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A Novel Human PACE4 Isoform, PACE4E Is an Active Processing Protease Containing a Hydrophobic Cluster at the Carboxy Terminus[1]

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PACE4 is a processing protease which processes the precursor protein to the mature protein. Currently, four PACE4 isoforms have been reported [Tsuji, A. *et al.* (1994) *Biochem. Biophys. Res. Commun.* 200, 943-950]. In this study, we have cloned cDNA encoding a novel isoform, PACE4E, by screening the human brain cerebellum cDNA library and reverse transcriptase-polymerase chain reaction analysis of total RNA from human hepatoma HepG2 cells. The PACE4E cDNA encoded an amino acid sequence of 975 residues. The sequence from the amino terminus to Arg^{900} of PACE4E was identical to the corresponding sequence of PACE4A, but the carboxy terminal sequence (75 residues) was unique and contained a hydrophobic cluster (Leu^{952}-Gly^{968}). PACE4E cDNA was transiently transfected in COS-1 cells, and the expressed proteins were a 112-kDa precursor form and a 105-kDa mature form. They were secreted into the culture medium, but their secretion was retarded compared with that of PACE4A. The expression of a mutant of PACE4E truncated up to the hydrophobic cluster from the carboxy terminus resulted in a remarkable increase in secretion level, suggesting that PACE4E tends to be retained intracellularly due to interaction with the membrane through the hydrophobic cluster. On the contrary, the transient expression experiment of PACE4C showed that only 68-kDa protein (precursor form) was detected in the cell and not secreted into the medium. In addition, coexpression experiment revealed that PACE4E was able to process the precursor form of von Willebrand factor to the mature form, but PACE4C did not process it.

Key words: kexin-like protease, PACE4E, PACE4 isoform, proprotein processing, subtilisin like proprotein convertase 4.

Many bioactive peptides and proteins are initially synthesized as inactive precursors, which undergo post-translational modification to generate biologically active products. An important step in this modification is the limited endoproteolytic cleavage of larger precursors, such as peptide hormones, growth factors, plasma proteins, receptors, and viral envelope proteins, and these proteolytic cleavages occur mostly at the site of paired basic amino acid residues (*1-3*). Kexin, encoded by the *KEX2* gene of *Saccharomyces cerevisiae*, was first identified as a processing protease with such cleavage specificity (*4-7*). It is a Ca^{2+}-dependent serine protease with a subtilisin-like catalytic domain and involved in processing of pro-α-mating factor and pro-killer toxin in yeast. Currently, seven mammalian kexin-like proteases have been identified by homology-based polymerase chain reaction methodology. These include furin (*8-10*), PC1 (also called PC3) (*11, 12*), PC2 (*11, 13*), PACE4 (*14-16*), PC4 (*17, 18*), PC6 (also called PC5) (*19-21*), and PC7 (also called PC8) (*22-24*), all of which share structural similarities that are marked by a signal peptide, a propeptide, a subtilisin-like catalytic domain, and a HomoB domain as the basic domain structure. Individual kexin-like proteases exhibit unique tissue distributions, suggesting that each has a tissue-specific function.

PACE4 (renamed as PACE4A) was first cloned from the cDNA library of human hepatoma HepG2 cells (*14*). The PACE4A protein possesses a cysteine-rich region at its carboxy terminal in addition to the basic domain structure of kexin-like protease. Kiefer *et al.* showed the wide tissue distribution of PACE4 as well as furin by Northern blotting (*14*). However, a high level of expression of PACE4 mRNA is found in both specific endocrine and non-endocrine tissues, such as pituitary, heart, and liver (*25*). In particular, PACE4 mRNA is expressed in high level in the anterior pituitary, and its expression is regulated by thyroid status (*15*). Detailed mapping by *in situ* hybridization showed

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; pro-C3, precursor form of C3; pro-renin, precursor form of renin; pro-vWF, precursor form of von Willebrand factor; SSC, standard sodium citrate.

that PACE4 expression is specifically restricted with high level in both neuronal and non-neuronal cells in the central nervous system (*26*). In contrast, furin is expressed in a ubiquitous manner.

Previously, we have isolated cDNAs encoding two isoforms, PACE4C and PACE4D, from a human placenta cDNA library (*27*). PACE4C lacks only the cysteine-rich region, while PACE4D additionally lacks the signal peptide and propeptide. Recently, we have determined the organization of human PACE4 gene and shown that these multiple isoforms are products of alternative splicing of the primary transcript of a single gene (Tsuji, A., unpublished results). Immunohistochemistry of rat tissues showed that each PACE4 isoform exhibits a cell-specific expression. For example, PACE4C is strongly and specifically expressed in the B-cells of the Langerhans islets in the pancreas, but no expression of PACE4A is found in the islets (*28*). These data suggest that each isoform plays a role in particular cells and that their expressions are highly regulated.

In this study, we have identified a cDNA encoding a novel PACE4 isoform from human brain cerebellum cDNA library. Interestingly, the predicted PACE4E protein contains a hydrophobic cluster at the carboxy terminus. Other isoforms do not contain such a hydrophobic cluster. It is known that the unique carboxy terminal domain of kexin-like protease contains a factor for intracellular localization. For example, furin contains a cytoplasmic tail that localizes it within the *trans*-Golgi network (*29, 30*). The carboxy terminal domain of PC1 plays an important role in the routing or storage of the molecule in the secretory granules (*31*). In view of this point, we investigated the biosynthesis of PACE4E by using the overexpression in COS-1 cells to define the function of its hydrophobic cluster.

MATERIALS AND METHODS

Screening of cDNA Library and DNA Sequence Analysis—About one million plaques from a human brain cerebellum λgt11 cDNA library (CLONTECH, CA, USA) were screened by plaque hybridization with the ^{32}P-labeled probe using the Colony/Plaque Screen (DuPont/NEN Research Products, MA, USA). Screening probe was PACE4A *Sac*I-*Pst*I probe (nucleotides 1951-2359 in the PACE4A-I cDNA). Inserts of positive clones were subcloned into the *Eco*RI site of pUC18. Both strands were sequenced by dideoxy-termination method using *Bca* BEST™ Dideoxy Sequencing Kit (Takara, Kyoto, Japan) and automated A.L.F. DNA Sequencer II (Pharmacia, Uppsala, Sweden).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)—Total RNA from HepG2 cells was isolated using ISOGEN (Nippon Gene, Tokyo, Japan) and then reverse-transcribed with SUPER SCRIPT™ II (Gibco BRL, MD, USA). PCR was performed using *TaKaRa Ex Taq* (Takara). The sense primer sequences were as follows: P-5′NCR, 5′-GAGCGGCTTTAAAAGGCGGCACTC-3′ (corresponding to nucleotides from −74 to −51 in the PACE4A-I cDNA); P-Pro, 5′-TACCTCAACTTGGGCCAGATT-3′ (nucleotides 280-300); P-SCD, 5′-GACGACGGCAAGACGGTGGACGGG-3′ (nucleotides 928-951); P-HomoB, 5′-CCCTCGGGAACCAAGTCTCAACTT-3′ (nucleotides 1687-1710). The sequence of antisense primer was P-E, 5′-GCTGGACACAGTTGCCTGCCGTTC-3′ (nucleotides 2719-2742 in the PACE4E-I cDNA). The reaction conditions were as follows: 98°C for 1 min, then 30 cycles of 98°C for 20 s, 60°C for 1 min, and 72°C for 10 min. PCR products were analyzed by Southern blotting with the PACE4A *Sac*I-*Pst*I probe and sequencing.

Construction of Expression Plasmids—The full-length human PACE4 isoform cDNAs (PACE4A-I, A-II, E-I, and E-II) were constructed by combining the HPL-11, 12 (*27*) and HBC-27, 66. Carboxy terminal truncation of PACE4E-I was performed by replacing Phe^{945} (TTT) of PACE4E-I to a stop codon (TAG). The mutation was performed using the U.S.E. Mutagenesis Kit (Pharmacia). The sequence of the target mutagenic primer was 5′-CAGGAGCCCATAGACAGAGCTGC-3′. The resulting clone was designated as PACE4E-I⊿945. The resulting cDNAs were cloned into the *Xho*I site of eukaryotic expression vector pSVL (Pharmacia) and pRc/CMV (Invitrogen, CA, USA) after confirmation of their sequences. The expression plasmid for the human pro-von Willebrand factor (pSVLvWF) (*32*), the rat complement pro-C3 (*33*) and the mouse pro-renin mutant (M2R^{-4} pro-renin) (*3*) were kindly provided by Dr. J.A. van Mourik (Central Laboratory of the Netherlands Red Cross), Dr. Y. Ikehara (Fukuoka University, Fukuoka), and Drs. K. Nakayama and K. Murakami (University of Tsukuba, Tsukuba), respectively.

Preparation of Anti-Human PACE4 Antibody—Anti-PACE4 subtilisin-like catalytic domain (SCD) antiserum was raised in rabbit against the glutathione *S*-transferase-PACE4 SCD (Cys^{263}-Leu^{448}) fusion protein, which was expressed and purified using the pGEX-2T prokaryotic expression system (Pharmacia). The specificity of the antiserum was confirmed by immunoprecipitation of recombinant PACE4A-I secreted from CHO cells stably expressing human PACE4A. A single band consistent with the size of the PACE4A-I in the culture medium was detected. Antibodies for human von Willebrand factor and rat complement C3 were purchased from DAKO (Glostrup, Denmark) and CAPPEL (NC, USA), respectively. Anti-mouse renin serum was kindly provided by Drs. K. Nakayama and K. Murakami (University of Tsukuba).

Transfection Analysis—Transfection was performed using the protocol of Lopata *et al.* (*34*) with minor modifications. Subconfluent cells (~3×10^5 cells) in a 35-mm dish were incubated for 1 h with DEAE-dextran (125 μg) and the expression plasmid (5 μg) in 3 ml of DMEM. Then chloroquine (52 μg/ml) in 1.2 ml of DMEM supplemented with 10% FCS was added, and these cells were incubated for 5 h. The transfected cells were washed with PBS, then incubated in DMEM supplemented with 10% FCS. After transfection for 60 h, the cells were labeled with 200 μCi/ml Pro-Mix (a mixture of ~70% [^{35}S]-methionine and ~30% [^{35}S]cysteine, Amersham, England) in methionine and cysteine-free DMEM supplemented with 10% dialyzed FCS for 6 or 12 h. For pulse-chase analysis, the transfected cells were labeled for 1 h and then chased in DMEM supplemented with 10% FCS, 1 mM methionine, and 1 mM cysteine for the indicated period.

The radiolabeled cells were lysed in the immunoprecipitation buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, and 2 mM EDTA) containing various protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A). These protease

inhibitors were also added to the culture medium. Appropriate antiserum was added to the cell lysate and the culture medium. After 1 h, protein A-Sepharose (Pharmacia) was added and the mixture was incubated with rotary mixing at 4°C for 1 h. The sample was centrifuged, and the pellet was washed four times with washing buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 100 μg/ml PMSF, 1 μg/ml aprotinin). The sample was boiled in the 2× sample buffer and resolved on SDS-polyacrylamide gel by electrophoresis under reducing conditions by the method of Laemmli (*35*). The gel was then treated with Amplify (Amersham) and dried. The gel bands of radioactivity were analyzed using the BAS-1500 bioimaging analyzer.

RESULTS

cDNA Cloning of Novel Human PACE4 Isoform; PACE-4E—To isolate novel PACE4 isoforms, human brain cerebellum cDNA libraries were screened with the PACE4A *Sac*I-*Pst*I probe, and we thereby isolated five clones (HBC-27, 1.3 kbp; HBC-50, 1.0 kbp; HBC-64, 1.3 kbp; HBC-65, 1.3 kbp; and HBC-66, 2.5 kbp) which contain a unique nucleotide sequence at the 3′ end, although their 5′ ends were identical to PACE4A cDNA (Fig. 1A). These clones were derived from the same mRNA, and we designated the protein encoded by these clones as PACE4E, a novel PACE4 isoform. Three and five clones of PACE4E were also isolated from human pituitary and placenta cDNA libraries (data not shown).

To show the presence of the full-length PACE4E cDNA, we performed RT-PCR using the total RNA from HepG2 cells. The sense primers were designed based on the sequences of several regions of PACE4A. The antisense primer corresponds to the specific region of PACE4E (Fig. 1A). As shown in Fig. 1B, each resulting DNA fragment of the predicted size (P-5′NCR, 2,816 bp; P-Pro, 2,463 bp; P-SCD, 1,815 bp; P-HomoB, 1,056 bp) was amplified, and all PCR products were hybridized with PACE4A *Sac*I-*Pst*I probe. The identity of these products was confirmed by DNA sequencing. These results indicate that the nucleotide sequence of the missing 5′-terminal part of PACE4E cDNA is completely the same as the corresponding part of PACE4A cDNA, in contrast to that of PACE4D. A smaller PCR product (1.4 kbp) compared with predicted size was also amplified with P-5′NCR primer (Fig. 1B). Since no smaller PCR product was amplified with other sense primers, the 1.4-kbp product may encode a shorter PACE-4E isoform that lacks at least part of the region between P-Pro and P-HomoB primers, but this fragment was not analyzed further. Figure 2A shows the predicted amino acid sequences of human PACE4E(-I). The carboxy terminal amino acid sequence (75 residues) of PACE4E(-I) showed no significant homology with those of other PACE4 isoforms. PACE4E contains only one potential glycosylation site (Asn^{259}), which is conserved among PACE4 isoforms in the catalytic domain, whereas PACE4A contains three sites. From the hydropathy profile of the unique carboxy terminal domain (data not shown), which is generated according to Kyte and Doolittle (*36*), PACE4E contains a hydrophobic cluster (Leu^{952}-Gly^{968}).

PACE4E Is Further Classified into Two Isoforms, Type-I and Type-II—DNA sequence analysis revealed that HBC-27 lacks the 39-bp nucleotide sequence (2039-2077) at the boundary between the HomoB and the cysteine-rich region (Fig. 2B). Therefore, the amino acid sequence (Ala^{680}-Ser^{693}) of this clone is substituted for Gly by this deletion. These data indicated that PACE4E is further classified into two isoforms, type-I and type-II (Fig. 2B). In a previous study (*27*), we obtained the cDNA fragment for PACE4A (HPL-11) from a human placenta cDNA library. This clone also had a deletion in the same region, and therefore PACE4A can be also classified into two isoforms. Our results revealed that PACE4 consists of seven species of isoform (A-I, A-II, B, C, D, E-I, E-II) as shown in Fig. 3.

Biosynthesis of PACE4 Isoforms in COS-1 Cells—The COS-1 cells transiently transfected with the full-length isoform cDNA construct were used to study the biosynthesis of PACE4 isoforms. As shown in Fig. 4A, the diffuse 110-kDa (major) and 103-kDa (minor) species for PACE-4A-I, 68-kDa species for PACE4C, and 112-kDa (major) and 105-kDa (minor) species for PACE4E-I were detected in the cell lysate. Digestion of these proteins with *N*-gly-

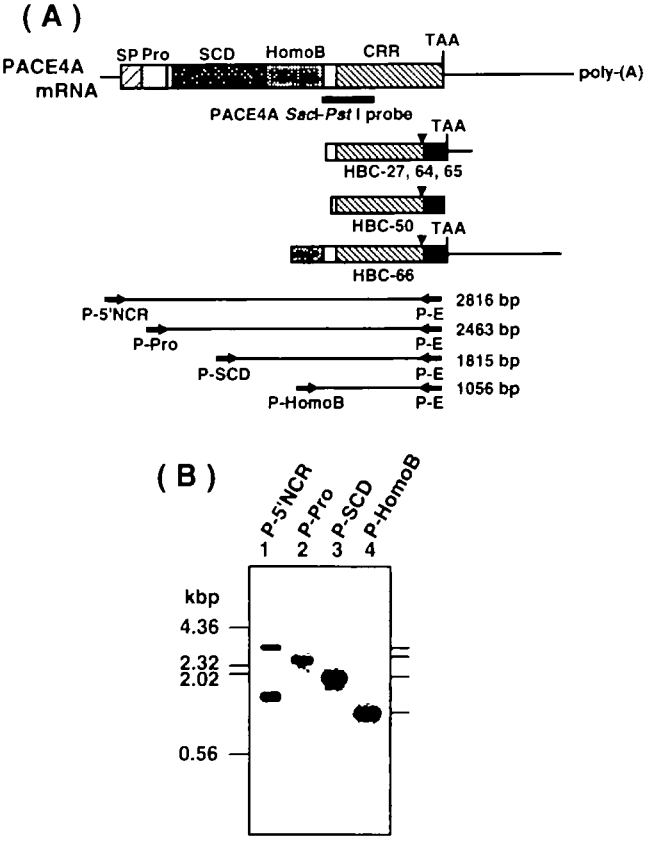

Fig. 1. **Schematic representation of the PACE4E cDNA clones and PCR analysis of PACE4E transcript in the HepG2 cells.** (A) Structural comparison of PACE4E cDNA isolated from human brain cerebellum cDNA library with PACE4A cDNA. SP, signal peptide; Pro, propeptide; SCD, subtilisin-like catalytic domain; CRR, cysteine-rich region. The black box shows the PACE4E-specific region. The positions of PCR primers are indicated by arrows and the theoretical size corresponding to each primer combination is also indicated. (B) Southern blot analysis of RT-PCR products obtained with the HepG2 RNA as template using primers common to PACE4 isoforms (P-5′NCR, P-Pro, P-SCD, P-HomoB) and PACE4E-specific primer (P-E). The position of the probe (PACE4A *Sac*I-*Pst*I) used to hybridize the Southern blot is indicated in (A).

(A)

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  1: MPPRAPPAPG PRPPPRAAAA TDTAAGAGGA GGAGGAGGPG FRPLAPRPWR
 51: WLLLLALPAA CSAPPPRPVY TNHWAVQVLG GPAEADRVAA AHGYLNLGQY
101: GNLEDYYHFY HSKTFKRSTL SSRGPHTFLR MDPQVKWLQQ QEVKRRVKRQ
151: VRSDPQALYF NPPIWSNMWY LHCGDKNSRC RSEMNVQAAW KRGYTGKNVV
201: VTILDDGIER NHPDLAPNYD SYASYDVNGN DYDPSPRYDA SNENKHGTRC
251: AGEVAASANN SYCIVGIAYN AKIGGIRMLD GDVTDVVEAK SLGIRPNYID
301: IYSASWGPDD DGKTVDGPGR LAKQAFEYGI KKGRQGLGSI FVWASGNGGR
351: EGDYCSCDGY TNSIYTISVS SATENGYKPW YLEECASTLA TTYSSGAFYE
401: RKIVTTDLRQ RCTDGHTGTS VSAPMVAGII ALALEANSQL TWRDVQHLLV
451: KTSRPAHLKA SDWKVNGAGH KVSHFYGFGL VDAEALVVEA KKWTAVPSQH
501: MCVAASDKRP RSIPLVQVLR TTALTSACAE HSDQRVVYLE HVVVRTSISH
551: PRRGDLQIYL VSPSGTKSQL LAKRLLDLSN EGFTNWEFMT VHCWGEKAEG
601: QWTLEIQDLP SQVRNPEKQG KLKEWSLILY GTAEHPYHTF SAHQSRSRML
651: ELSAPELEPP KAALSPSQVE VPEDEEDYTA QSTPGSANIL QTSVCHPECG
701: DKGCDGPNAD QCLNCVHFSL GSVKTSRKCV SVCPLGYFGD TAARRCRRCH
751: KGCETCSSRA ATQCLSCRRG FYHHQEMNTC VTLCPAGFYA DESQKNCLKC
801: HPSCKKCVDE PEKCTVCKEG FSLARGSCIP DCEPGTYFDS ELIRCGECHH
851: TCGTCVGPGR EECIHCAKNF HFHDWKCVPA CGEGFYPEEM PGLPHKVCRR
901: YGPPGGERQA TVSSKGVPGG QSLSASSPGA GEGMLHHPTV DRSPFTELLR
951: GLRPFVHWMH LICWVPAVGR HRAAAG
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(B)

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PACE4 type-I   680: A Q S T P G S A N I L Q T S
(PACE4-I)     2039: CTCAATCCACCCAAGGCTCTGCTAATATTTTACAGACCA

PACE4 type-II 2029: GATTACACAGGTGTGTGCCAT
(PACE4-II)     677: D Y T G V C H
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Fig. 2. **Predicted amino acid sequence of human PACE4E type-I and type-II.** (A) Predicted amino acid sequence of PACE4E. The putative cleavage sites of signal peptide and propeptide are indicated by an arrowhead and an arrow, respectively. The active-site Asp, His, Ser, and Asn are indicated by closed circles. The potential Asn-linked glycosylation site is shown by a closed square. The unique sequence of PACE4E is double underlined. The hydrophobic cluster is shaded. The sequence (residues 680-693) which is deleted in PACE4E type-II is indicated by a single underline. The nucleotide sequence data will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with accession numbers D87993 (PACE4E-I) and D87994 (PACE4E-II). (B) The deletion of amino acid sequence (residues 680-693) in PACE4E and PACE4A type-II cDNA. Because this deletion does not cause a frame-shift, the amino acid sequence downstream of the deletion is the same in type-I and type-II.

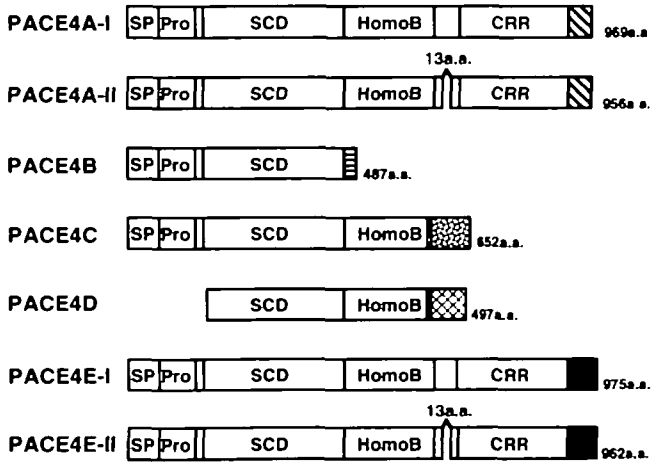

Fig. 3. **Comparison of the domain structure of PACE4 isoforms.** PACE4 exhibits seven species of isoforms as indicated. The isoform-specific sequences located at the carboxy termini are indicated by various patterns.

canase F, which hydrolyzes all types of Asn-linked glycan chain from proteins, resulted in 97.4- and 90-kDa species for PACE4A-I, 60-kDa species for PACE4C, and 98- and 90-kDa species for PACE4E-I (data not shown). These molecular masses estimated after *N*-glycanase F digestion were coincident with the predicted sizes from their cDNA structures, suggesting that 110- and 112-kDa species for PACE4A-I and E-I and the 68-kDa species for PACE4C are precursor forms, and that 103- and 105-kDa species for PACE4A-I and E-I are the mature forms.

In the culture medium, PACE4A proteins were clearly detected as the 103-kDa mature form and minor forms with lower molecular weights that seem to be degradation products, although Rehemtulla *et al.* reported that PACE-4A is not secreted and is expressed as cell-associated protein (*37*). PACE4E proteins were also detected, but the secretion level of PACE4E was half of that of PACE4A. Most of the secreted protein of PACE4E had lower molecular weight than the intracellular species, suggesting that the mature form of PACE4E is degraded quickly. On the other hand, PACE4C protein was not secreted at all into the culture medium, in spite of high level expression in the cells. Further analysis by pulse-chase experiment showed that the precursor forms of PACE4E and PACE4A are

converted into mature forms with the same efficiency at a chase period of 0.5 h and that the PACE4E species is secreted into the culture medium after a chase period of 3 h, whereas the PACE4A species is secreted after 0.5 h (data not shown). Thus, PACE4E seemed to mature but to be retained intracellularly for a short while.

Deletion Analysis of PACE4E-Specific Region at Its Carboxy Terminus—As mentioned above, the secretion of PACE4E was retarded compared with that of PACE4A. To confirm that retardation of PACE4E secretion is caused by a structural feature of its carboxy terminus, a mutant of PACE4E-I (PACE4E-I⊿945) in which the carboxy terminal sequence (Phe^{945}-Gly^{975}) containing the hydrophobic cluster is truncated was expressed in COS-1 cells and its secretion into the culture medium was analyzed. As shown in Fig. 4B, expression of PACE4E-I⊿945 resulted in a remarkable increase in secretion level of PACE4E protein compared with the wild type. These results suggested that PACE4E protein probably interacts with intracellular membranes or some protein through the hydrophobic cluster at its carboxy terminus and is thereby retained intracellularly.

Processing Activities of PACE4 Isoforms—PACE4E-I and E-II are considered to be functional proteases like PACE4A, because they have most of the basic domain structures (signal peptide, propeptide, subtilisin-like catalytic domain, HomoB domain) that are essential for the expression of proteolytic activity of kexin-like protease. To assess the processing activity of these isoforms, we performed the transient coexpression of each PACE4 isoform with precursor proteins (human pro-vWF, rat complement pro-C3, mouse pro-renin mutant $M2R^{-4}$) in COS-1 cells and analyzed the processing of the secreted precursor proteins. These precursor proteins contain an Arg-*X*-Lys/Arg-Arg furin-like protease cleavage motif at the processing site, but the amino acid sequences of the processing sites are not the same. When either pro-vWF, pro-C3, or pro-renin $M2R^{-4}$ alone was expressed in COS-1 cells, these precursor proteins were processed to mature forms (220-

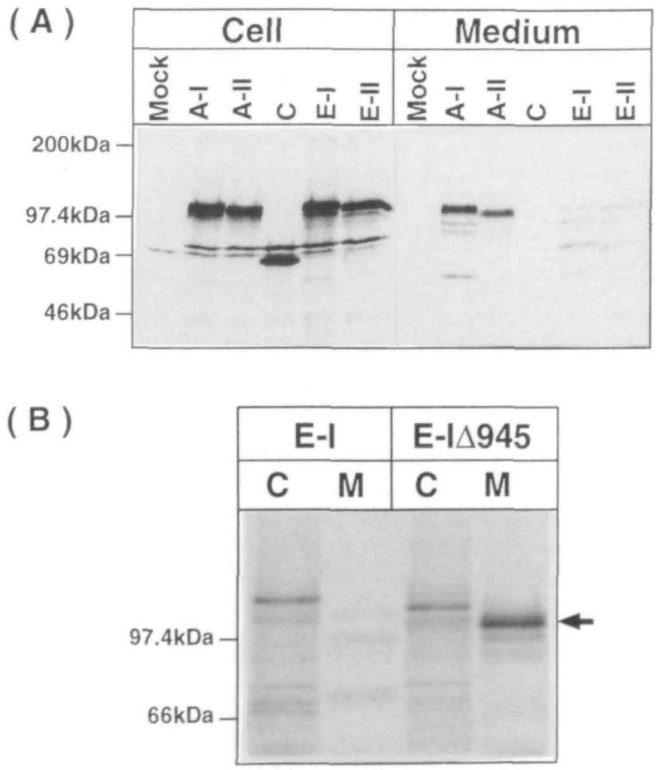

Fig. 4. **Biosynthesis of the PACE4 isoform (A) and PACE4E mutant (B) in transiently transfected COS-1 cells.** (A) COS-1 cells were transfected with each cDNA cloned into pSVL. PACE4 isoforms were radio-labeled for 6 h and immunoprecipitated from the cell lysate as described under "MATERIALS AND METHODS." The secreted enzymes were also immunoprecipitated from the culture medium. As a negative control, COS-1 cells were transfected with pSVL vector which had no insert (Mock). Immunoprecipitated proteins were analyzed by 7% SDS-polyacrylamide gel electrophoresis and visualized using bioimaging analyzer. (B) COS-1 cells were transfected with full-length PACE4E-I and its deletion mutant (PACE4E-I) cDNA cloned into pRc/CMV. The respective enzymes were immunoprecipitated from the cell lysate and the culture medium. Arrow indicates the mature form of PACE4E-I⊿945. C, cell lysate; M, culture medium.

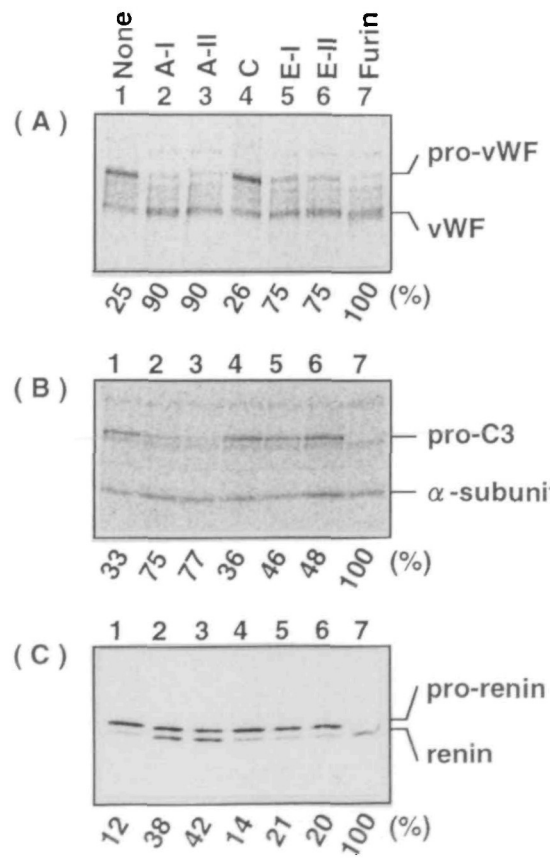

Fig. 5. **Analysis of the processing activities of PACE4 isoforms.** COS-1 cells were transfected with expression plasmids for pro-vWF (panel A), complement pro-C3 (panel B), or renin mutant $M2R^{-4}$ (panel C) in either the absence (lane 1) or the presence of the expression plasmids for PACE4A-I (lane 2), PACE4A-II (lane 3), PACE4C (lane 4), PACE4E-I (lane 5), PACE4E-II (lane 6), or furin (lane 7). After transfection for 60 h, the cells were radio-labeled for 12 h, then the resulting culture medium was immunoprecipitated as described under "MATERIALS AND METHODS." The immunoprecipitants were resolved on SDS-polyacrylamide gel (5%, vWF; 7.5%, C3; 10%, renin). The processing efficiency is indicated below each panel. The positions of precursor (pro-) and mature forms of the substrate are indicated on the right. Two independent experiments provided similar results.

kDa, 115-kDa α-subunit and 38-kDa, respectively) inefficiently (25, 33, or 12%, respectively) by endogenous furin-like activity (Fig. 5, lane 1). However, these precursor proteins were processed to mature forms completely by coexpression with furin (Fig. 5, lane 7). As shown in Fig. 5A, PACE4A-I and A-II were able to process pro-vWF, which possesses an Arg-Ser-Lys-Arg cleavage site (each 90%; lanes 2 and 3). PACE4E-I and E-II also processed it (each 75%; lanes 5 and 6). We then examined the processing of complement pro-C3, which is converted into α- and β-subunits by limited proteolysis at the Arg-Arg-Arg-Arg cleavage site. As shown in Fig. 5B, 75 and 77% of pro-C3 was processed by PACE4A-I and A-II, respectively (lanes 2 and 3). PACE4E-I and E-II also processed it with low efficiency (46 and 48%, respectively; lanes 5 and 6). Finally, as shown in Fig. 5C, pro-renin M2R^{-4}, which possesses an Arg-Thr-Lys-Arg cleavage site, was processed slightly by PACE4A-I and A-II (38 and 42%, respectively; lanes 2 and 3). PACE4E-I and E-II also processed it with low efficiency (21 and 20%, respectively; lanes 5 and 6). On the other hand, no processing activity of PACE4C for these precursor proteins was detectable (Fig. 5, lane 4). These data suggest that the substrate specificities of PACE4A and E are similar but not identical.

DISCUSSION

In this study, we identified an active PACE4 isoform, PACE4E, from a human brain cerebellum cDNA library. The predicted amino acid sequence of PACE4E is identical to the corresponding sequence of PACE4A except for the carboxy terminal sequence of 75 residues (Fig. 2A). Interestingly, this specific sequence contains a hydrophobic cluster (Leu^{952}-Gly^{968}). PACE4E mRNA was expressed not only in HepG2 cells but also in human neuroblastoma IMR-32 cells (data not shown). The existence of PACE4E transcript was confirmed by analysis of human PACE4 genomic DNA. The gene analysis revealed that the exon encoding the PACE4E-specific sequence is located downstream of the exon encoding the cysteine-rich region, indicating that PACE4E transcript is generated *via* alternative splicing of the same primary transcript of PACE4 (Tsuji, A., unpublished results). We also showed that PACE4E is further classified into two isoforms, type-I and type-II, which are defined by deletion of a 39-bp nucleotide sequence located just before the cysteine-rich region (Fig. 2B). This 39-bp deletion was confirmed by RT-PCR analysis of total RNA from IMR-32 cells and rat brain (*38*). On the other hand, human PACE4A cDNA, first reported by Kiefer *et al.*, contained this 39-bp sequence (*14*), but HPL-11 cDNA clone (*27*), which we isolated from a human placenta cDNA library, and which encodes the carboxy terminal region of PACE4A, did not. The cDNA sequences of rat and mouse PACE4A reported previously (*15, 16*) had a 39-bp deletion of nucleotide sequence in the same region. The gene analysis also demonstrated that type-II of PACE4 is generated by the skipping of a single exon encoding its 39-bp sequence. These results indicate that type-I and type-II isoforms are present in both PACE4E and PACE4A cDNAs.

PACE4E and PACE4A were expressed efficiently in transiently transfected COS-1 cells. However, they differed in the degree of secretion and the molecular size of secreted protein. PACE4E expressed in COS-1 cells was secreted into the culture medium to a lesser extent than PACE4A. Most of the secreted PACE4E had lower molecular weight than the intracellular protein. Furin, PC6B, and PC8/7 possess a hydrophobic cluster as the transmembrane domain at the carboxy terminus, which causes them to be retained intracellularly (*8-10, 19-23, 29, 30*). PACE4E also possesses a hydrophobic cluster (Leu^{952}-Gly^{968}) at the carboxy terminus. The hydrophobicity scale of this cluster is low compared with those of the transmembrane domains of furin, PC6B, and PC8/7 based on the hydropathy profile according to Kyte and Doolittle (*36*). However, PACE4E seemed to interact with intracellular membranes or some protein through this cluster. This possibility is supported by the finding that the carboxy terminal-truncated mutant, PACE4E-IΔ945, was well secreted (Fig. 4B). On the other hand, no PACE4C was secreted into the culture medium. It is likely that PACE4C expressed in COS-1 cells remains in the endoplasmic reticulum without activation, as do precursor forms of furin and PC1 (*39, 40*). Recently, Zhong *et al.* reported that the carboxy-terminally shortened version of PACE4C, PACE4CS, is also expressed as an inactive precursor form in BSC40 cells (*41*). These data suggested that each PACE4 isoform is localized intracellularly at a different site.

Coexpression experiments of PACE4 isoforms with several precursor proteins revealed that PACE4E is definitely a functional protease, but its processing activity seems to be weaker than that of PACE4A under these conditions.

To elucidate the physiological role of PACE4E, it is also important to determine its tissue distribution. Dong *et al.* showed the distribution of PACE4 mRNA in rat central nervous system by *in situ* hybridization analysis (*26*). Although PACE4 mRNA was expressed at low or undetectable levels in neuronal and glial cells, a high level of expression was found in particular cells, such as medial habenular and cerebellar Purkinje cells. However, the distribution of each PACE4 isoform was not determined, because the probe used in their experiments hybridized to mRNAs of all PACE4 isoforms. Our results from *in situ* hybridization analysis with the PACE4E-specific probe revealed that PACE4E was expressed predominantly in some restricted cells (Akamatsu, T. *et al.*, unpublished results).

In conclusion, the novel isoform, PACE4E, differed from the previously identified PACE4 isoforms in two important aspects. (1) Only PACE4E possesses a hydrophobic cluster through which it is retained intracellularly. (2) PACE4E has similar but not identical processing activity to PACE4A. These data suggest that PACE4E may have a distinct and important functional role in PACE4 physiology. Currently, PACE4 has been reported to be responsible for processing of various precursor proteins within the constitutive secretory pathway. However, PACE4 may process precursor protein within the regulated secretory pathway, because a high level of expression of PACE4 is found not only in non-endocrine but in endo/neuroendocrine tissues and cells (*25, 26, 28*). It is, therefore, important to investigate further the intracellular localization of each PACE4 isoform in the regulated cells.

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