Functional analysis of human PACE4-A and PACE4-C isoforms: identification of a new PACE4-CS isoform

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Abstract There are seven known subtilisin/kexin-like proprotein convertases responsible for the processing of numerous precursors at either pairs or specific single basic residues. Three members, PACE4, PC4 and PC5, exhibit alternative splicing of their RNAs resulting in the generation of multiple isoforms differing in their C- or N-terminal segments. In this study we examined the biosynthesis, functional activity and cellular localization of two of these isoforms, namely the full length PACE4-A and the C-terminally truncated PACE4-C which lacks 11 amino acids at the end of its chaperone-like P-domain. We report the existence of a new isoform, termed PACE4-CS, which is a C-terminally shortened version of PACE4-C. Cellular expression results demonstrated that PACE4-A codes for a functional secretable enzyme capable of cleaving pro7B2 into 7B2. In contrast, PACE4-CS is not secreted since it remains in the endoplasmic reticulum as an inactive zymogen form, thereby emphasizing the importance of the integrity of the P-domain. Microsequencing of the intracellular PACE4-CS protein in two cell lines revealed that it is proPACE4-CS with an N-terminal trimming reminiscent of the action of a dipeptidylpeptidase recognizing the motifs X-Ala and X-Pro.

Key words: PACE4-A; PACE4-C; PACE4-CS; Convertase; Alternative splicing; 7B2

1. Introduction

Processing of proproteins and prohormones at either single or pairs of basic residues is a general mechanism to generate bioactive proteins and peptides from inactive precursors [1,2]. The subtilisin/kexin-like proteinases responsible for such intracellular cleavages have recently been described and are called 'proprotein convertases' (PCs). So far, seven members of this family have been identified: furin, PC1, PC2, PC4, PACE4, PC5 and PC7 (for reviews see [1–4]). All of these PCs exhibit an N-terminal signal peptide, followed by a pro-segment, a catalytic domain, a P-domain and an enzyme-specific C-terminal segment [5]. Variant cDNA structures, possibly arising from alternative gene splicing, have been reported for PC4 [6,7], PC5 [8] and PACE4 [9,10]. In the case of PC5, the isoform PC5-A is directed to secretory granules while PC5-B localizes to the trans Golgi network (TGN) [11]. For human PACE4 four different isoforms have so far been reported; they are named PACE4-A (full length), PACE4-B (also known as PACE4.1), PACE4-C and PACE4-D [9,10]. Furthermore, as compared to human PACE4-A [9], the rat and mouse homologues [12,13] exhibit an extra Leu residue close to the predicted signal peptidase cleavage site and additionally show three deletions within the signal peptide, the N-terminal of the catalytic domain and the C-terminal segment which follows the conserved P-domain. From the reported importance of the pro-segment acting as an intramolecular chaperone in subtilisin-like enzymes [14], it was suggested that PACE4-D would code for an inactive enzyme [10]. Similarly, as the integrity of the P-domain has been reported to be critical for the activity of yeast kexin [15] and mouse furin [16], it was also predicted that PACE4-B, which lacks the P-domain, would be inactive [9]. In contrast, PACE4-C (652 aa), which is truncated at the C-terminus compared to PACE4-A (969 aa), has a distinct sequence at the end of the P-domain, resulting in a shorter form in which the 32 amino acids following Gly^{620} are different. While the C-terminal border of the P-domain has been suggested to be close to Gly^{631} [5,15,16], its exact site is not known with certainty and could be convertase-dependent. Therefore, it was suggested that PACE4-C could code for an active convertase [10]. Furthermore, it was reported that PACE4-C is expressed in a tissue-specific manner, based on RT-PCR [17] and immunocytochemical [18] criteria.

In this work, we isolated a fifth isoform called PACE4-CS (shortened form of PACE4-C), which led us to address the question of the integrity of the P-domain, the tissue distribution of the PACE4 isoforms and to suggest that PACE4-A is the only active proteinase of the isoforms so far known.

2. Materials and methods

2.1. Cellular expression of PACE4 isoforms

The mRNA expression of PACE4 isoforms was analyzed by RT-PCR on 5 μg of total RNA [19] isolated from established cell lines including: Caco2 (colon carcinoma, human), LoVo (colon adenocarcinoma, human), HepG2 (hepatocellular carcinoma, human), and SKNM (neuroepithelioma, human). The sense oligonucleotides used were the general PACE4 oligonucleotides: oligo II: TTCATGACTGTCCACTGCTGGGGAG, oligo VI: GGGACCTCAGTCTCTGCCCCCATG; and the PACE4-C/CS-specific oligo IV: CCTGGACTAAAACACGT. The antisense oligonucleotides were either the PACE4-A-specific oligo I: CAGACAGTACATTTCTCA; or the PACE4-C/CS-specific oligo III: CTACTGGAGATACAGAACCGAC, and the [*Hin*dIII]-containing oligo V: [AAGCTT]GCGGGAGCTGAGAGATCCAGCTCTGGAC. The 30 cycle PCR

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Abbreviations: VV, vaccinia virus; PC, precursor convertase; RT-PCR, reverse transcriptase polymerase chain reaction

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reactions were performed at an annealing temperature of 58°C for PACE4-A-specific reactions (oligo pair II/I) and for PACE4-C/CS-specific PCRs at either 50°C (oligo pair II/III) or 55°C for the pair VI/V and for the nested PCR using the pair IV/III.

2.2. Vaccinia virus (VV) expression of PACE4-A and PACE4-CS

The RT-PCR product of a PACE4-C-specific sequence using the oligo VI/V pair allowed the isolation of a 770 bp PACE4-C-like fragment. Transfer to the PCR™II vector and sequencing revealed that this segment is an alternatively spliced product of PACE4-C called PACE4-CS (Fig. 2). This allowed us to construct a PACE4-CS cDNA by ligation of the 5′ *Nco*I and 3′ *Hin*dIII digested PCR product (745 bp) into the original PMJ601 VV transfer vector containing the full length PACE4-A cDNA digested with the same enzymes [20]. The isolation of the recombinant VV was obtained as previously described [21]. Green monkey kidney epithelial BSC40 cells or mouse corticotroph AtT20 cells were infected with 4 pfu/cell of either VV:hPACE4-A, VV:hPACE4-CS or VV:wild type (VV:WT) [21]. Following overnight growth, the cells were washed and then pulse-labeled with [^{35}S]methionine, [^{3}H]tyrosine or [^{3}H]valine for 4 h, as previously described [20,21]. The media and cell extracts were then purified on a lentil lectin column (Pharmacia) and analyzed by autoradiography of an SDS-PAGE separation. The identified PACE4-CS (77 kDa) [^{3}H]Tyr- and [^{3}H]Val-labeled bands were then excised and the underlying protein microsequenced on an Applied Biosystem model 470A sequenator, as previously described [20–22].

2.3. Bioactivity of PACE4 isoforms

The enzymatic activity of PACE4-A or PACE4-CS was gauged by its capacity to process the neuroendocrine precursor pro7B2 [20]. Accordingly, BSC40 cells infected with 2 pfu/cell of VV:m7B2 were co-infected with 4 pfu/cell of either VV:PACE4-A, VV:PACE4-CS or VV:wild type. Following overnight growth, the cells were pulse-labeled for 30 min (P30) with [^{35}S]methionine and then chased with cold methionine for 60 min (C60). The cell extracts and media were immunoprecipitated with a 7B2-specific antibody and the precipitates analyzed by SDS-PAGE as described elsewhere [20].

3. Results

3.1. Cellular expression of PACE4 isoforms

Previous results using a general PACE4 probe recognizing all isoforms demonstrated the widespread tissue and cellular mRNA expression of PACE4 [3,9,12,13,17,23]. Aside from the major 4.4 kb transcript, a 3.9 kb mRNA was also detected in some rat tissues such as the jejunum, duodenum and kidney [3], suggesting the presence of alternatively spliced mRNA forms of PACE4. Recent reports of Tsuji et al. [10,17] suggest that an alternatively spliced human PACE4-C isoform can be isolated from human placenta. However, none of these isoforms could be detected by screening mouse liver, kidney or brain cDNA libraries [13]. In order to probe for the specific expression of the PACE4-A and PACE4-C isoforms, we first attempted to perform Northern blots on cell lines and rat tissues with human PACE4-A and PACE4-C-specific probes obtained by PCR using the oligo pairs II/I and IV/III, respectively (Fig. 1A). Although similar results to those obtained in [3] were found for PACE4-A expression, we could not detect PACE4-C-specific mRNAs in the tissues or cells analyzed in [3] (not shown). Therefore, we opted for the use of RT-PCR as an alternative and more sensitive approach for the detection of PACE4-C-specific mRNA. Accordingly, two consecutive PCR amplifications were needed in order to detect PACE4-C-like transcripts. In the first PCR, no visible amplification product was obtained with the oligo pair II/III. However, nested PCR of an aliquot of this first amplification using the oligo pair IV/III allowed the detection of PACE4-C-like expression in the human cell lines Caco2, LoVo, HepG2 but not in SKNM cells (Fig. 1A,B). In contrast, PACE4-A expression was easily detected by RT-PCR using the single oligo pair II/I (Fig. 1A,C). This result suggests that in the cells analyzed, PACE4-A transcripts are much more abundant than those of PACE4-C.

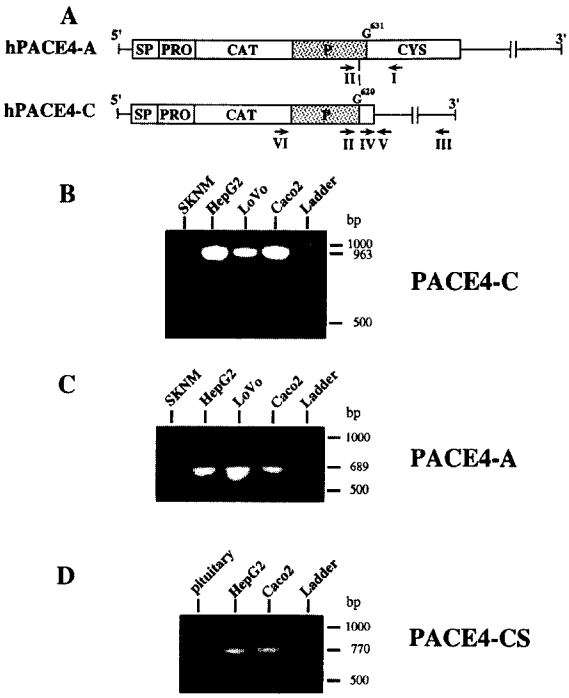

Fig. 1. Tissue and cell expression of PACE4. A: Diagrammatic representation of human hPACE4-A and hPACE4-C DNA structures including the signal peptide (SP), pro-segment (PRO), catalytic domain (CAT), P-domain (P) and C-terminal Cys-rich domain (CYS). The 5′ and 3′ non-coding segments are represented by a horizontal line. The positions of the oligonucleotides used in the RT-PCR amplifications are shown. The putative Gly^{631} at the C-terminal end of the P-domain as well as the Gly^{620} where splicing caused the generation of PACE4-C and PACE4-CS, are emphasized. B: RT-PCR of PACE4-C in the human cell lines SKNM, HepG2, LoVo and Caco2. The first RT-PCR done with the oligo pair II/III (30 cycles) was followed by a nested PCR with the pair IV/III (30 cycles). C: RT-PCR of PACE4-A done with the oligo pair II/I (30 cycles). D: RT-PCR of PACE4-CS done on cell lines and human pituitary RNA with the oligo pair VI/V (35 cycles).

In order to obtain a construction of the full length PACE4-C, it was necessary to amplify the variant 3′ end using the oligo pair VI/V starting from either HepG2 or Caco2 cells (Fig. 1A,D). With this pair it was possible to detect the expected PACE4-C-like product of 770 bp on the first amplification reaction by using 35 cycles of amplification instead of the usual 30 (Fig. 1D). In contrast, the same RT-PCR done on human pituitary RNA did not yield any product (Fig. 1D). To our surprise, the DNA sequence of this PCR product was itself a spliced form of PACE4-C, where the gt/ag donor/acceptor splice sites were derived from an exon of PACE4-C (Fig. 2). Accordingly, this new variant, called PACE4-CS, is a 3′ shortened version of PACE4-C with a different C-terminal sequence. Thus, both PACE4-C and PACE4-CS diverge from PACE4-A at Gly^{620} within the predicted P-domain (Fig. 2). However, PACE4-C and PACE4-CS are C-terminally extended by 32 and 3 amino acids to give proteins composed of 652 and 623 residues, respectively. Analysis of 11 different clonal inserts in PCR™II (see Section 2) gave the same DNA sequence and did not reveal the presence of an authen-

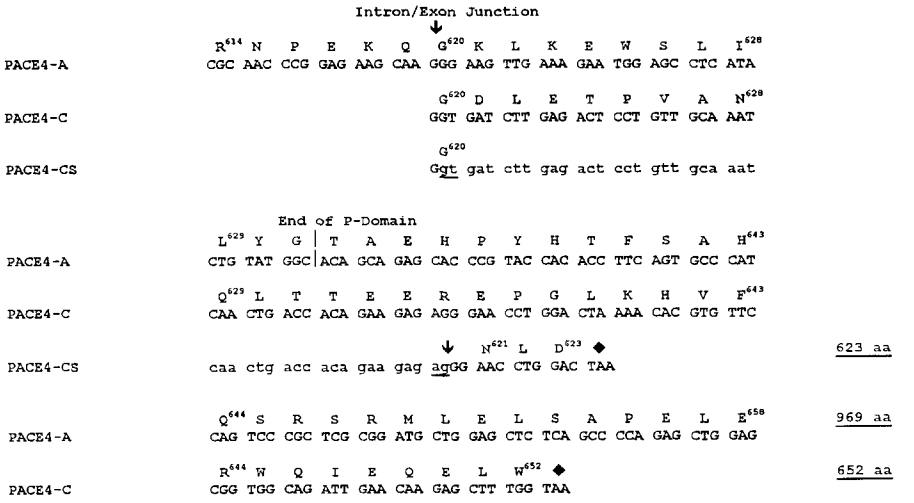

Fig. 2. Generation of PACE4-A, PACE4-C and PACE4-CS isoforms by alternative splicing of the human PACE4 gene. The positions of the intron-exon junction (↓) and the end of the P-domain (|) are shown. The small letter notations in PACE4-CS emphasize the splicing of this segment at the canonical gt/ag sites resulting in the production of the PACE4-CS isoform.

the PACE4-C product. This 770 bp product was then digested with appropriate restriction enzymes (see Section 2) and ligated to the equivalently digested PACE4-A cDNA, resulting in a full length PACE4-CS construct which was used to obtain a VV:PACE4-CS recombinant.

3.2. Biosynthetic analysis of PACE4-A and PACE4-CS

Cellular expression of PACE4-A and PACE4-CS was analyzed after infection of the constitutively secreting cell line BSC40 with either VV:PACE4-A or VV:PACE4-CS and followed by a 4 h pulse-labeling with [^{35}S]methionine. The labeled products could not be immunoprecipitated as PACE4-specific antibodies are not yet available. As an alternative, since PACE4 is predicted to be *N*-glycosylated [9], we used a lentil lectin column [24] to concentrate *N*-glycosylated proteins from cell extracts and media. As shown in Fig. 3, this technique revealed that PACE4-CS encodes a non-secretable protein (even after very long exposure of the gel, no protein is detected in the medium) migrating with an apparent M_r of 77 kDa on SDS-PAGE. In contrast, VV:PACE4-A expression results in the intracellular labeling of a number of bands migrating with M_r of 160–180 kDa but releasing only the 150 kDa product. Therefore, although PACE4-A is a secretable protein, PACE4-CS is not detectable in the medium. Furthermore, digestion of the 77 kDa PACE4-CS with either endoglycosidase H or *N*-glycanase F [25] revealed that this form is insensitive to both enzymes (not shown). In contrast, the secreted 150 kDa PACE4-A is only digested by *N*-glycanase F resulting in a 5 kDa decrease in apparent molecular mass (not shown). Therefore, both PACE4-CS and PACE4-A migrate with apparent molecular masses much higher than predicted from their amino acid sequences and this is not primarily due to carbohydrate attachment, but could in part be due to the Cys-rich domain in PACE4-A, as observed for *Drosophila* furins [26]. These data suggest that PACE4-CS is localized within a cellular compartment where trimming of carbohydrate chains does not proceed to completion, most likely the endoplasmic reticulum itself, whereas PACE4-A can exit the cell through the constitutive secretory pathway of BSC40 cells.

In order to unequivocally define the nature of the 77 kDa PACE4-CS product, we microsequenced the [^{3}H]Val- and [^{3}H]Tyr-labeled proteins obtained from both AtT20 and BSC40 cells. The N-terminal sequence of the intracellular 77 kDa PACE4-CS product reveals a Tyr^7 in AtT20 cells (Fig. 4A) and a Tyr$^{6.7}$ in BSC40 cells (Fig. 4B). This surprising result led us to examine the sequence of valine-labeled PACE4-CS from BSC40 cells. The data obtained suggest the presence of two chains differing by one amino acid, i.e. of sequence Val^{5,12,14} and Val^{6,13,15} (Fig. 4C). This interpretation is based on the much lower carry-over of radioactivity from cycle to cycle obtained for labeled proteins which were sequenced before and after PACE4-CS (not shown). From the predicted protein structure of PACE4 [9], these results suggest that the 77 kDa sequence starts at either Pro^{64} or Pro^{65} in BSC40 cells and at Pro^{64} in AtT20 cells. Furthermore, the data demonstrate that proPACE4-CS is not processed into PACE4-CS.

3.3. Processing of pro7B2 by PACE4-A and PACE4-CS

In order to demonstrate enzymatic activity of the PACE4 isoforms, the medium of BSC40 cells overexpressing PACE4-A or PACE4-CS was partially purified on a DEAE column as reported for PC1 [27]. Accordingly, although appreciable activity towards the fluorogenic substrate pGluArgThrLysArg-MCA was observed for PACE4-A (Munzer et al., in preparation), none was obtained for PACE4-CS (not shown). This result is in agreement with the biosynthetic data (Fig. 3) which revealed that only PACE4-A could be detected in the medium. In order to further probe the intracellular enzymatic activity of either PACE4-A or PACE4-CS, we co-expressed these isoforms with pro7B2, a PC2-specific binding protein [20,28,29] known to be processed in the TGN by furin-like enzymes [20,30]. As shown in Fig. 5, only PACE4-A was able to increase the intracellular processing of pro7B2 (30 kDa) into 7B2 (23 kDa), which is then secreted. No increased processing over background control (pro7B2/wild-type virus) was observed when PACE4-CS was co-expressed with pro7B2 (Fig. 5). In conclusion, our data demonstrate that PACE4-CS does not exhibit intracellular (ex vivo) or extracellular (in vitro) enzymatic activity. Accordingly, we suggest that only PACE4-A is an enzymatically active convertase.

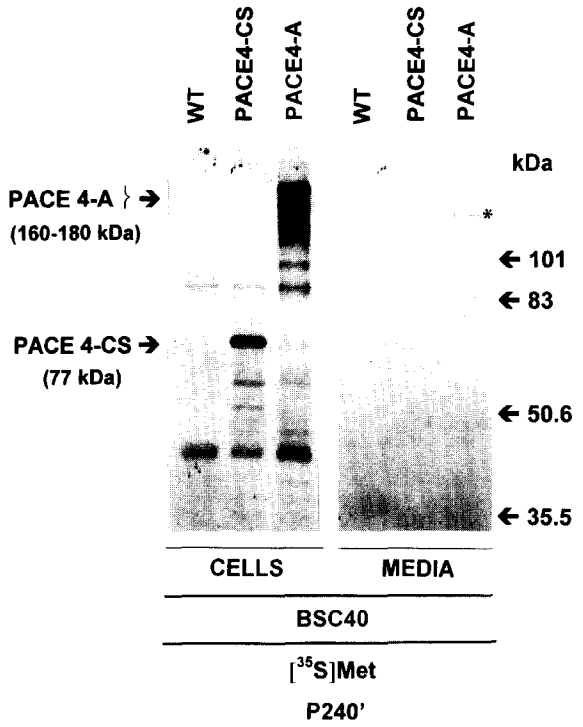

Fig. 3. SDS-PAGE analysis of the biosynthesis of PACE4-A and PACE4-CS. BSC40 cells were infected with 4 pfu/cell of either VV:WT, VV:PACE4-CS or VV:PACE4-A and then pulse-labeled for 240 min (P240) with [^{35}S]methionine. The media and cell extracts were then purified on a lentil lectin column and analyzed by SDS-PAGE (8% T, 2.7% C). Based on the migration of the molecular standards the molecular weights of PACE4-CS (77 kDa) and PACE4-A (160–180 kDa) were estimated.

4. Discussion

Tissue-specific processing of inactive precursors into active polypeptides is a general mechanism to generate and regulate the level of biological diversity achieved with a given proprotein. The role of the PCs in the generation of such diversity is now well accepted and is the subject of intense study aimed at defining the specific role of each of the seven known convertases in this process [1–5]. Another level of diversity can also be achieved by alternative splicing of either the proprotein substrate or its cognate convertase(s). For example, the differential intracellular localization of the two PC5-A and PC5-B isoforms is expected to affect the fate of different sets of precursors since PC5-B would cleave constitutively secreted proteins, whereas PC5-A could also process proteins sorted to dense-core secretory granules [11]. In a similar fashion, it was thought that the isoforms of PACE4 could also lead to different subcellular localizations of the resultant enzymes. Indeed, our results clearly show that whereas PACE4-A is secreted and active, PACE4-CS is retained intracellularly (likely in the ER) as an inactive proPACE4-CS zymogen. Therefore, analogous to profurin [31] and proPC1 [21] which remain in the endoplasmic reticulum and are not secreted, it is likely that the 77 kDa proPACE4-CS undergoes a similar fate. What then is the function of a seemingly inactive convertase in the ER? One possibility is that it may act as a dominant negative and affect the level of active PACE4-A, e.g. by interfering with its transport through the cellular secretory pathway. However, cellular co-expression of PACE4-A and PACE4-CS together with pro7B2 did not affect its processing to 7B2 by PACE4-A (S. Benjannet and N.G. Seidah, unpublished results). Therefore, although we still do not know the role of the PACE4 isoforms, it is possible that it is an accident of genetic noise as a result of evolutionary pressures.

The P-domain found at the C-terminus of the catalytic segment in the PCs is not found in the ancestral bacterial subtilisins and has been acquired by the eukaryotic PC genes. The function of this domain is not well known, but recent data suggest that it may play an important chaperone-like role in the folding of the zymogen, hence allowing autocatalytic pro-

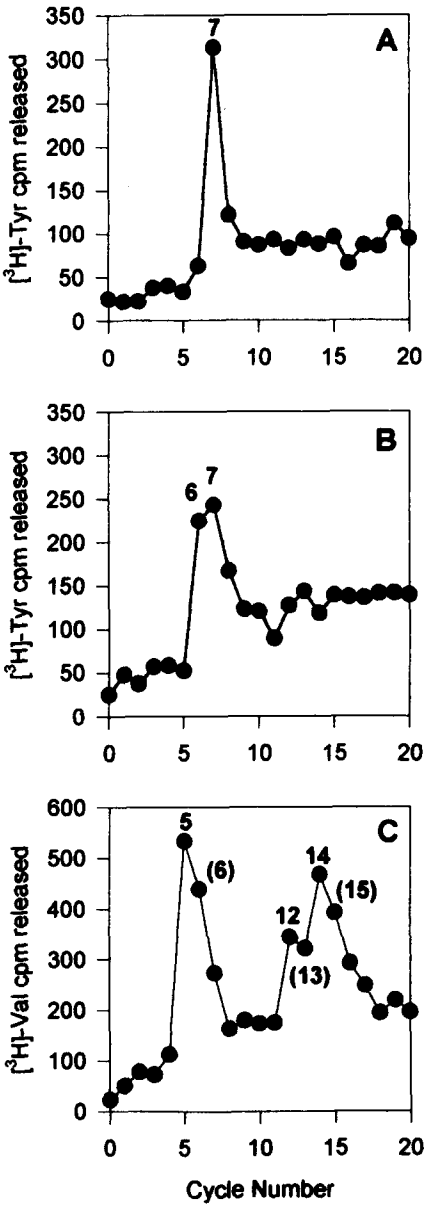

Fig. 4. Microsequence analysis of PACE4-CS. Radiolabeled 77 kDa PACE4-CS was obtained from the cell extracts of AtT20 (A) and BSC40 (B,C) cells pulsed for 4 h with either [^{3}H]tyrosine (A,B) or [^{3}H]valine (C). The labeled proteins were separated by SDS-PAGE (8% T, 2.7% C), and the gel was then sliced (1 mm) and the eluted 77 kDa proteins were sequenced for 20 cycles. Cycle 0 corresponds to a full sequencing cycle performed in the absence of the coupling reagent phenylisothiocyanate. The numbers above the peaks represent the deduced sequence positions.

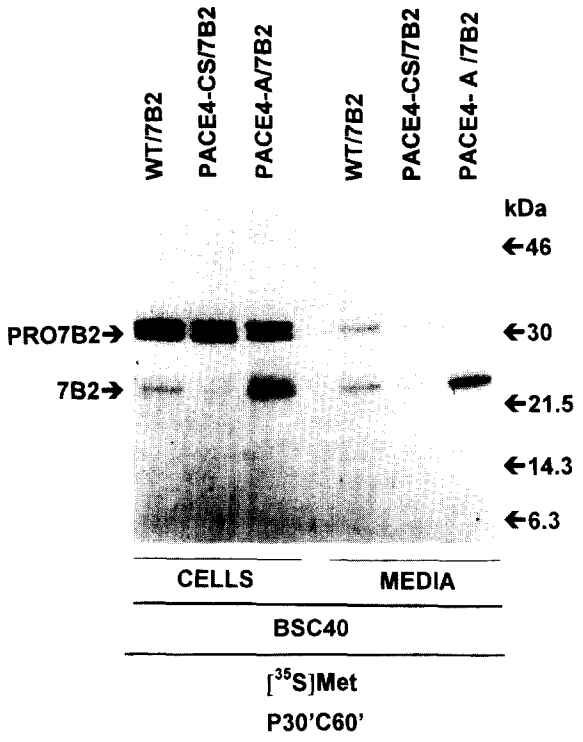

Fig. 5. Comparative processing of pro7B2 by PACE4-CS and PACE4-A. BSC40 cells were co-infected with VV:7B2 (2 pfu/cell) and with 4 pfu/cell of either VV:WT, VV:PACE4-CS or VV:PACE4-A and then pulse-labeled for 30 min (P30) with [35S]methionine, followed by a chase of 60 min (C60) in the presence of cold methionine. The media and cell extracts were then immunoprecipitated with a 7B2 antibody [20] and analyzed by SDS-PAGE (15% T, 1.3% C). The positions of pro7B2 (30 kDa) and 7B2 (23 kDa) are emphasized. The band above pro7B2 was observed previously [20] and is a non-specific protein which is not secreted.

cessing of the proPCs [14–16]. The integrity of this domain is obviously affected in the PACE4-C/CS isoforms as it lacks at least 11 C-terminal amino acids which are replaced by 36 or 3 a of different composition, respectively. This may explain why proPACE4-CS is not processed to PACE4-CS (Fig. 3), rendering the enzyme functionally inactive (Fig. 5). By analogy, we believe that PACE4-C should also code for an inactive enzyme.

In certain rat tissues such as the jejunum, duodenum and kidney, aside from the major 4.4 kb transcript of PACE4-A, a 3.9 kb mRNA was also detected [3]. However, Northern blot analyses of those same tissues with a PACE4-C/CS-specific probe did not reveal any labeling of specific transcripts (not shown), demonstrating that the 3.9 kb form is not PACE4-C/CS. In agreement with the mouse data of Hosaka et al. [13], the mRNA level of PACE4-C/CS seems to be low compared to PACE4-A in all the cell lines and rat tissues examined (Fig.). Intriguingly, a recent report suggested that PACE4-C immunoreactivity could be detected in rat pancreatic β-cells [18], which are also known to express PC1 and PC2 [2,32]. Furthermore, the authors claim that PACE4-A immunoreactivity was undetectable in pancreatic islets [18]. The peptide antigen used to obtain this PACE4-C-specific antibody [18] would not recognize PACE4-CS as these two isoforms differ at their C-terminus (Fig. 2). It remains to be seen whether PACE4-CS is also expressed in β-cells, as we did not study the expression of PACE4 in the pancreas [3]. However, we believe that the PACE4-C/CS isoforms would be functionally inactive and hence would not directly participate in the processing of proproteins in this tissue.

Using the von Heijne criteria [33], we estimated the best predicted signal peptidase cleavage site of human proPACE4 as ACS↓APPPRPVYTNHWAVQV and of rat/mouse PACE4 as ACSA↓LPPPRPVYTNHWAVQV [12,13]. The second best predicted site occurs at the AC↓SAPPP sequence. Interestingly, if this site was recognized by the signal peptidase, the N-terminal sequence of human PACE4-CS would reveal the absence of either the dipeptide AlaPro ($Val^{5,12,14}$ and Tyr^{6} in BSC40 cells) or SerAla ($Val^{6,13,15}$ in BSC40 cells and Tyr^{7} in AtT20 cells) (Fig. 4), suggesting an aminodipeptidase activity endogenous to BSC40 and AtT20 cells which recognizes these sequences. It is a matter of speculation whether rat/mouse proPACE4 would also lose its N-terminal LeuPro dipeptide. It has been reported that the secretory pathway of honeybee, amphibian skin and yeast [34] expresses a dipeptidylaminopeptidase with an X-Pro > X-Ala > X-Gly specificity, resembling the mammalian dipeptidylpeptidase enzyme. However, the restrictions on the nature of the X-residue are not yet clear. It would be informative to pursue this observation in the future and identify the putative mammalian homologue which could be related to the enzyme responsible for the reported stepwise processing of canine gastrin releasing peptide at the N-terminal ValPro-LeuPro sequence [34].

In conclusion, our data demonstrate that PACE4-A is likely to be the only active form of the PACE4 isoforms so far known. This does not exclude the possibility that other C-terminal variant forms which retain the integrity of the P-domain may turn out to be active enzymes. Thus far, differential splicing of convertase transcripts has resulted in multiple active forms only for PC5 where PC5-A and PC5-B were demonstrated to be functionally active convertases derived from a single *PC5* gene [11,20,22].

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