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The cDNA structure of the porcine pro-hormone convertase PC2 and the comparative processing by PC1 and PC2 of the N-terminal glycopeptide segment of porcine POMC

N.G. Seidah[a], Hélène Fournier[c], Guy Boileau[c], Suzanne Benjannet[b], Normand Rondeau[b] and Michel Chrétien[b]

J.A. DeSève Laboratories of [a]Biochemical and [b]Molecular Neuroendocrinology, Clinical Research Institute of Montreal, 110 Pine Ave West, Montreal, Que., H2W 1R7, Canada, and *the [c]Department of Biochemistry, University of Montréal, Montréal, P.Q. H4P 2R2, Canada*

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The complete cDNA structure of the porcine (p) pro-protein and pro-hormone convertase PC2 (pPC2) was obtained from a cDNA library of pituitary neurointermediate lobes mRNA. The deduced amino acid sequence revealed that pPC2 exhibits a 99–97% sequence identity to the human, mouse and rat homologues. The 3′ end of the 2.1 kb cDNA is the least conserved segment. On Northern blots of pars intermedia poly A⁺ RNA two transcripts of 3 and 5 kb were detected. Molecular analysis of the N-terminal glycopeptide products of porcine pro-opiomelanocortin (pPOMC) co-expressed with vaccinia virus recombinants of PC1 or PC2, revealed that in cells devoid or containing secretory granules both convertases can cleave pPOMC with PC1 releasing the 1–80, 1–107 and 1–148 glycopeptide fragments, and PC2 cleaving pPOMC directly into pPOMC 1–107.

Porcine PC2; Pro-hormone convertase; Vaccinia virus expression; POMC N-terminal glycopeptide cleavage

1. INTRODUCTION

Polypeptide hormones, certain hormonal receptors, growth factors and many viral surface glycoproteins are activated by the specific processing of their precursor by endogenous intracellular proteinases. The mammalian enzymes responsible for this process have recently been identified and were found to belong to the subtilisin family of serine proteinases [1]. At least 5 distinct members of this family have now been identified and sequenced. These include PC1 [2,3], PC2 [2,4], furin [5], PACE4 [6] and PC4 [7,8]. These enzymes fall into three groups: those which are ubiquitously expressed such as furin [5] and PACE4 [6], those which are expressed only in endocrine and neuroendocrine cells, such as PC1 and PC2 [2–4,9], and finally PC4 which is only expressed within testicular [7,8] and ovarian germ cells [8].

Cellular co-expression of furin, PC1 and PC2 with polypeptide precursors revealed that these enzymes exhibit exquisite selectivity of cleavage post-pairs of basic residues [10,11]. The POMC processing by PC1 and PC2 has now been well documented for the ACTH/αMSH and βLPH/βEndorphin segments of this precursor [10]. Accordingly and in agreement with in situ cellular localization studies [9], PC1 would be responsible for the production of ACTH and βLPH with some βEndorphin while PC2 would produce βEndorphin and together with PC1, carboxypeptidase E, the amidation enzyme and an *N*-acetylating enzyme would then generate αMSH [10]. The manufacture of αMSH is favored in cells containing secretory granules.

In this paper, we undertook the complete cDNA characterization of porcine pituitary pars intermedia PC2 and show that the evolutionary conservation of sequence for PC2 is greater than that for PC1 [12]. We also compared the cleavage selectivity of PC1 and PC2 for the N-terminal glycopeptide segment of the POMC molecule and show that both convertases can process the N-terminal segment of porcine POMC with PC1 generating pPOMC 1–80, 1–107 and 1–148 and PC2 producing pPOMC 1–107 directly.

Correspondence address: N.G. Seidah, J.A. DeSève Laboratory of Biochemical Neuroendocrinology, Clinical Research Institute of Montreal, 110 Pine Ave West, Montreal, Que. H2W 1R7, Canada. Fax: (1) (514) 987 542.

2. MATERIALS AND METHODS

2.1. *Cloning of porcine pituitary neurointermediate lobe pPC2*

Using 100 μg of poly A⁺ RNA from porcine pituitary neurointermediate lobes, a size selected (>1 kb) cDNA library was established in a pcDNA II vector. This cDNA library was screened with a full-length mouse PC2 cDNA probe radiolabeled with [α-^{32}P]dCTP as described [2]. Fifteen positive clones were isolated out of a total of 800,000 colonies screened. The longest clone isolated contained a 2.1 kb insert and was further analysed for complete DNA sequence determination as described [2].

2.2. *Vaccinia virus recombinants of mPC1, mPC2, pPOMC and cellular infections*

The purified recombinant vaccinia viruses (VV) using the full-length cDNA inserts of mouse PC1 [3] (VV:mPC1) and mouse PC2 [2] (VV:mPC2) were the same as those previously reported [10]. The porcine POMC vaccinia virus recombinant (VV:pPOMC) was prepared from the cDNA of porcine POMC [13] using the PMJ-601 transfer vector [14]. The cellular infections of BSC40 and PC12 cells were done as described [10], consisting of the co-infection of the VV:pPOMC (2 pfu/cell) with either 4 pfu/cell of the wild type virus (VV:WT) or the recombinants VV:mPC1 or VV:mPC2 or 2 pfu/cell of each VV:mPC1 and VV:mPC2. 17 h post-infections, the cells were washed and incubated for 7 h, and the culture media analysed.

2.3. *Immunoblotting*

Proteins in culture media were separated by SDS-PAGE using a 14% (w/v) acrylamide slab gel system, and then transfered electrophoretically to nitrocellulose membranes, which were incubated for 4 h with two monoclonal antibodies, Mab 2-197 and Mab 1-244 directed against the NH_2-terminal glycopeptide segment of porcine POMC, as described [15]. The porcine markers pPOMC 1-241 and pPOMC 1-148 were obtained by expression of full length and truncated pPOMC cDNAs in COS-1 cells.

3. RESULTS AND DISCUSSION

3.1. *cDNA sequence analysis of pPC2*

From the 2140 nucleotides (nts) sequence presented in Fig. 1, we observe an open-reading frame containing 1914 bp. A variant polyadenylation signal AATACA is present as well as the poly A tail appearing 15 nts later. Similar to mPC2 [2,11] a 25 amino acids signal peptide is predicted (Fig. 1), the cleavage of which would result

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                                 -25                 -20                                      -10                                 -1 ↓ 1
                                 MetLysGlyGlyCysValSerGlnTrpLysAlaAlaAlaGlyLeuLeuPheCysValThrValPheAlaSerAlaGluArgProValPheThr
AACACCACTTTTCACTCCCAAAGAAAGATGAAAGGTGGCTGTGTCTCCCAGTGGAAGCGGCCCCCGGGCTCCTCTTCTGTGTCACGGTTTTGCGTCTGCCCGAAAGACCGGTCTTCACA  120
   10                            20                            30                            40
AsnHisPheLeuValGluLeuHisLysGlyGlyGluGluGluAlaArgGlnValAlaAlaGluHisGlyPheGlyValArgLysLeuProPheAlaGluGlyLeuTyrHisPheTyrHis
AATCATTTTCTTGTGGAGTTGCATAAAGGGGGAGAGGAAGAAGCTCGCCAAGTTGCAGCAGAACACGGCTTTGGAGTCCGAAAGCTTCCTTTTGCTGAAGGCCTGTACCATTTTTATCAC  240
   50                            60                            70                            80
AsnGlyLeuAlaLysAlaLysArgArgArgSerLeuHisHisLysGlnArgLeuGluArgAspProArgValLysArgAlaLeuGlnGlnGluGlyPheAspArgLysLysArgGlyTyr
AATGGCCTGGCAAAGGCCAAGAGAAGACGCAGCCTACACCATAAGCAGCGGCTGGAGAGAGACCCCAGGGTAAAGAGAGCTTTGCAACAGGAAGGGTTCGATCGGAAAAAGCGAGGGTAT  360
   90                            100                           110                           120
ArgAspIleAsnGluIleAspIleAsnMetAsnAspProLeuPheThrLysGlnTrpTyrLeuIleAsnThrGlyGlnAlaAspGlyThrProGlyLeuAspLeuAsnValAlaGluAla
AGAGACATCAATGAAATCGACATCAACATGAATGACCCTCTTTTTACGAAGCAGTGGTATCTGATCAATACTGGACAAGCTGATGGTACCCCTGGCCTTGACTTGAATGTGGCAGAAGCC  480
   130                           140 ■                         150                           160
TrpGluLeuGlyTyrThrGlyLysGlyValThrIleGlyIleMetAspAspGlyIleAspTyrLeuHisProAspLeuAlaSerAsnTyrAsnAlaGluAlaSerTyrAspPheSerSer
TGGGAGCTCGGATACACTGGGAAAGGTGTTACCATTGGAATTATGGACGATGGAATCGACTATCTCCACCCGGACCTGGCCTCGAACTACAATGCGGAAGCGAGCTACGACTTTAGCAGC  600
   170                           180   ■                       190                           200
AsnAspProTyrProTyrProArgTyrThrAspAspTrpPheAsnSerHisGlyThrArgCysAlaGlyGluValSerAlaAlaAlaAsnAsnAsnIleCysGlyValGlyValAlaTyr
AACGACCCCTACCCTTACCCTCGCTACACAGACGACTGGTTTAACAGCCACGGGACCCGATGTGCCGGGGAGGTTTCTGCCGCAGCCAACAACAATATCTGCGGGGTTGGAGTGGCCTAC  720
   210                           220                           230                           240
SerSerLysValAlaGlyIleArgMetLeuAspGlnProPheMetThrAspIleIleGluAlaSerSerIleSerHisMetProGlnLeuIleAspIleTyrSerAlaSerTrpGlyPro
AGCTCCAAGGTTGCAGGTATCCGGATGCTGGACCAGCCCTTCATGACGGACATCATCGAGGCCTCTTCCATCAGCCACATGCCTCAGCTAATTGACATCTACAGCGCCAGCTGGGGCCCC  840
   250                           260                           270                           280        ■
ThrAspAsnGlyLysThrValAspGlyProArgGluLeuThrLeuGlnAlaMetAlaAspGlyValAsnLysGlyArgGlyGlyLysGlySerIleTyrValTrpAlaSerGlyAspGly
ACCGACAACGGGAAGACGGTGGACGGGCCGCGGGAGCTCACGCTTCAGGCGATGGCCGACGGTGTGAACAAGGGCCGAGGGGGCAAAGGCAGCATCTACGTGTGGGCCTCCGGGGACGGT  960
   290                           300                           310                           320
GlySerTyrAspAspCysAsnCysAspGlyTyrAlaSerSerMetTrpThrIleSerIleAsnSerAlaIleAsnAspGlyArgThrAlaLeuTyrAspGluSerCysSerSerThrLeu
GGCAGCTACGACGACTGCAACTGTGACGGCTATGCCTCCAGCATGTGGACCATCTCCATCAACTCTGCCATCAACGACGGCAGGACCGCCCTGTACGACGAGAGCTGCTCCTCCACCTTG  1080
   330                           340                           ***                         ■ 360
AlaSerThrPheSerAsnGlyArgLysArgAsnProGluAlaGlyValAlaThrThrAspLeuTyrGlyAsnCysThrLeuArgHisSerGlyThrSerAlaAlaAlaProGluAlaAla
GCCTCCACCTTCAGCAATGGGAGGAAGAGGAACCCCGAGGCCGGCGTGGCAACCACAGATTTGTACGGCAACTGCACTCTGAGGCATTCTGGGACATCCGCGGCTGCTCCTGAGGCAGCT  1200
   370                           380                           390                           400
GlyValPheAlaLeuAlaLeuGluAlaAsnLeuGlyLeuThrTrpArgAspMetGlnHisLeuThrValLeuThrSerLysArgAsnGlnLeuHisAspGluValHisGlnTrpArgArg
GGAGTGTTTGCACTGGCTTTAGAGGCTAACCTGGGTCTGACCTGGAGAGACATGCAGCATCTGACCGTGCTCACTTCCAAACGGAACCAACTTCATGATGAGGTCCATCAGTGGCGGAGA  1320
   410                           420                           430                           440
AsnGlyValGlyLeuGluPheAsnHisLeuPheGlyTyrGlyValLeuAspAlaGlyAlaMetValLysMetAlaLysAspTrpLysThrValProGluArgPheHisCysValGlyGly
AACGGAGTCGGCCTAGAGTTTAATCACCTCTTTGGCTATGGAGTCCTTGACGCAGGGGCCATGGTGAAAATGGCTAAAGACTGGAAAACCGTGCCTGAGAGATTCCACTGTGTGGGAGGC  1440
   450                           460                           470                           480
SerValGlnAspProGluLysIleProSerThrGlyLysLeuValLeuThrLeuThrThrAspAlaCysGluGlyLysGluAsnPheValArgTyrLeuGluHisValGlnAlaValIle
TCCGTACAGGACCCTGAAAAAATACCATCCACTGGCAAGCTGGTGCTGACGCTCACCACCGATGCATGTGAGGGGAAGGAAAATTTCGTCCGCTACCTGGAGCACGTCCAAGCTGTCATC  1560
***490                       ***500                            510                           520
ThrValAsnAlaThrArgArgGlyAspLeuAsnIleAsnMetThrSerProMetGlyThrLysSerIleLeuLeuSerArgArgProArgAspAspAspSerLysValGlyPheAspLys
ACGGTCAATGCGACCCGGAGAGGAGACCTGAACATTAACATGACATCCCCCATGGGCACCAAGTCCATCCTGCTGAGCCGGCGCCCGAGGGACGACGACTCCAAGGTGGGCTTCGACAAG  1680
   530                           540                           550                           560
TrpProPheMetThrThrHisThrTrpGlyGluAspAlaArgGlyThrTrpThrLeuGluLeuGlyPheValGlySerAlaProGlnLysGlyAlaValLysGluTrpThrLeuMetLeu
TGGCCTTTCATGACCACCCACACGTGGGGGGAGGATGCCCGAGGCACCTGGACCCTGGAGCTAGGCTTTGTGGGGAGCGCACCCCAGAAGGGGGCTGTGAAGGAGTGGACGCTGATGCTG  1800
   570                           580                           590                           600
HisGlySerGlnSerAlaProTyrIleAspGlnValValArgAspTyrGlnSerLysLeuAlaMetSerLysLysGluGluLeuGluGluGluLeuAspGluAlaValGluArgSerLeu
CACGGCAGCCAGAGCGCCCCTTACATCGACCAGGTGGTGAGGGATTACCAGTCCAAGCTGGCCATGTCCAAGAAGGAGGAGCTGGAGGAAGAGCTGGACGAAGCAGTGGAGAGAAGCCTG  1920
   610      613
LysSerIleLeuGlyLysHis
AAGAGCATCCTGGGCAAGCACTAGCGCCACCTCCTGGCCGCCGCCGCCGCCCTGCCCGACTCTGCCTCTGTCCTCGCTCCACATCCCACCCATCGCCTCGCAATCACTGTCCCCCGACCC  2040

ACGATTCCTGATCCGCCTTCGCTCACTGTCGGTGATTATTTTCATTACCATGAAAGCAAGCTTTTTTATTCTGTGCCCCAAATACAGTGTTCCCACCCAC(A)n                 2100
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Fig. 1. Complete cDNA and the deduced amino acid sequence of pPC2. The active site **Asp**■, **His**■, and **Ser**■, and the important **Asp**■ residue, as well as the three potential *N*-glycosylation (***) sites and the RGD sequence are emphasized. The predicted sites of the signal peptidase cleavage and zymogen activation [2,11] are depicted by an arrow and an inverted triangle, repsectively. The 6 pairs of basic residues, a tribasic and the two tetrabasic residues are emphasized. The variant polyadenylation signal AATACA is underlined.

in a 613 amino acids long proteinase. Recently, we showed that PC1 and PC2 are first synthesized as precursor enzymes (zymogens) which undergo post-translational modifications leading to the excision of their N-terminal pro-segment via the cleavage at a specific LysArg-sequence [11]. Therefore, similar to mPC2, we expect that pPC2 would be first synthesized as a zymogen with an 84 amino acid pro-segment which is then cleaved at the ArgLysLysArg^{84}↓ sequence to release the active enzyme. The mature enzyme would therefore contain 529 amino acids and three potential Asn-glycosylation sites (Fig. 1). The protein sequence deduced from the cDNA structure of pPC2 shows the presence of the three active site amino acids Asp^{142}, His^{183} and Ser^{359} at similar positions to the equivalent residues found in PC2 of mouse [2], human [4], rat [16] and *Xenopus* [17] and in subtilisins [18]. Furthermore, pPC2 also contains the important Asp^{285}, which replaces the usually found Asn reported to play a crucial role in stabilizing the oxyanion hole in subtilisins [19]. From Fig. 1, we notice the presence of an Arg**ArgGlyAsp**Leu sequence (residues 492–496), which is identical to that found in PC1 [3,12] PC2 [2,4], furin [5], PACE4 [6] and PC4 [7,8]. The tripeptide **ArgGlyAsp** (RGD) represents the minimal recognition sequence for cell surface receptors known as integrins, which are important for functions such as cell adhesion [20]. The role of this conserved RGD sequence in any of the identified convertases is still unknown. Finally, as in all PC2 structures reported and similar to carboxypeptidase E [21] we also observe the presence of a C-terminal amphipathic structure which may allow pPC2 to be membrane associated in a pH-dependent manner. It is interesting to note that the C-terminal amino acid of pPC2 is His while all other PC2 structures reported end in Asn [2,4,16,17]. The pPC2 protein structure is highly conserved exhibiting an overall sequence identity to human, mouse, rat and *Xenopus* PC2 of 97.5%, 95.8%, 95.6% and 86.7%, respectively. This homology of sequence of PC2 is more pronounced than that of PC1 which exhibits a 92.6% sequence identity between human, mouse and rat PC1 [5,16,22].

Similar to what was already described for mouse [2], human [4] and rat [9] PC2, Northern blot analysis of the poly A+ RNA obtained from porcine neurointermediate lobes demonstrated the presence of a major 3 kb and a minor 5 kb transcript (not shown). By analogy to what was reported regarding the 5 kb form of rat [22] and human [12] PC1, this mRNA size for pPC2 could also represent a 3' extended form of the 3 kb, probably due to the utilization of an alternative polyadenylation site.

3.2. *Processing of pPOMC by PC1 and PC2*

In order to complete our earlier studies on the processing of POMC by PC1 and PC2, we undertook the analysis of the N-terminal glycopeptide cleavage pattern of pPOMC by PC1 and PC2 in both BSC-40 cells which are devoid of secretory granules and in PC12 cells which contain secretory granules. For this purpose, we co-infected these cells with vaccinia virus recombinants of pPOMC and of either mPC1 or mPC2 or both. The media were analysed for the N-terminal glycopeptide fragments using two monoclonal antibodies: Mab 1–244 which recognizes an epitope located within the c3-melanotropin sequence (resi-dues 51–80 of pPOMC) and Mab 2–197 which binds specifically to a determinant in the 1–49 segment of pPOMC [13,15], and both antibodies recognize the pPOMC 1–80 or the bovine

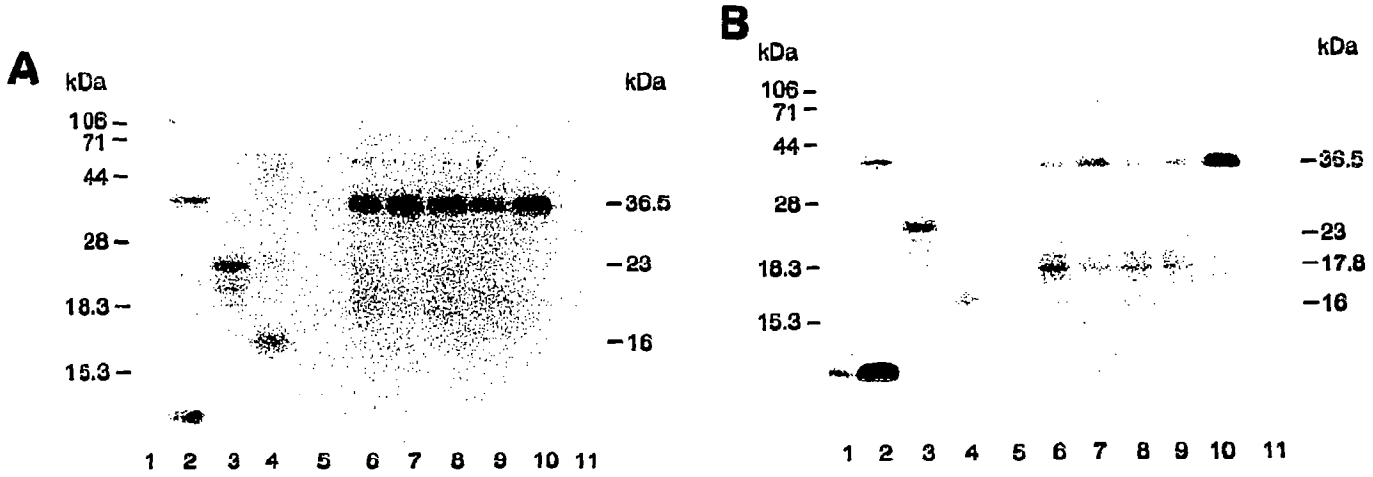

Fig. 2. SDS-PAGE/Western blot analysis of culture media proteins secreted from either BSC40 (A) or PC12 (B) cells infected with VV:pPOMC at 2 pfu/cell and VV:mPC1 and/or VV:mPC2 as indicated. The PC1- or PC2-specific pPOMC N-terminal glycopeptide (NT) digestion products were revealed using two NT-pPOMC-specific monoclonal antibodies, as described in the methods and in [15]. Lane 1, culture medium from control COS-1 cells; lanes 2,3 porcine pPOMC 1–241 and 1–148 markers; lanes 4,5 bovine bPOMC 1–77 and 1–49 markers; lane 6, VV:mPC1 at 4 pfu/cell; lane 7, VV:mPC2 at 4 pfu/cell; lane 8, VV:mPC1 and VV:mPC2 each at 2 pfu/cell; lane 9, VV:mPC1 at 1 pfu/cell and VV:mPC2 at 4 pfu/cell; lane 10, the control vaccinia virus wild type VV:WT at 4 pfu/cell; lane 11, culture medium from either BSC40 (A) or PC12 (B) cells.

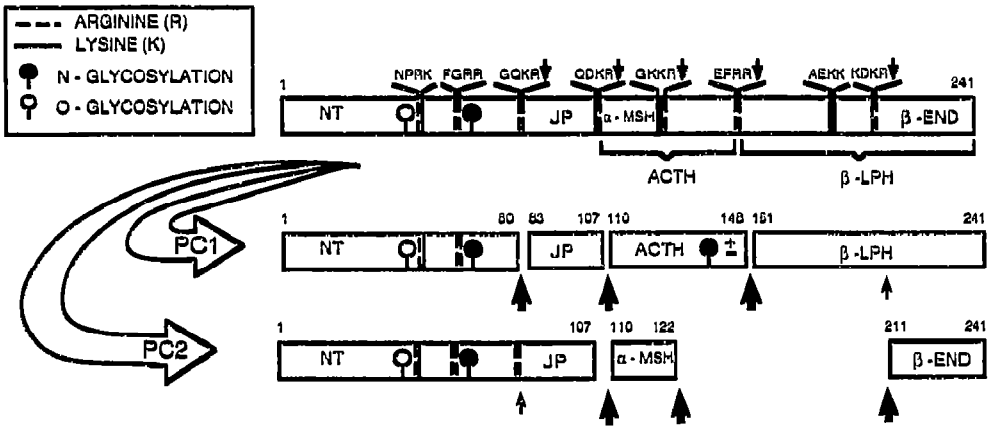

Fig. 3. Composite model representing the end products of POMC processing by PC1 and PC2. The major and minor cleavage sites following pairs of basic residues, are represented by heavy and light arrows, respectively. The amino acid sequence at the cleavage sites is also shown. The numbers represent the start and end positions of the processed peptides based on the reported POMC sequence of pPOMC [15]. The *N*- and *O*-glycosylation (CHO) sites as well as the pairs of basic residues are emphasized.

bPOMC 1–77 [15]. In general, the data in Fig. 2A and B show that PC1 and PC2 cleaved the N-terminal glycopeptide segment of pPOMC with higher efficiency in PC12 cells as compared to BSC-40 cells. This is in agreement with our earlier analysis of the cleavage of the C-terminal ACTH/βLPH segment of POMC by these convertases [10]. Fig. 2A and B show the following: (a) a doublet migrating close to pPOMC 1–148 (i.e. ending with the ACTH sequence) only seen with PC1 in both BSC-40 and PC12 cells (lane 6); (b) in both BSC-40 and PC12 cells with PC1 (lane 6) and only in PC12 cells with PC2 (Fig. 2B, lane 7) we see a major doublet with a molecular weight intermediate between bPOMC 1–77 and pPOMC 1–148, most probably representing pPOMC 1–107 which ends with the joining peptide sequence [13,23]; (c) only with PC1 in PC12 cells can we detect a band migrating at the position of bPOMC 1–77, most probably representing pPOMC 1–80 [13,23,24]; (d) with the conditions and cell types used we could not detect an immunoreactive peptide migrating close to bPOMC 1–49 with either enzyme; (e) PC1 and PC2 act independently, since the co-expression of both enzymes at equal levels (lane 8) or 4 times more of PC2 over PC1 (lane 9) did not change the relative abundance of the products as compared to those obtained had we summed both enzymatic activities, i.e. the sum of the products in lanes 6 and 7.

The conclusions drawn from the above experiment suggest that PC1 is more efficicent than PC2 in cleaving pPOMC into pPOMC 1–80, while PC2 cleaves pPOMC with the direct production of pPOMC 1–107 without detectable intermediate pPOMC 1–148 (Fig. 2B, compare lanes 6,7). Therefore, the combination of these results and those obtained previously [10] allow us to propose the model shown in Fig. 3, for the POMC processing by either PC1 or PC2. It is interesting to note that under the conditions chosen of vaccinia virus expression and using either BSC-40 or PC12 cells, we did not observe the production of pPOMC 1–49, known to be inefficiently produced in the pituitary pars intermedia [25]. Since this cleavage occurs late during the intracellular transport route [25], it may therefore be affected by the vaccinia virus expression system which does change somewhat the intracellular organization of the cell. Alternatively, the intracellular and intragranular environment conditions within the pituitary pars intermedia are not mimicked in either the BSC-40 or PC12 cells used in this study, and hence the cleavage conditions may not be optimal for such an inefficient cleavage reaction, which in vivo in the pars intermedia never exceeds 50% [25]. Finally, it is also possible that the AsnProArg|Lys cleavage needed for the production of pPOMC 1–49 and Lys-γ_3MSH [13] in the pituitary pars intermedia is effected by another enzyme not yet identified.

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