ala was performed by an asymmetric polymerase chain reaction (PCR) technique, which relies on two PCR reactions using a sense wild type and one of three overlapping antisense mutant oligonucleotides [19]: sense 5′-GACCCCTACCCATACCCTCGATA-3′; and antisense 5′-TAT*CTCGGG*TATGG(GTC; GAG; GGC)GGGGTC-3′ for Tyr_{194} to Asp, Leu or Ala mutations, respectively. Recombinant vaccinia viruses (VV:) were then generated. The VV:PC2-D309N [5,11], VV:pro7B2^{186} (consisting of aa 1–186) [13,14] and VV:7B2^{150} (consisting of aa 1–150) [13,22] were previously reported.

2.2. Cellular infections, metabolic labeling and immunoprecipitations

The constitutively secreting green monkey kidney BSC40 cells and the endocrine pituitary somatolactotroph GH4C1 cells were infected with the vaccinia virus recombinants VV:mPC2 or its mutants, VV:m7B2, or VV:mPOMC (provided by G. Thomas, Vollum Institute, Portland, OR, USA) as described [5,10,11]. Following VV infections (2 pfu/cell, for each recombinant) and 17-h incubations, the cells were pulse-labeled with either [^{35}S]methionine or $Na_2[^{35}SO_4]$. Immunoprecipitations under denaturing [5] or non-denaturing [10,11] conditions were then performed on media and cell extracts. The antibodies used were a C-terminally directed mPC2 fusion protein antibody [5], a 7B2 antibody directed against the segment 23–39 [8] and a β-endorphin-recognizing antibody [20]. The immunoprecipitates were separated by SDS-PAGE followed by autoradiography. For β-endorphin immunoreactivity, gel lanes were sliced every 1 mm and eluted in radioimmunoassay (RIA) buffer where analysis was performed [20].

2.3. In vitro activity of PC2

Enzymatic activity was assessed using the fluorogenic synthetic peptide substrate pERTKR-MCA [21] as follows: the enzyme preparation (usually 5 μl) was added to the assay reaction mixture consisting of 50 mM sodium acetate (pH 5) and 5 mM $CaCl_2$. The reaction (in a total volume of 100 μl) was initiated at room temperature by the addition of pERTKR-MCA to a final concentration of 0.1 mM. Fluorescence was monitored every 30 min over 4 h, as described [21].

3. Results

3.1. Zymogen processing of PC2 and its Tyr_{194} and Asp_{309} mutants

In this work, we generated vaccinia virus recombinants of the mutants Y194D, Y194L, and Y194A, as well as D309N. The first two mutants represent the substitutions of Tyr_{194} observed in other PCs and in kexin, respectively, whereas the Y194A mutant represents a neutral amino acid substitution. Analyses of the cellular expression of PC2 and its mutants were done in constitutively secreting BSC40 cells and in the regulated GH4C1 cell line (Fig. 1). Following a 30-min pulse labeling with [^{35}S]methionine and a chase of 180 min in the absence of label, we note the intracellular presence of both proPC2 and PC2 in both cell lines, although as previously reported for native PC2 [5], proPC2 predominates over PC2, especially in BSC40 cells. We also document that Y194D-proPC2 and Y194D-PC2 migrate slightly slower than the native enzyme. Furthermore, we observe the presence of a PC2 doublet in GH4C1 cells, which may represent either heterogeneous glycosylation or alternative processing at the secondary cleavage site within the pro-segment [5]. However, even though in all cases the intracellular levels of proPC2 and/or PC2 are similar, in the medium the level of accumulation of either proPC2 (BSC40 cells) or PC2 (GH4C1 cells) is by far highest for the Y194D mutant (see overexposed BSC40 media). The much higher level of secreted [^{35}S]methionine-labeled proPC2 or PC2 from both cell types could be due either to a higher rate of exit of Y194D-proPC2 from the ER, which is known to be limiting [5,23,24] and/or to its preferred exit from the TGN and secretion from the cell.

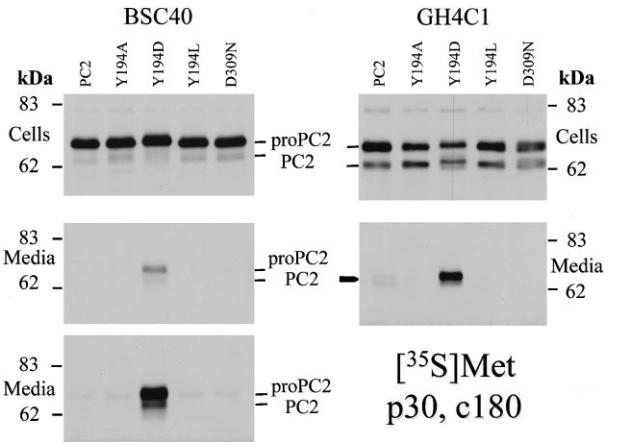

Fig. 1. Biosynthesis of PC2 and its Tyr_{194} and Asp_{309} mutants. (Left) BSC40 or (Right) GH4C1 cells were infected with VV:recombinants of either native PC2, or its mutants Y194A, Y194D, Y194L, D309N at a multiplicity of 2 plaque forming units/cell (pfu/cell). Following overnight culture, the cells were washed and then pulse-labeled for 30 min (p30) with [^{35}S]methionine, and then chased for 180 min (c180) in absence of radiolabel, as described [5]. Cell extracts and media were subjected to immunoprecipitation with a C-terminal PC2 antibody [5], analysed by SDS-PAGE (8% T, 2.7% C), and fluorographed as described [5]. At the bottom, a BSC40 media autoradiogram obtained after 4 days of exposure (as opposed to 1 day) is also presented, in order to emphasize the low level of secretion of proPC2 from either native PC2, or its Y194A, Y194L and D309N mutants. The migration positions of proPC2 and PC2 are indicated. In the media of GH4C1 cells, native PC2 is secreted both as a 71-kDa partially processed PC2 and a 68-kDa fully processed PC2 [5]. Notice that the Y194D-PC2 and Y194D-proPC2 migrate slightly slower than the other isoforms in both cell extracts and media. This may be due to a structural and/or negative charge difference caused by the mutation.

In order to differentiate between the above two alternatives, BSC40 cells expressing PC2 and its mutants were labeled with $Na_2[^{35}SO_4]$ for 90 min (Fig. 2A), and, in order to compare the rate of proPC2 to PC2 processing of the native vs. that of the Y194D mutant, these cells were also pulse-labeled for 15, 45, and 90 min (Fig. 2B). This choice of pulse times and type of label was based on the documented slow exit of proPC2 from the ER [5,23,24], and on the knowledge that protein sulfation occurs in the TGN [25]. In BSC40 (Fig. 2A,B) and GH4C1 (not shown) cells, we noted the presence of sulfate-labeled PC2 and/or proPC2 in all cases, demonstrating that PC2 as well as its mutant isoforms reach the TGN. Since the levels of secreted sulfated proPC2 and/or PC2 are very low (even after 17 days of exposure), in order to see all of the bands we had to overexpose the film, using an HP ScanJet 6100C Scanner and the Adobe Photoshop 4.0.1 program. The data (Fig. 2A; Media) revealed that only the native, Y194D, and D309N $^{35}SO_4$-labeled enzymes significantly accumulate PC2 in the medium, whereas the Y194L and Y194A mutants mostly secrete proPC2 from BSC40 cells. It is also evident that at all pulse times proPC2 to PC2 conversion is more effective for the Y194D mutant than for native PC2 (Fig. 2B). Remarkably, as estimated by densitometric scanning of films, the percent total radioactivity of sulfated proPC2 and/or PC2 se-

creted into the medium is about equal (10–12% at p90) for both Y194D-PC2 and native PC2. We therefore suggest that the major reason for the comparatively much higher accumulation of proPC2 and/or PC2 in the medium of cells expressing the Y194D-PC2 mutant (Fig. 2) is not due to a differential exit rate from the TGN. Unfortunately, in view of the limited sensitivity of proPC2 to endoglycosidase H treatment [5], we could not use this type of digestion to compare the rate of exit of proPC2 and its mutant from the ER. Thus, we can only speculate that the Y194D mutant may fold more efficiently in the ER than native proPC2 [26].

Interestingly, although the percent of total of $^{35}SO_4$-labeled proPC2 and/or PC2 secreted into the medium of the Y194D mutant is about equal to that of native PC2 (i.e. 11 ± 1%; Fig. 2A), it is at least 12-fold higher in the case of [^{35}S]methionine-labeled proPC2 and/or PC2 (Fig. 1). This may be due to the fact that the cellular sulfotransferase(s) is present in limiting amounts, and that vaccinia virus infections significantly reduce the overall levels of endogenous proteins [5]. Indeed, analysis of total sulfate labeled proPC2 and PC2 at 5 h vs. 17 h post infection revealed that the ratio of [$^{35}SO_4$]/[^{35}S]methionine is higher at 5 h post infection (not shown).

In order to further test the fate of PC2 and its mutants we analysed by Western blots the molecular forms of PC2 obtained from BSC40 and GH4C1 cells infected with the corresponding vaccinia virus recombinants. Intracellularly, the levels of proPC2 and/or PC2 were about the same for all infections (not shown). Moreover, only the native-, Y194D- and D309N-PC2 exhibited the presence of significant amounts of PC2 in the media of either cell type, with notably higher levels in the case of the Y194D-PC2. These data confirmed that the differences observed in our previous biosynthetic experiments (Figs. 1 and 2) are not due to variable infection efficiencies, but rather reflect the intrinsic properties of each enzyme isoform.

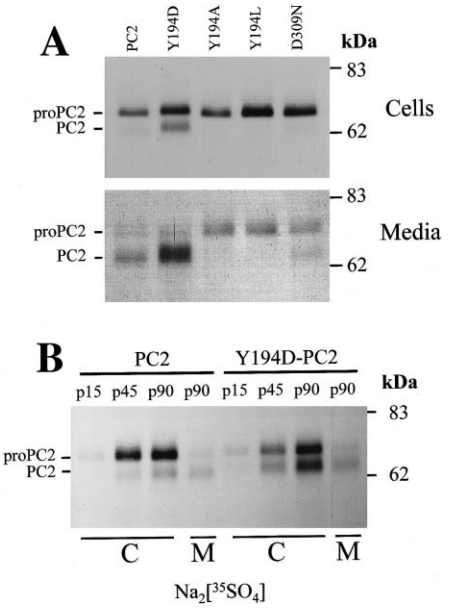

Fig. 2. Zymogen processing of PC2 and its Tyr_{194} and Asp_{309} mutants. BSC40 cells were infected with VV:recombinants of either native PC2, or its mutants Y194A, Y194D, Y194L, and D309N at a multiplicity of 2 pfu/cell. Following overnight culture, the cells were washed and then pulse-labeled with $Na_2[^{35}SO_4]$ for either (A) 90 min, or (B) 15 (p15), 45 (p45) or 90 (p90) min. Cell extracts (C) and media (M) were subjected to immunoprecipitation with a C-terminal PC2 antibody [5] and analysed by SDS-PAGE as in Fig. 2.

3.2. *Comparative ex vivo and in vitro bioactivity of PC2 and its Tyr_{194} and Asp_{309} mutants*

The composite picture deduced from the above data (Figs. 1 and 2) suggests that the level of proPC2 to PC2 conversion is highest for the Y194D mutant, followed by the native enzyme and then by the D309N mutant. Both the Y194A and

Table 1
POMC processing by native PC2 and its Tyr_{194} and Asp_{309} mutants in BSC40 and GH4C1 cells

BSC40 cells						
Enzyme		Cells		Media		% of total β-end-sized product
		POMC	β-End	POMC	β-End	
POMC	Wt	1076	9	134	0	0.1%
	PC2	1279	12	368	42	3.1%
	Y194D	1637	250	236	1243	44.3%
	Y194L	1158	12	256	0	0.1%
	Y194A	745	20	160	0	0.1%
	D309N	1123	10	357	0	0.1%
GH4C1 cells						
Enzyme		Cells		Media		% of total β-end-sized product
		POMC/LPH	β-End	POMC/LPH	β-End	
POMC	Wt	5903	96	2718	1	1.1%
	PC2	5702	646	1596	786	16.4%
	Y194D	5016	659	1658	1186	21.7%
	Y194L	3798	79	1198	115	3.7%
	Y194A	2745	91	1026	3	2.3%
	D309N	3334	310	1218	1227	25.2%

β-Endorphin RIAs were performed on SDS-PAGE-purified fractions. The total immunoreactivity of [POMC] (2×10^6 BSC40 cells) or [POMC+β-LPH] (2×10^6 GH4C1 cells)-related fractions was compared to that of β-endorphin-sized products. The numbers represent pg of immunoreactive β-endorphin/pooled fraction estimated from duplicate experiments. The % of total β-endorphin-sized products is obtained by the sum of the β-endorphin-sized immunoreactivity (cells+medium) divided by the total immunoreactivity. In BSC40 cells, we only observed POMC and β-endorphin, whereas in GH4C1 cells β-LPH is additionally present due to the endogenous presence of PACE4 [2]. Co-expression of POMC with the wild type (Wt) vaccinia virus is presented as control.

Y194L mutants exhibit drastically reduced levels of PC2. In order to test the intracellular enzyme activity of each PC2 isozyme, we co-expressed the substrate proopiomelanocortin (POMC) with PC2 or its mutants in either BSC40 or GH4C1 cells, neither of which endogenously express PC1 or PC2 [2]. Cell extracts and media obtained from 6 h post overnight infections were first fractionated on SDS-PAGE gels. Each lane was then sliced into 1-mm sections and the eluted polypeptides analysed using a β-endorphin-specific RIA (Table 1). Clearly, in BSC40 cells only the Y194D mutant and, to a lesser extent, native PC2 exhibit significant β-endorphin converting activity above background in this constitutively secreting cell line. In contrast, in the regulated GH4C1 cells, which endogenously express large amounts of 7B2 [2], the D309N, Y194D and native PC2 show similar activities. However, as in BSC40 cells, the Y194L and Y194A seem inactive. These data suggest that the intracellular environment of GH4C1 cells is favorable for the expression of active D309N, Y194D and native PC2, whereas in BSC40 cells only the Y194D isoform is significantly active.

In order to test whether 7B2 is one of the major factors contributing to the productive activation of PC2 and its mutants, we next compared the processing of POMC by the native PC2 and Y194D-PC2 in the presence or absence of 7B2 in BSC40 cells (Table 2). The data demonstrate that co-expression of PC2 or its Y194D mutant with $7B2^{150}$, lacking the C-terminal inhibitory CT-peptide [15], increase the level of β-endorphin produced by about 8- and 2-fold, respectively. Thus, our data suggest that only the Y194D mutant can exhibit significant activity in the absence of 7B2, and that 7B2 increases the level of enzymatically active native- and Y194D-PC2. Co-expression of $7B2^{150}$ and POMC with either Y194L- or Y194A-PC2 did not result in significant β-endorphin converting activity (not shown).

We also analysed the in vitro enzymatic activity of secreted PC2 isozymes, using the fluorogenic pentapeptide (pyroE)RTKR-MCA. Of the media obtained from native PC2- or mutant PC2-expressing BSC40 cells, only that of Y194D-PC2 exhibited detectable activity (0.2 pmol/min$\times 10^6$ cells). In contrast, in the presence of co-expressed $7B2^{150}$ (as well as $pro7B2^{186}$), both native- (5.5 pmol/min$\times 10^6$ cells) and Y194D-PC2 (2.5 pmol/min$\times 10^6$ cells) were able to cleave the pentapeptide substrate. However, and in agreement with the data of Figs. 1 and 2, triplicate Western blot analyses demonstrated that the level of 68-kDa PC2 was much higher in the case of the Y194D mutant as compared to the native enzyme (not shown). Accordingly, in the presence of co-expressed 7B2, the calculated specific activities demonstrated a 6-fold higher value for native PC2 (2.5 pmol/min/integrated level of 68 kDa PC2$\times 10^6$ cells) as compared to Y194D-PC2 (0.4 pmol/min/integrated level of 68 kDa PC2$\times 10^6$ cells). When $7B2^{150}$ was added exogenously to the medium of native PC2 expressing cells, we still did not detect any activity (not shown). In contrast, addition of $7B2^{150}$ to the medium of Y194D-PC2 resulted in a substantial reduction in the loss of enzymatic activity observed over time (not shown). This suggests that, as reported for native PC2 [26], 7B2 can stabilize the Y194D-PC2 activity and protect it from inactivation at pH 5.

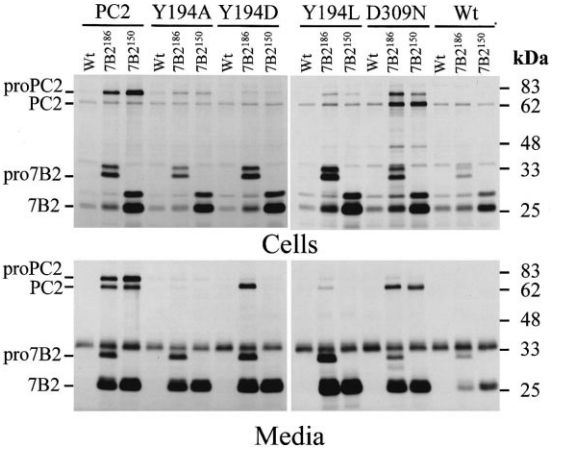

Fig. 3. Binding of 7B2 to PC2 and its Tyr_{194} mutants. Autoradiograms of SDS-PAGE slab gels (12% T; 1.3% C) of immunoprecipitated media and extracts of BSC40 cells infected with VV:$pro7B2^{186}$ or VV:$7B2^{150}$ and VV:PC2, its Asp_{309} or Tyr_{194} mutants (each at 1 pfu/cell), obtained following a pulse of 2 h with [^{35}S]methionine. Immunoprecipitation reactions were done under non-denaturing conditions using a $7B2_{23-39}$ antibody [8]. The positions of proPC2, PC2, pro7B2 and 7B2 are emphasized, as well as those of the molecular weight markers. Controls consisted of cells individually infected with either native VV:PC2, $7B2^{150}$, or $pro7B2^{186}$. In the media, most of the protein migrating just above pro7B2 seems to be non-specific since we can also observe this band in the absence of 7B2 (Wt).

3.3. Binding of 7B2 to PC2 and its Tyr_{194} mutants

We previously reported that the D309N mutation decreases the ability of proPC2 to bind pro7B2 within the ER, but still allows binding of 7B2 and PC2 within the TGN/ISG [10]. Accordingly, we co-expressed in BSC40 cells either full length 7B2 ($pro7B2^{186}$) or the N-terminal 150-aa 7B2 ($7B2^{150}$) lacking the CT-peptide [15,22], together with either native PC2, its Tyr_{194} or its Asn_{309} mutants. Analyses of the interactions of pro7B2/7B2 with proPC2 and/or PC2 were performed on SDS-PAGE-fractionated immunoprecipitated [^{35}S]methionine-labeled 7B2-containing complexes under non-denaturing conditions (Fig. 3). These data confirmed

Table 2
POMC processing by native PC2 and its Y194D mutant in BSC40 cells in the absence or presence of $7B2^{150}$

BSC40 cells						
Enzyme		Cells		Media		% of total β-end-sized product
		POMC	β-End	POMC	β-End	
POMC	PC2/Wt	305	0	178	51	9.5%
	PC2/$7B2^{150}$	735	172	155	2144	72.2%
	Y194D/Wt	817	46	124	612	41.1%
	Y194D/$7B2^{150}$	531	248	103	2195	79.4%

Similar to Table 1, the values represent pg of immunoreactive β-endorphin/pooled fraction (estimated from duplicate experiments) obtained from the cells and media of BSC40 cells expressing either native- or Y194D-PC2 together with $7B2^{150}$ and POMC or Wt and POMC (control).

that pro7B2^{186} and 7B2^{150} (doublets in BSC40 cells; [10,11]) co-immunoprecipitate with native PC2 and proPC2. Furthermore, in the medium Y194D-PC2 selectively binds pro7B2^{186} and does not bind to 7B2^{150}. In addition, the Y194L- and Y194A-proPC2 and/or PC2 only weakly bind pro7B2^{186} and much less so 7B2^{150} (see medium of Fig. 3). This strongly suggests that Tyr_{194} is critical for the effective interaction of PC2 with 7B2^{150}. The data also reveal that whereas pro7B2^{186} and 7B2^{150} interact preferentially with the native pro-enzyme, they prefer binding to the mature enzyme in the D309N mutant, even though proPC2 predominates in the cells and media (Fig. 1). Thus, the oxyanion hole Asp_{309} seems to enhance the interaction of proPC2 with pro7B2^{186}. Finally the above results were independently confirmed by Western blot analysis using a PC2-specific antiserum (not shown). Thus, the data in Fig. 3 suggest that Tyr_{194} participates in the interaction of mature PC2 with 7B2^{150}, and that Asp_{309} improves the binding of proPC2 to pro7B2^{186}.

4. Discussion

Characterization of the repertoire of dibasic processing endoproteases within endocrine and neural cells revealed that PC1 and PC2 are the major enzymes responsible for the processing of most polypeptide hormone precursors into bioactive peptides [1–4]. Biosynthetic analyses demonstrated that except for PC2, all other PCs tested undergo zymogen processing within the ER. Indeed, autocatalytic proPC2 to PC2 conversion is a late event which starts in the TGN and continues in the ISG (reviewed in [2–4]). The observation that PC2 is weakly active in constitutively secreting cells, but is quite potent in regulated cells, led to the identification of an intracellular PC2-specific binding protein, 7B2 [2,10–12,15–17]. Thus, PC2 exhibits at least two regulatory mechanisms. The first implicates its pro-segment which acts as an intramolecular chaperone guiding the folding of the enzyme which, when cleaved, acts as an intermolecular inhibitor. The second involves the participation of pro7B2/7B2 which regulate the intracellular location and time at which PC2 becomes optimally active. In contrast, the activity of PC1 is regulated by its inhibitory pro-segment and C-terminal sequence [27].

In this work, we pursued our systematic investigation of the critical residues involved in the regulation of the enzymatic activity of PC2 and of its binding to 7B2 [10,11]. We hypothesized that PC2 must contain unique residues which are responsible for its special properties. Of the six candidate amino acids within the catalytic domain, the oxyanion hole Asp_{309} and Tyr_{194} caught our attention. The former had previously been reported to affect the pH optimum of the proPC2 to PC2 processing [28]. In addition, some of our previous data suggested that the D309N mutation markedly diminished the interaction of pro7B2 with proPC2 in the ER [10,11]. Our present study further shows that the D309N mutation dramatically decreases the ability of PC2 to process POMC into β-endorphin in cells devoid of 7B2 (Table 1). However, in GH4C1 cells which endogenously express 7B2, or in BSC40 cells co-infected with VV:7B2^{150}, the D309N-mutant is active and still exhibits a pH optimum of 5.0 (not shown). Since the pro-segment of PC2 is expected to be a very potent inhibitor of this enzyme, as reported for subtilases [1,3], it likely interacts with residues lining the catalytic pocket of PC2. In accordance to such a model, the preferential interaction of 7B2^{150} with the mature form of PC2 observed for the D309N mutant (Fig. 3) suggests that this mutation affected the intramolecular binding of the pro-segment of PC2 to its catalytic pocket in such a way as to diminish the ability of 7B2^{150} to interact with proPC2. Upon autocatalytic cleavage of the pro-segment, a possible conformational change may then explain the observed similar binding of both pro7B2^{186} and 7B2^{150} to mature D309N-PC2 (Fig. 3). In contrast, for native PC2, pro7B2 and 7B2 are both able to interact with proPC2 (Fig. 3), suggesting that they can compete with the pro-segment of PC2 for the catalytic domain.

Turning our attention to the second candidate position in PC2, namely Tyr_{194}, the data revealed that the Y194D mutation did not cause an earlier zymogen activation of Y194D-PC2 as compared to native PC2. Indeed, we observed no POMC maturation nor processing of proPC2 to PC2 when BSC40 cells infected with VV:Y194D-PC2 in the presence or absence of VV:POMC were pulse labeled for 3 h with [^{35}S]methionine at 20°C (not shown), a temperature which prevents exit of PC2 from the TGN [11]. Furthermore, our data clearly show that Y194D-PC2 is enzymatically active, even in the absence of 7B2, both ex vivo where it still preferentially cleaves POMC into β-endorphin (Tables 1 and 2) and in vitro. In the presence of 7B2, the specific activity of the Y194D-PC2 is about 6-fold less than that of native PC2. This suggests that stabilization of PC2 into its active conformation by 7B2 is most efficient for native PC2 [26]. Interestingly, the Y194D mutation also resulted in an increased level of basal secretion of PC2, suggesting that aside from affecting the overall ability of PC2 to interact with 7B2, Tyr_{194} may also influence its intracellular level.

Pro7B2^{186} selectively binds to Y194D-PC2, as opposed to 7B2^{150} which does not bind to this enzyme (Fig. 3). This suggests that the inhibitory CT-peptide of pro7B2^{186} cannot compete with the pro-segment of this mutant, whereas it can with native-PC2 and less so with D309N-PC2. Our data thus reveal that Tyr_{194} is critical for the binding of PC2 to 7B2^{150}, possibly via the latter's proline rich Src homology 3 (SH3)-like segment [16]. SH3 domains are structurally well-characterized as monomeric or dimeric modular units of protein structure that mediate protein-protein recognition in numerous signal transduction proteins (reviewed in [29]). Interestingly, the polyproline sequence D**P(Y/D)**PXPRY(T/D) surrounding PC2's Tyr_{194}, or Asp_{194} found in all the other PCs bears some similarity to the SH3-like sequence YPDPPNPCP found within 7B2 (aa 88–95; [24]) and reported to be critical for its binding to PC2 [16]. A common characteristic of SH3 domain binding sites is the presence of the core motif **PXXPX(R)** [30]. In PC2, the critical 7B2-binding sequence seems to center around the motif **PYPYPR** (this work); whereas in 7B2 it is **PDPPNP** [16]. The identified polyproline structure in PC2 may thus play a critical role in the binding of PC2 to 7B2, presumably via an essential hydrophobic interaction of Tyr_{194}.

In evolutionary terms, it is tempting to speculate that the presumed ancestral form of PCs [4] may have had a hydrophobic/aliphatic amino acid at the position equivalent to aa 194 and an Asn at position 309 (e.g. yeast kexin (Leu) and subtilisin BPN′ (Val)). Point mutations into either Asp_{194}/Asn_{309} or Tyr_{194}/Asp_{309} may have led to two branches of convertases: (i) the Asp_{194}/Asn_{309} family which includes the enzymes PC1, Furin, PACE4, PC4, PC5 and PC7 which have not been shown to require a specific binding protein; (ii) the

Tyr_{194}/Asp_{309} family which so far only includes PC2, would have lost its ability to function without the help of an accessory binding protein. Thus, it is probable that a regulatory advantage was conferred to PC2 by its specific binding to 7B2, allowing it to be optimally active in its presence and virtually inactive in its absence (Tables 1 and 2). This interaction would tightly couple the regulation of PC2 activity to the level of 7B2 expression, thus ensuring that PC2 becomes active only at the necessary location and to the required extent.

In conclusion, the data presented in this work reinforce the notion that the highly conserved structure of PC2 evolved to retain specific amino acids which, together with the participation of 7B2, ensure the proper spatial and temporal functioning of PC2. Future work targeting the other unique PC2 residues should further elucidate the complex regulatory mechanisms of PC2 folding, activation and cellular localization.

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