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 expression results demonstrated that PACE4-A codes for a functional secretable enzyme capable of cleaving pro7B2 into expression results demonstrated that PACE4-A codes for a functional secretable enzyme capable of cleaving pro7B2 into expression results demonstrated that PACE4-A codes for a functional secretable enzyme capable of cleaving pro7B2 into expression results demonstrated that PACE4-A codes for a functional secretable enzyme capable of cleaving pro7B2 into expression results demonstrated that PACE4-A codes for a functional secretable enzyme capable of cleaving pro7B2 into expression results demonstrated that PACE4-A codes for a functional secretable enzyme capable of cleaving pro7B2 into expression results demonstrated that PACE4-A codes for a functional secretable enzyme capable of cleaving pro7B2 into expression results demonstrated that PACE4-A codes for a functional secretable enzyme capable of cleaving pro7B2 into expression results demonstrated that PACE4-A codes for a functional secretable enzyme capable of cleaving pro7B2 into expression results demonstrated that PACE4-A codes for a functional secretable enzyme capable of cleaving pro7B2 into expression results demonstrated that PACE4-A codes for a functional secretable enzyme capable of cleaving pro7B2 into

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 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 expressing the motifs X-Ala and X-Pro.

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: TT-CATGACTGTCCTAAGTGGGAG, oligo VI: -GGACCTCAGTCCCTGCACGATC, and the PACE4-C/CS-specific oligo IV: CCTGGACATAAACGACG, the [HindIII]-containing oligo V: [AAGCCT]-GCGGAGCTAGAGATCCGCTGGAGA. The 30 cycle PCR

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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/Cys-specific PCRs at either 50°C (oligo pair II/III) or 55°C for the pair VII/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 pair II/V 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' NcoI and 3' HindIII 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 corticophrof 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 [35S]methionine, [3H]tyrosine or [3H]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) [3H]Tyr- and [3H]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 (VV:WT) [21]. Following overnight growth, the cells were washed and then pulse-labeled with [35S]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.

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 VII/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 itself a spliced form of PACE4-C, where the gt/ag donor/ac-

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PACE4-C is an enzymatically active convertase. 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 PCI [27]. Accordingly, although appreciable activity towards the fluorogenic substrate pGluArgThrLysArgMCA was observed for PACE4-A (Munzer et al., in preparation), none was obtained for PACE4-CS (not shown). From the predicted protein sequence 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 G4 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 PCI [27]. Accordingly, although appreciable activity towards the fluorogenic substrate pGluArgThrLysArgMCA 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 (30 kDa) product, which is known to be processed in the TGN by furin-like enzymes [20,30]. Accordingly, although appreciable activity was observed for PACE4-A (Munzer et al., in preparation), none was obtained for 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 G4 in AtT20 cells. Furthermore, the data demonstrate that proPACE4-CS is not processed into PACE4-CS.

In order to unequivocally define the nature of the 77 kDa PACE4-CS product, we microsequenced the [3H]Val- and [3H]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 in AtT20 cells (Fig. 4A) and a Tyr 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 Val1214 and Val1213,15 (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 Pro64 or Pro65 in BSC40 cells and at Pro64 in AtT20 cells. Furthermore, the data demonstrate that proPACE4-CS is not processed into PACE4-CS.

The 77 kDa product of PACE4-C is secreted, whereas PACE4-A is not. In BSC40 cells, the PACE4-A product is secreted, whereas PACE4-A is not. Therefore, 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 PCI [27]. Accordingly, although appreciable activity towards the fluorogenic substrate pGluArgThrLysArgMCA 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 (30 kDa) product, which is 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.
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. 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 [35S]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

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 [3H]tyrosine (A,B) or [3H]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.
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. 3). However, the restrictions on the nature of the X-residue recognition 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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