Endoprotease PACE4 is Ca^{2+}-dependent and temperature-sensitive and can partly rescue the phenotype of a furin-deficient cell strain

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PACE4 is a member of the eukaryotic subtilisin-like endoprotease family. The expression of human PACE4 in RPE.40 cells (furin-null mutants derived from Chinese hamster ovary K1 cells) resulted in the rescue of a number of wild-type characteristics, including sensitivity to Sindbis virus and the ability to process the low-density-lipoprotein receptor-related protein. Expression of PACE4 in these cells failed to restore wild-type sensitivity to *Pseudomonas* exotoxin A. Co-expression of human PACE4 in these cells with either a secreted form of the human insulin pro-receptor or the precursor form of von Willebrand factor resulted in both proproteins being processed; RPE.40 cells were unable to process either precursor protein in the absence of co-expressed PACE4. Northern analysis demonstrated that untransfected RPE.40 cells express mRNA species for four PACE4 isoforms, suggesting that any endogenous PACE4 proteins produced by these cells are either non-functional or sequestered in a compartment outside of the secretory pathway. In experiments *in vitro*, PACE4 processed diphtheria toxin and anthrax toxin protective antigen, but not *Pseudomonas* exotoxin A. The activity of PACE4 *in vitro* was Ca^{2+}-dependent and, unlike furin, was sensitive to temperature changes between 22 and 37 °C. RPE.40 cells stably expressing human PACE4 secreted an endoprotease with the same Ca^{2+} dependence and temperature sensitivity as that observed in membrane fractions of these cells assayed *in vitro*. These results, in conjunction with other published work, demonstrate that PACE4 is an endoprotease with more stringent substrate specificity and more limited operating parameters than furin.

Key words: furin, preprotein processing, secretion.

INTRODUCTION

A family of eukaryotic, subtilisin-like endoproteases that processes precursor proteins at either single or paired basic amino acid residues has recently been discovered. These enzymes process a variety of precursor proteins, including hormones and their receptors, growth factors and their receptors, coagulation factors, bacterial toxins and viral glycoproteins [1–4]. Furin, the best characterized of these endoproteases, is the product of the *fur* gene. Furin is expressed in a wide variety of mammalian cells and in virtually all eukaryotic organisms; furin is believed to function in the constitutive secretory pathway where it processes proproteins on the C-terminal side of the consensus sequence Arg-Xaa-Xaa-Arg [5–9]. PACE4 is another member of this family of endoproteases [10] that is predominantly, but not exclusively, expressed in neural and endocrine tissue in a developmentally regulated fashion [11–16]. Results on the sequence motif necessary for cleavage by PACE4 are conflicting. Some reports suggest that a basic amino acid is required in the P2 position [17,18], but others have shown that PACE4 can recognize the Arg-Xaa-Xaa-Arg sequence [19] and sequences that lack the P4 arginine residue [20,21]. Several isoforms of PACE4, generated via alternative RNA processing, have been identified [10,15,22–24]. However, PACE4 has yet to be characterized to the same extent as furin; subsequently, less is known about the biochemical properties and physiological role(s) of PACE4.

Among the most useful tools used to study furin and related endoproteases are mutant cell strains that lack furin activity. One such cell strain, RPE.40, was developed in our laboratory by mutagenizing Chinese hamster ovary (CHO)-K1 cells with ethyl methane sulphonate [25]. RPE.40 cells are resistant to *Pseudomonas* exotoxin A (PEA) and several alpha viruses owing to an inability to process and activate PEA or the relevant viral envelope glycoproteins [25–28]. RPE.40 cells are also unable to process their insulin pro-receptor and show a diminished ability to process their low-density-lipoprotein receptor-related protein (LRP) compared with wild-type CHO-K1 cells [29]. We have recently shown that the defect in proteolytic processing in these cells is due to mutations in both *fur* alleles; therefore these cells produce no functional furin [30,31]. Here we report the use of RPE.40 cells to examine the role of PACE4 in processing several proproteins in a furin-null background. When PACE4 was expressed in RPE.40 cells, the phenotype of these cells was partly restored to wild type. The restoration of distinct wild-type characteristics *in vivo* was correlated with the activity of PACE4 *in vitro*. We also present some biochemical characterization of the PACE4 activity. Like furin, PACE4 is a Ca^{2+}-dependent enzyme. Unlike furin, PACE4 exhibits an interesting temperature sensitivity, showing diminished activity at 22 °C when compared with activity at 30 or 37 °C.

EXPERIMENTAL

Materials

All restriction enzymes were purchased from Gibco-BRL (Gaithersburg, MD, U.S.A.). Calf intestinal alkaline phosphatase

Abbreviations used: CHO, Chinese hamster ovary; DTX, diphtheria toxin; G418, geneticin; GST–39 protein, glutathione S-transferase-conjugated 39 kDa protein; LRP, low-density-lipoprotein receptor-related protein; PA, anthrax toxin protective antigen; PEA, *Pseudomonas* exotoxin A; SbV, Sindbis virus strain AR339; vWF, von Willebrand factor.

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was also purchased from Gibco-BRL. T4 DNA ligase was obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Western blotting reagents were obtained from Promega (Madison, WI, U.S.A.). Unless otherwise indicated, all other reagents were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). The construct encoding the precursor to von Willebrand factor (pro-vWF) was generously provided by Dr. Jan A. van Mourik and Dr. Jan Voorberg of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). The expression plasmid encoding the secreted human insulin pro-receptor was a gift from Dr. Eric Schaefer and Dr. Leland Ellis of Texas A&M University (Houston, TX, U.S.A.).

Cell culture and virus propagation

CHO-K1 cells were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). The cell strains used in this work and some of their characteristics are listed in Table 1. Cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium (Sigma) containing 5% (v/v) fetal bovine serum (hereafter referred to as growth medium) at 37 °C in an air/CO_2 (19:1) atmosphere. The propagation of the AR339 strain of Sindbis virus (SbV) and the determination of titres of infectious virus by plaque assays were done as described previously [26].

Construction of the PACE4 expression vector

The human PACE4 cDNA cloned into pBluescript was a gift from Dr. Steven Smeekens of the Chiron Corporation (Emeryville, CA, U.S.A.). The PACE4 cDNA was excised from pBluescript by using an *Eco*RV/*Xho*I double digest. The PACE4 cDNA was then purified from agarose by using the GeneClean system (Bio 101, Vista, CA, U.S.A.) in accordance with the manufacturer's instructions, then directionally ligated into the expression vector pSVL (Pharmacia) by using the *Xho*I and *Sma*I sites of the vector. The blunt ends generated by both *Eco*RV and *Sma*I allowed directional ligation, and the vector was treated with calf intestinal alkaline phosphatase before the ligations. Ligation products were transformed into *Escherichia coli* XL1-Blue cells and transformants harbouring the construct were selected by growth on Ampicillin-containing Luria–Bertani medium. The presence of the construct was verified by standard plasmid miniprep analysis. Large-scale purification of the recombinant construct, designated pSVL-PACE4, was done by using a plasmid isolation kit obtained from Qiagen (Chatsworth, CA, U.S.A.).

Transfections

The generation of RPE.40 cell strains stably expressing mouse furin has been described previously [32]. The generation of RPE.40 cell strains stably expressing human PACE4 was done by co-transfecting the pSVL-PACE4 construct, which does not contain a selectable marker, and pSV2Neo, which harbours a gene conferring resistance to geneticin (G418); transfections were done with the Transfectam reagent (Promega) in accordance with the manufacturer's instructions. Clones that were resistant to G418 were selected and screened for the ability to process pro-vWF with either transfection assays or processing assays *in vitro* (see below). Cell strains capable of processing pro-vWF were propagated in growth medium supplemented with 850 μg/ml G418. Recombinant vectors with cDNA species for the various proproteins were transiently introduced into cells by using the Lipofectamine reagent (Gibco-BRL) in accordance with the manufacturer's protocol; for all of these transient transfections, 10^6 cells were seeded in 3 ml of growth medium in 60 mm × 15 mm tissue culture dishes and incubated for 16 h before the transfections were initiated. After transfections, cells were incubated in growth medium until radiolabelling or were incubated in serum-free medium (HyQ-CCM5, from Hyclone Laboratories, Logan, UT, U.S.A.) for experiments involving the expression of pro-vWF.

Intact cell assay for inhibition of protein synthesis by PEA

Cells (2×10^4) were seeded in 1 ml of growth medium in 6 ml flint glass scintillation vials and incubated overnight. Triplicate vials were changed to medium with or without serial dilutions of PEA and incubated for 24 h. Cultures were changed to serum-free Eagle's basal medium with a 1:20 dilution of amino acids and 0.05 M Tris/HCl, pH 7.4, containing 0.4 μCi/ml Trans^{35}S-label (ICN Biochemicals). Cells were washed with 5% (w/v) trichloroacetic acid; acid-insoluble radioactivity was determined in a scintillation spectrometer. The concentration of toxin that inhibited protein synthesis by 50% was determined from dose–response curves generated from the results of these assays [32].

Virus infectivity assay

Cells were seeded in 16 mm well plates at 4×10^4 cells per well and allowed to grow overnight to approx. 50% confluence. Growth medium was removed from the wells; serial dilutions of SbV were applied in 0.2 ml of growth medium. After 1 h of incubation at 37 °C, cells were washed to remove unadsorbed viruses and 1 ml of growth medium was added. Maximum cytopathic effect was scored by microscopic observation after incubation for 72 h.

Ligand blotting of LRP

The procedure for ligand-blot analysis of LRP, with ^{125}I-labelled glutathione S-transferase-conjugated 39 kDa (GST–39) protein, a recombinant receptor-associated protein that binds to LRP [33], has been described previously [34]. To prepare partly purified cell membranes, 10^6 cells were lysed in 200 μl of 0.1% saponin in PBS, membranes were pelleted in a Microfuge and supernatants containing all soluble cell proteins were removed. Membrane proteins were separated by SDS/PAGE, transferred to Immobilon-P membranes (Millipore) and exposed to the labelled ligand; the LRP bands were detected by autoradiography.

Western blotting to analyse pro-vWF processing

Conditioned HyQ-CCM5 medium from cells transfected with the pro-vWF construct was collected 60 h after transfection. Samples of medium were subjected to SDS/PAGE [5% (w/v) gels under denaturing conditions] and the resolved proteins were transferred to Immobilon-P membranes. The membranes were blocked for 1 h with 2% (w/v) milk in PBS, then incubated overnight with anti-vWF antibody (Dako Corporation, Carpinteria, CA, U.S.A.) diluted 1:2500 in 0.6% (w/v) milk/PBS. The membranes were washed three times (3 min each) with 0.6% (w/v) milk/PBS, blocked for an additional 30 min, then subjected to an anti-rabbit IgG alkaline phosphatase conjugate [diluted 1:5000 in 0.6% (w/v) milk/PBS] for 1.5 h. After two more washes in 0.6% (w/v) milk/PBS and one in substrate buffer [0.1 M Tris/HCl (pH 9.5)/0.1 M NaCl/5 mM $MgCl_2$], each for 3 min, colour development was initiated by adding 40 μl of Nitro Blue Tetrazolium (50 mg/ml) and 20 μl of 5-bromo-4-chloro-indol-3-yl phosphate (50 mg/ml) into 20 ml of substrate buffer and placing this mixture on the membrane.

Metabolic labelling and immunoprecipitations

Metabolic labelling was used to examine the processing of the secreted human insulin pro-receptor. Cells transfected with the insulin pro-receptor construct were incubated for 40 h in growth medium, then starved of cysteine and methionine for 30 min in Dulbecco's modified Eagle's medium without L-cysteine, L-methionine or fetal bovine serum (hereafter referred to as labelling medium). This medium was removed and the cells were pulse-labelled for 5 h with labelling medium (2 ml per 60 mm × 15 mm dish) containing 200 μCi of Trans^{35}S-label. After the pulse-labelling, immunoprecipitations were set up on samples of medium removed from the labelled cells exactly as described by Robertson et al. [35]. Immunoprecipitated proteins were analysed by SDS/PAGE followed by autoradiography with Kodak XAR X-ray film.

RNA isolation and Northern blotting

Total cellular RNA was isolated from cells by the method of Salvatori et al. [36]. Poly(A)$^+$ RNA was selected by using the Oligotex system (Qiagen). Recovered RNA was quantified by spectrophotometric analysis; approx. 5 μg of RNA from each cell strain was resolved by denaturing agarose electrophoresis as described by Sambrook et al. [37]. The resolved RNA was transferred by capillary action to a Hybond N nylon membrane (Amersham) by using the procedure of Virca et al. [38] and then covalently cross-linked to the membrane by using a Stratalinker UV Crosslinker (Stratagene). Prehybridization, probe hybridization and washes to remove non-specifically bound probe were done by using the procedure of Virca et al. [38]. The 2.8 kb human PACE4 cDNA was used as a probe; it was excised from pBluescript, labelled by random priming with the use of the High Prime system of Boehringer Mannheim, and hybridized to the blot at 57 °C. After washing as described by Virca et al. [38], the membrane was wrapped in plastic wrap and used to expose Dupont Reflection autoradiography film.

Processing assays *in vitro*

The isolation of membrane fractions from cell strains was done as described previously [27]. Membrane proteins were quantified by spectrophotometric analysis at 280 nm. The processing reactions *in vitro* were set up essentially as described previously [27]. Unless otherwise indicated, the buffer used for all the processing reactions contained 0.1 M Hepes (pH 7.0), 0.1 % (v/v) Triton X-100 and 3 mM $CaCl_2$, and the reactions were incubated for 16 h at 30 °C. The source of the substrate pro-proteins varied. The pro-vWF was derived from conditioned medium from RPE.40 cells transfected with the pro-vWF construct; typically, 25 μl of this medium was used in a total reaction volume of 40 μl. The processing of pro-vWF in these reactions was analysed by Western blotting, as described above. The secreted human insulin pro-receptor was derived from conditioned medium from RPE.40 cells that were pulse-labelled, as described above, after transfection with the insulin pro-receptor construct. This medium was concentrated approx. 10-fold by using Centricon concentrators (Millipore); typically, 25 μl of concentrated medium was used in a total reaction volume of 40 μl. Processing in these reactions was analysed by immunoprecipitation of the insulin receptors from the reactions, followed by SDS/PAGE and autoradiography. Reactions involving the various toxins were set up with the use of ^{125}I-labelled toxins, as described previously [25]. In some cases, purified, non-labelled diphtheria toxin (DTX) was used as a substrate. Processing in these reactions was assayed by running the entire reaction volume on SDS/PAGE, followed by either autoradiography (radiolabelled toxin) or staining with Coomassie Blue (non-labelled DTX). To examine the processing activity of secreted endoproteases, the cell strains were incubated for approx. 40 h in HyQ-CCM5 medium; samples of the medium were then collected and concentrated 10-fold with the use of Centricon concentrators; 2–5 μl of each concentrated medium sample was used in the place of membrane proteins in the processing reactions.

RESULTS

Expression of PACE4 in cells lacking furin can partly restore wild-type characteristics

We used RPE.40 cells to examine the role of PACE4 in processing several mammalian pro-proteins by stably expressing human PACE4 in these cells; the transfected cells were designated 40.P4.T34. CHO-K1 cells, untransfected RPE.40 cells and RPE.40 cells stably expressing mouse furin (40.fur.5g) were employed as controls. These cell strains were initially examined for sensitivity to SbV and PEA (Table 1). RPE.40 cells show a pronounced resistance to SbV, owing to an inability to process the PE2 glycoprotein. The 40.fur.5g and 40.P4.T34 cells were both as sensitive to SbV as wild-type CHO-K1 cells. Therefore the expression of PACE4 in RPE.40 cells compensated for the lack of furin activity and restored wild-type sensitivity to SbV. RPE.40 cells are also more than 7000-fold as resistant to PEA as are parental CHO-K1 cells. Wild-type sensitivity to PEA was restored in 40.fur.5g cells, in contrast with RPE.40 cells expressing PACE4. Some increase in sensitivity to PEA was noted in 40.P4.T34 cells but these cells were still well below wild-type levels of sensitivity. Therefore the expression of PACE4 in RPE.40 cells failed to restore wild-type sensitivity to PEA.

Because the SbV glycoprotein PE2 transits through the secretory pathway, we speculated that the recombinant PACE4 protein was active in the secretory pathway. To examine this possibility in more detail, we performed experiments in which two secreted proteins (pro-vWF, a known substrate for PACE4 [17,18,39], and a truncated, soluble form of the human insulin pro-receptor) were expressed in 40.P4.T34 cells (Figure 1). CHO-K1 cells processed approx. 50 % of the expressed pro-vWF, whereas RPE.40 cells processed essentially none of the expressed vWF; pro-vWF expressed in 40.fur.5g and 40.P4.T34 cells was processed to levels in CHO-K1 cells or greater (Figure 1A). Figure 1(B) shows that CHO-K1 cells processed the truncated insulin pro-receptor, whereas RPE.40 cells did not. Both 40.fur.5g and 40.P4.T34 cells processed the truncated insulin pro-receptor as efficiently as CHO-K1 cells. LRP also transits through the secretory pathway, and RPE.40 cells are impaired in the processing of endogenous LRP [29]. We examined the processing of endogenous LRP in 40.P4.T34 cells by exposing radioactively labelled GST–39 protein, which binds specifically to LRP, to LRP resolved from cell membrane fractions (Figure 1C). In CHO-K1 cells the 600 kDa precursor form of LRP was entirely processed: the GST–39 protein bound only to the 515 kDa subunit of mature LRP. In RPE.40 cells, 50 % or more of the precursor remained unprocessed; the processing of LRP in these cells occurs via an unknown endoprotease during the recycling of LRP from the cell surface [29]. PACE4 (40.P4.T34 cells) restored wild-type processing of LRP.

The results presented in Table 1 and Figure 1 show that the expression of PACE4 can correct the endoprotease deficiency of RPE.40 cells in SbV infection and in the processing of endogenous LRP. These results also demonstrate that the expressed PACE4 protein was active in the secretory pathway. However, resistance to PEA was largely unaltered by the expression of PACE4. The

Table 1 Sensitivity of CHO-K1 cell strains to SbV and PEA

Sensitivity to SbV is expressed as the number of infectious particles required to produce the maximum cytopathic effect (80–100% killing of cells) in a 72 h exposure. ID_{50} is the concentration of PEA (ng/ml) that decreased protein synthesis by 50% after 24 h of exposure. Results are averages for triplicate samples. Results for sensitivity to PEA are given $\pm$S.D.

Cell strain	Transfected cDNA	Characteristics	Sensitivity to SbV infectious particles	Sensitivity to PEA (ID_{50})
CHO-K1	None	Wild type	4	6 ± 2
RPE.40	None	Furin-null mutant	4000	$45\,000 \pm 8500$
40.fur.5g	*fur*	Transfected RPE.40	4	12 ± 2
40.P4.T34	PACE4	Transfected RPE.40	4	4750 ± 400

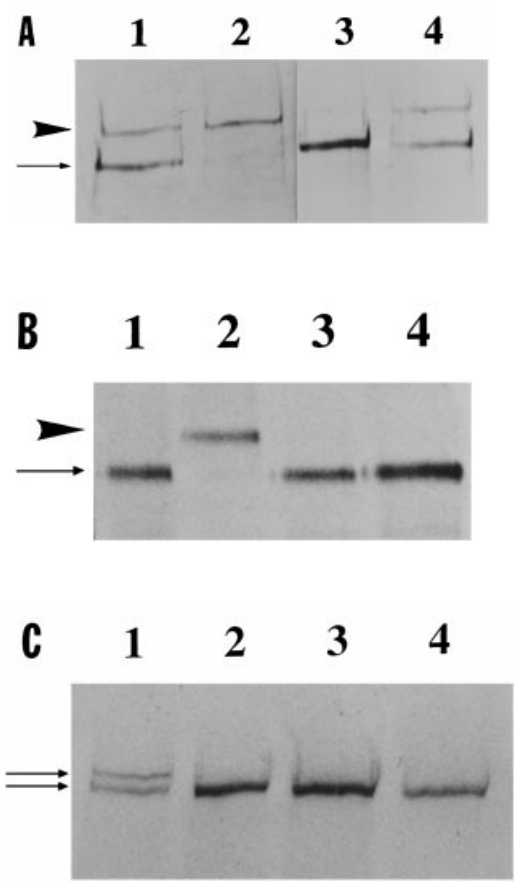

Figure 1 Processing of pro-vWF, a secreted form of the human insulin pro-receptor, and endogenous LRP by CHO-K1 cell strains

Expression constructs containing cDNA species for pro-vWF or a secreted form of the human insulin pro-receptor were introduced into CHO-K1, RPE.40, 40.fur.5g and 40.P4.T34 cells. (**A**) Conditioned medium from cells transfected with pro-vWF was collected 60 h after transfection; proteins in the medium were resolved by SDS/PAGE. The state of vWF in the medium samples was determined by Western blotting with an anti-vWF antiserum. (**B**) The human insulin pro-receptor was metabolically labelled with ^{35}S-amino acids and immunoprecipitated from the medium of the labelled cells. Immunoprecipitated proteins were resolved by SDS/PAGE; bands were detected by autoradiography. In (**A**) and (**B**): lane 1, results obtained with CHO-K1 cells; lane 2, results obtained with RPE.40 cells; lane 3, results obtained with 40.fur.5g cells; lane 4, results obtained with 40.P4.T34 cells. Precursor proteins are indicated by arrowheads; the processed proteins are indicated by arrows. (**C**) Partly purified membrane fractions were prepared from RPE.40 (lane 1), CHO-K1 (lane 2), 40.fur.5g (lane 3) and 40.P4.T34 (lane 4) cells. Membrane proteins were resolved by SDS/PAGE, transferred to a nylon membrane filter and exposed to radioactively labelled GST–39 protein, which binds specifically to LRP. The binding of the GST–39 protein to LRP was detected by autoradiography. The two arrows indicate the position of the 600 kDa LRP precursor (upper arrow) and the 515 kDa subunit of the mature receptor (lower arrow).

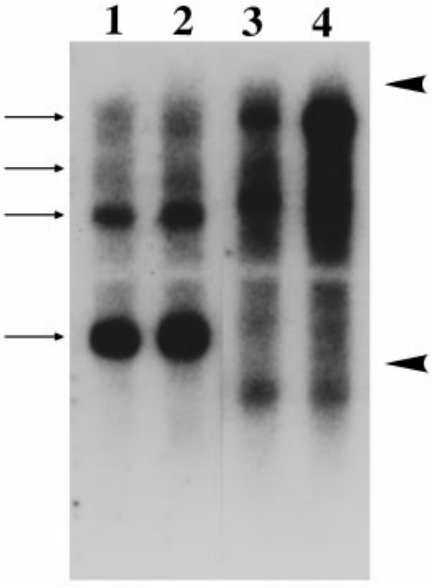

Figure 2 Northern blot analysis of PACE4 mRNA in CHO-K1 and RPE.40 cells

Total RNA was isolated from cells and subjected to poly(A)$^+$ selection. The poly(A)$^+$ RNA was resolved by denaturing agarose electrophoresis and transferred to a nylon membrane by capillary action. The membrane was probed with a radiolabelled 2.8 kb PACE4 cDNA, washed, then exposed to X-ray film. Lane 1, RNA isolated from CHO-K1 cells; lane 2, RNA from RPE.40 cells; lane 3, RNA from LoVo cells; lane 4, RNA from HepG2 cells. The arrowheads indicate the positions of the 28 S and 18 S rRNA bands (run in parallel with the samples transferred to the nylon filter but stained with ethidium bromide). The arrows indicate the four mRNA species in CHO-K1 and RPE.40 cells that hybridized to the PACE4 cDNA.

failure of PACE4 to restore wild-type sensitivity to PEA in RPE.40 cells suggests that PACE4 does not have a physiological role in processing PEA; we shall address this possibility in more detail below.

RPE.40 cells express mRNA species for four PACE4 isoforms

Because the expression of PACE4 in RPE.40 cells compensated for the lack of furin activity (Table 1 and Figure 1), we decided to examine these cells for expression of endogenous PACE4. We hypothesized that these cells might lack endogenous PACE4 expression, because recombinant PACE4 expressed in these cells largely corrected their deficiency in endoproteolytic processing. Efforts to detect any endogenous PACE4 protein with Western blotting and metabolic labelling were unsuccessful (results not shown). We utilized Northern analysis to examine whether RPE.40 cells expressed any PACE4 mRNA species. RNA

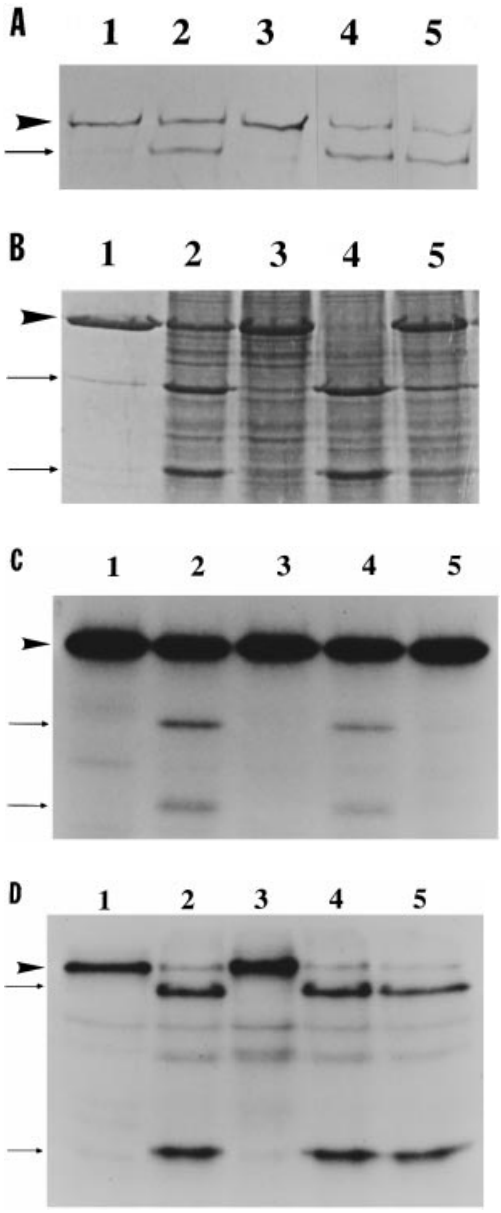

Figure 3 Processing of pro-proteins by PACE4 *in vitro*

Membrane fractions were prepared from CHO-K1, RPE.40, 40.fur.5g and 40.P4.T34 cells; 50 μg of total membrane protein from each sample was added to processing reactions *in vitro*. Pro-protein substrates used were: pro-vWF (**A**), DTX (**B**), PEA (**C**) and PA (**D**). All reactions were incubated at 30 °C for 16 h; the reactions were at pH 7.0 except for the PEA reactions, which were at pH 5.4. Results of processing *in vitro* were analysed by SDS/PAGE followed by either Western blotting (vWF processing), Coomassie Blue staining (DTX) or autoradiography (all other reactions). In all panels, lane 1 is a negative control with no membrane fraction added (proprotein only); the other lanes contained membrane fractions derived from CHO-K1 cells (lane 2), RPE.40 cells (lane 3), 40.fur.5g cells (lane 4) or 40.P4.T34 cells (lane 5). The arrowheads indicate the precursor proteins; the processed proteins are indicated by arrows.

samples isolated from LoVo and HepG2 cells, which are human colon carcinoma and human hepatoma cells respectively, were used as positive controls [3,10]. CHO-K1 and RPE.40 cells expressed four mRNA species that hybridized to the PACE4 cDNA (Figure 2). These four mRNA species might correspond to PACE4 isoforms that have been identified in other cells [10,15,22–24]. Whether any of these presumptive endogenous PACE4 mRNAs produces functional PACE4 protein was not resolved. However, on the basis of the results in Table 1 and Figure 1, it seems that no endogenous PACE4 is active in the secretory pathway of RPE.40 cells.

Processing activity of PACE4 *in vitro*: substrate specificity

It has been reported that PACE4 has a more limited substrate specificity than furin, perhaps because of more stringent cleavage sequence requirements [17,18]. Our results obtained with PEA and 40.P4.T34 cells could reflect this limited substrate specificity; that is, the PEA resistance of these cells could be the result of a furin-null background combined with an inability of PACE4 to cleave and activate PEA. We tested this hypothesis by examining the ability of PACE4, present in membrane fractions derived from transfected cells, to process several potential substrates [PEA, DTX and the protective antigen of anthrax toxin (PA)] *in vitro*; pro-vWF was used as a positive control for PACE4 activity (Figure 3). PACE4 processed DTX and PA, but not PEA. Because the processing of PEA by furin requires an acidic pH (5.4), we tested the processing activity of PACE4 at that pH to ensure that the acidic pH did not inactivate PACE4. Processing of pro-vWF by PACE4 at pH 5.4 was as effective as at pH 7, ruling out the possibility that PACE4 was inactivated by the acidic pH (results not shown). Table 2 shows the range of pro-proteins tested as substrates for PACE4 and their cleavage-site amino acid sequences. With the exception of pro-lactase-phlorizin hydrolase, furin can process all of the proproteins listed in Table 2.

The results obtained *in vitro* with PEA confirm those obtained *in vivo*. Thus, even if PACE4 encountered PEA in either RPE.40 (endogenous PACE4) or 40.P4.T34 (endogenous and/or transfection derived PACE4) cells, PEA does not seem to be a substrate for PACE4 and therefore the cells could not undergo intoxication.

PACE4 is Ca^{2+}-dependent and temperature-sensitive

To determine whether PACE4 is Ca^{2+}-dependent we examined the effect of EGTA on the processing of pro-vWF by PACE4 *in vitro*. Figure 4(A) shows that the addition of EGTA to the reactions *in vitro* blocked the ability of PACE4 to process pro-vWF. Figure 4(B) shows that the addition of excess Ca^{2+} to reactions *in vitro* containing EGTA reversed the inhibition of processing by EGTA. Figure 4(C) shows that the addition of Mg^{2+} under the same conditions could not reverse the EGTA inhibition of PACE4 activity. These results strongly suggest that PACE4, like furin, requires Ca^{2+} for activity.

Figure 5(A) shows that the processing of pro-vWF *in vitro* by PACE4 was temperature-sensitive. PACE4 efficiently processed pro-vWF at 37 °C but the processing was markedly decreased at 22 and 30 °C. Furin processed pro-vWF to the same extent at all three temperatures. To determine whether this effect of temperature reflected a property of PACE4 or was due to some aspect of the pro-vWF that rendered it unable to be processed by PACE4 at 22 °C, we examined the processing of DTX, the soluble insulin pro-receptor and PA by PACE4 *in vitro* at different temperatures. PACE4 processed all three substrates in a temperature sensitive fashion (Figures 5B–5D). Furin did not exhibit this sensitivity to temperature.

Pro-vWF processing activity is secreted by 40.P4.T34 cells

Because PACE4 lacks the transmembrane anchor found in other members of the furin family of endoproteases [10], we examined

Table 2 Pro-proteins examined as potential PACE4 substrates

Pro-protein	Cleavage site sequence	Processed by PACE4	References
Pro-(factor IX)	RPKR	No	[44]
DTX	RVRR	Yes	This study
Sindbis virus PE2	RSKR	Yes	This study*
Human insulin pro-receptor	RKRR	Yes	This study
Pro-vWF	RSKR	Yes	[17,18,39]
PEA	RHRQPR	No	This study; [19]
LRP	RHRR	Yes	This study
Influenza haemagglutinin	RKKR	No	[45]
Pro-(complement C3)	RRRR	Yes	[39]
PA	RKKR	Yes	This study; [19]
Pro-(nerve growth factor)	RSKR	Yes	[46]
Pro-(insulin-like growth factor IA)	KSAR or RAQR†	No	[47]
HIV gp160	REKR	Yes	[41,48,49]
Pro-(brain-derived neurotrophic factor)	RVRR	Yes	[50]
Neurotrophin-3 precursor	RRKR	Yes	[50]
Bovine leukaemia virus gp72	RVRR	Yes	[51]
Prosomatostatin (S-28 production)	RLELQR	Yes	[20]
pro-(lactase-phlorizin hydrolase)	RTPR	No	[52]
Proglucagon (glicentin production)	RNNIAKR	Yes	[21]

* PE2 processing by PACE4 inferred by restoration of sensitivity to SV in RPE.40 cells transfected with PACE4.
† Pro-(insulin-like growth factor IA) contains two cleavage sites and might be processed sequentially to yield mature insulin-like growth factor I.

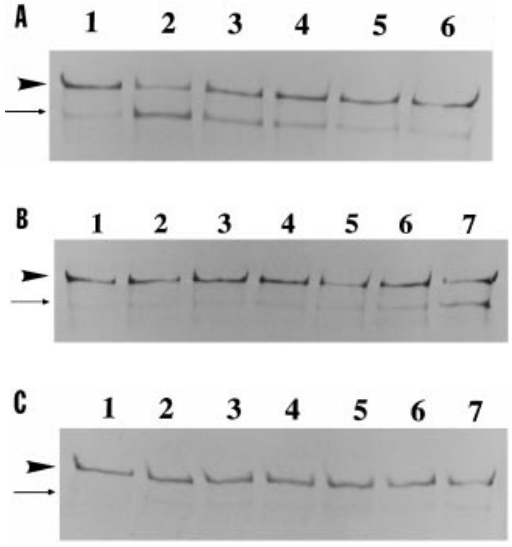

Figure 4 Ca^{2+}-dependent pro-protein processing by PACE4 *in vitro*

Processing reactions were set up *in vitro* with pro-vWF as a substrate; unless otherwise indicated, the processing buffer used for these reactions contained no exogenously added Ca^{2+}. The processing of pro-vWF was examined by Western blotting. (**A**) The effect of increasing concentrations of EGTA. Lane 1, untreated pro-vWF (no membrane proteins added); the reactions in all of the other lanes received 50 μg total membrane protein derived from 40.P4.T34 cells, plus no EGTA added (lane 2), 500 μM EGTA (lane 3), 1 mM EGTA (lane 4), 1.5 mM EGTA (lane 5) or 2 mM EGTA (lane 6). (**B**) Processing reactions containing 2 mM EGTA were supplemented with increasing levels of $CaCl_2$. Lane 1, no membrane proteins added; the reactions in all of the other lanes contained 50 μg of total membrane protein derived from 40.P4.T34 cells, plus no $CaCl_2$ (lane 2), 10 μM $CaCl_2$ (lane 3), 100 μM $CaCl_2$ (lane 4), 500 μM $CaCl_2$ (lane 5), 1 mM $CaCl_2$ (lane 6) or 3 mM $CaCl_2$ (lane 7). (**C**) Processing reactions containing 2 mM EGTA were supplemented with increasing levels of $MgCl_2$. Lane 1, no membrane proteins added; the reactions in all of the other lanes received 50 μg of total membrane protein derived from 40.P4.T34 cells, plus no $MgCl_2$ (lane 2), 10 μM $MgCl_2$ (lane 3), 100 μM $MgCl_2$ (lane 4), 500 μM $MgCl_2$ (lane 5), 1 mM $MgCl_2$ (lane 6) or 3 mM $MgCl_2$ (lane 7). In all panels, pro-vWF is indicated by an arrowhead; processed vWF is indicated by an arrow.

whether PACE4 activity could be detected in the conditioned medium from 40.P4.T34 cells. Conditioned medium from 40.P4.T34 cells was collected, concentrated and assayed for pro-vWF processing activity. Figure 6(A) shows that a strong pro-vWF processing activity was present in the medium derived from 40.P4.T34 cells; medium from RPE.40 cells exhibited no pro-vWF processing activity. Conditioned medium derived from 40.fur.5g cells also processed pro-vWF, a result that was expected because furin has been shown to be secreted as a result of an as yet uncharacterized C-terminal cleavage event. We speculated that the pro-vWF processing activity observed in the conditioned medium of 40.P4.T34 cells was secreted PACE4. To examine this possibility, the Ca^{2+} dependence of the secreted activity was examined. Figure 6(B) shows that the secreted activity was Ca^{2+}-dependent, as the addition of EGTA to the reactions inhibited pro-vWF processing. The secreted activity also demonstrated the temperature sensitivity observed for the membrane-associated PACE4 (results not shown). These results suggest that the recombinant PACE4 is both associated with the cellular membrane fraction and secreted.

DISCUSSION

The discovery of furin and furin-like endoproteases has spurred a great deal of interest and research dedicated to the analysis of the structure and function of these enzymes. One area that has been difficult to address, because of overlapping endoprotease activities in mammalian cells, has been the functional diversity and physiological role of these enzymes. We have utilized RPE.40 cells to examine the potential role of PACE4 in processing several mammalian pro-proteins in a furin-null background. We have shown that the expression of human PACE4 in RPE.40 cells resulted in the rescue of at least two wild-type characteristics: PACE4 restored sensitivity to SbV in these cells and corrected the deficient processing of LRP by them. The expression of human PACE4 in RPE.40 cells also endowed them with the ability to process a soluble form of the human insulin pro-

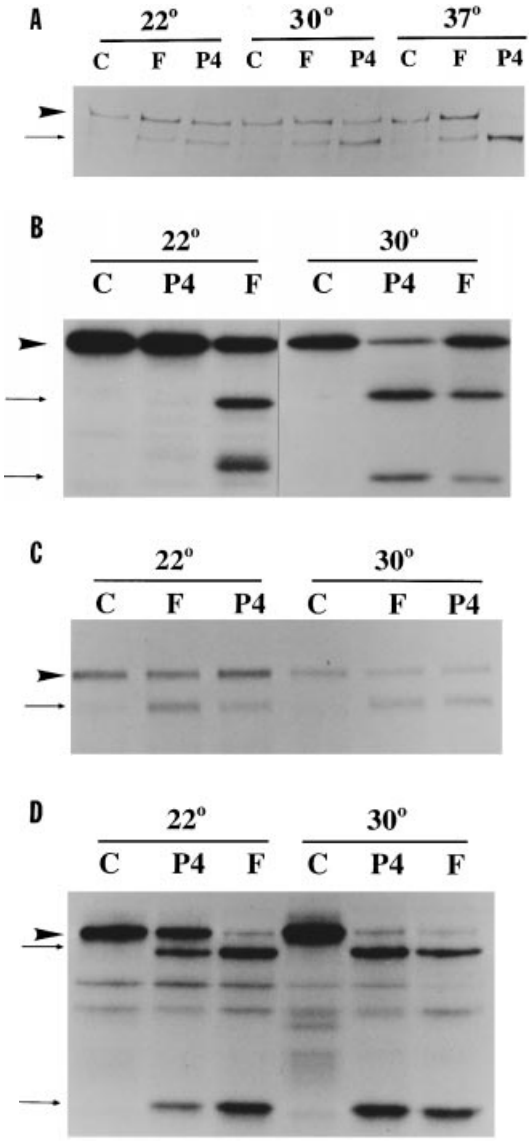

Figure 5 Temperature sensitive pro-protein processing by PACE4 *in vitro*

Processing reactions were set up *in vitro* with the following pro-proteins as substrates: pro-vWF (**A**), DTX (**B**), the secreted human insulin pro-receptor (**C**) and PA (**D**). The reactions were incubated at 22, 30 or 37 °C, as indicated. In all panels, lanes C were control reactions containing no added membrane proteins; lanes F were reactions containing 50 μg of total membrane protein derived from 40.fur.5g cells; lanes P4 were reactions containing 50 μg of total membrane protein derived from 40.P4.T34 cells. The precursor proteins are indicated by arrowheads; the processed proteins are indicated by arrows.

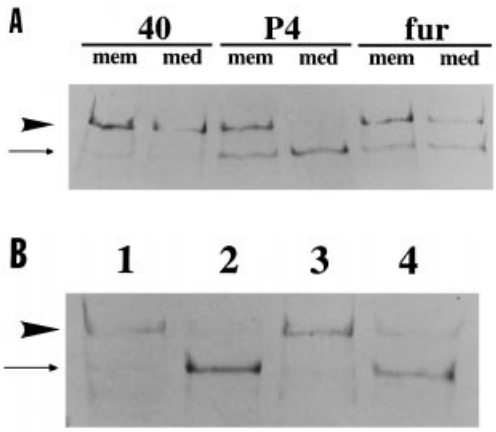

Figure 6 Processing of pro-vWF by an endoprotease activity secreted from 40.P4.T34 cells

Processing reactions were set up *in vitro* with pro-vWF as a substrate and samples of concentrated, conditioned medium from RPE.40, 40.fur.5g and 40.P4.T34 cells. Reactions with the use of membrane proteins derived from each of these cell strains were included as controls. (**A**) Lanes marked 'mem' were reactions containing 50 μg of total membrane protein derived from the indicated cells; lanes marked 'med' were reactions containing 5 μl of concentrated conditioned medium from the indicated cells; 40 indicates RPE.40 cells, fur indicates 40.fur.5g cells, and P4 indicates 40.P4.T34 cells. (**B**) The Ca^{2+} dependence of the secreted activity was examined. Lane 1 was a control and was derived from a reaction with the use of pro-vWF and medium derived from RPE.40 cells; lane 2 was a reaction with the use of concentrated medium from 40.P4.T34 cells. Exogenous Ca^{2+} (3 mM) was added to both of these reactions. The reactions in lanes 3 and 4 used conditioned medium from 40.P4.T34 cells and contained 2 mM EGTA to chelate Ca^{2+} present in the conditioned medium; the reaction in lane 4 was supplemented by 3 mM exogenous Ca^{2+}. Pro-vWF is indicated by an arrowhead; processed vWF is indicated by an arrow.

receptor and pro-vWF; RPE.40 cells lacking recombinant PACE4 processed neither of these pro-proteins. These results lead us to conclude that the PACE4 expressed from the transfected construct was active in the secretory pathway.

We demonstrated that RPE.40 cells express four mRNA species that hybridize to a radiolabelled 2.8 kb human PACE4 cDNA. These four mRNA species might correspond to PACE4 isoforms that have been identified by others working with different cells [10,15,20–24]. It is currently unclear whether functional proteins are produced from these transcripts. We have not been able to detect any PACE4 activity by using pro-vWF or any other PACE4 substrate in membrane extracts or conditioned medium derived from RPE.40 cells (Figures 3 and 6; some other results not shown). This finding, along with the results from our virus sensitivity and transfection experiments, might indicate that any PACE4 proteins translated from endogenous mRNA species are either non-functional or are sequestered in a compartment outside the secretory pathway. It is unclear why the endogenous mRNA species do not seem to give rise to an active protein product in the secretory pathway. The human cDNA expressed in RPE.40 cells encoded the PACE4A isoform, and it has been suggested that this isoform and the PACE4E isoform are the only catalytically active versions of PACE4 [23,40]. Our Northern analysis shows that the PACE4 mRNA large enough to encode either of these isoforms (the largest of the four mRNA species) is only weakly expressed in RPE.40 cells; this low level of expression could account for our inability to detect PACE4 activity. Alternatively, perhaps the hamster counterpart(s) of these PACE4 isoforms are non-functional, or perhaps the presence of the recombinant PACE4 in the secretory pathway in our transfection experiments reflects the fact that it is overexpressed and might be leaking out of its normal compartment. This latter possibility seems unlikely, given the strong pro-vWF processing activity secreted by 40.P4.T34 cells.

Although the expression of human PACE4 in RPE.40 cells restored some wild-type characteristics to these cells, we found that the expression of PACE4 in RPE.40 cells did not restore wild-type sensitivity to PEA. This finding probably reflects the limited substrate range of PACE4, and we investigated this possibility by examining the ability of PACE4 to process PEA and several other proteins *in vitro*. We found that PACE4 could

process the soluble human insulin pro-receptor, PA and DTX *in vitro*, but could not process PEA; furin is capable of processing all four proteins. These results confirm the results of our experiments *in vivo* and support the conclusion that PACE4 has a more limited substrate specificity than furin (see Table 2). Whereas PEA, with a cleavage site sequence of Arg-His-Arg-Gln-Pro-Arg, and the precursor of the insulin-like growth factor-IA (Lys-Ser-Ala-Arg or Arg-Ala-Gln-Arg) clearly deviate from the consensus Arg-Xaa-Lys/Arg-Arg sequence ascribed to PACE4 substrates [17,18,39], coagulation Factor IX (Arg-Pro-Lys-Arg) and influenza haemagglutinin (Arg-Lys-Lys-Arg) clearly do not. Because none of these proteins can be cleaved by PACE4, it is likely that additional structural characteristics of the putative substrate molecule, in addition to the cleavage site sequence, are important in determining whether the protein can be processed by this endoprotease. Curiously, PACE4 seems able to process two precursor proteins lacking the P4 arginine residue [20,21], and one of these proteins, prosomatostatin, also lacks a basic residue in the P2 position. The role of PACE4 in the activation of bacterial toxins has recently been investigated by others [19]. Although these researchers showed, as we have, that PACE4 processes PA but not PEA, they also reported that PACE4 could not cleave DTX, a result that conflicts with our findings.

Our results clearly indicate that PACE4, like furin, is a Ca^{2+}-requiring enzyme. Others investigating the biochemical properties of PACE4 have reported conflicting results on the Ca^{2+} requirements of PACE4. Decroly et al. [41] report that PACE4 activity towards a fluorogenic substrate was inhibited by 2 mM EDTA; others, however, claim that PACE4 is relatively independent of Ca^{2+} [42]. We cannot resolve this discrepancy at present. Putative Ca^{2+}-binding domains have been identified in a subtilisin [43], and one such domain is evident in mammalian furins {[42]; also analysis of sequences obtained from GenBank (J. F. Sucic, unpublished work)}. This domain is not observed in some non-mammalian furins or in mammalian PACE4 sequences (J. F. Sucic, unpublished work), although, as Mains et al. [42] point out, other structural determinants probably influence Ca^{2+} binding by these enzymes. We have also discovered an interesting aspect of PACE4 activity: an unusual temperature sensitivity. PACE4 showed greatly decreased processing activity below 30 °C. Furin did not exhibit this temperature sensitivity. Given that the temperature sensitivity was observed with multiple substrates, it seems to reflect a legitimate property of the PACE4 enzyme. Whether this temperature sensitivity is merely an interesting laboratory observation or has some physiological role is currently unclear, although in view of the mammalian nature of the enzyme used in these experiments it is difficult to postulate a physiological significance for this phenomenon.

We have shown that 40.P4.T34 cells secrete an endoprotease activity capable of processing pro-vWF with the same Ca^{2+} dependence and temperature sensitivity as the presumptively membrane-associated PACE4. This suggests that the recombinant PACE4 can be secreted, although the results from processing *in vitro* also clearly indicate that some PACE4 activity is membrane-associated. It is unlikely that the secreted and membrane-associated activities represent two unique endoproteases, given the overlapping Ca^{2+} and temperature dependences. Some of the processing of pro-vWF in the expression experiments (Figure 1A) might be the result of the secreted activity's building up in the conditioned medium over the 60 h time course; an accumulation of this activity could result in the processing of any pro-vWF released by the cells. The biochemical basis for the secreted activity is not well established but presumably results from the lack of a defined transmembrane domain in PACE4.

Studies on the cellular localization of furin have shown that it is localized in the secretory pathway, in the trans-Golgi network [9]. Work from our lab with RPE.40 cells suggests that furin has a physiological role in processing proteins that pass through the constitutive secretory pathway, as proteins such as vWF and the insulin receptor are not processed by RPE.40 cells. Results on the subcellular localization of PACE4 are less definitive [42]. Our results suggest that PACE4 is not active in the secretory pathway in RPE.40 cells. The role or roles of the various PACE4 isoforms are also unclear at present. It is clear that PACE4 has a more limited substrate range than furin's, as shown in this and other studies. It also seems that PACE4 has more limited operating parameters than furin's, although the physiological significance of this is not currently known.

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