PACE4 Is a Member of the Mammalian Propeptidase Family That Has Overlapping but Not Identical Substrate Specificity to PACE

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ABSTRACT: Proteins that transit the constitutive pathway of secretion frequently require proteolytic processing after a pair of basic amino acids to attain their full functional activity. A ubiquitously expressed calcium-dependent subtilisin-like serine protease, named PACE or furin, can cleave precursor polypeptides specifically at pairs of basic amino acids where an arginine residue is present in the P4 position. Another member of this protease family, PACE4, was cloned recently by a PCR-based strategy and was also shown to be ubiquitously expressed. We have expressed PACE4 by transient DNA transfection of COS-1 cells and have shown that the cDNA encodes a 120-kDa polypeptide that is present in cell extracts but not in conditioned medium of transfected cells. The substrate specificities of PACE and PACE4 for cleavage of pro-von Willebrand factor were studied in parallel using a transient DNA cotransfection system. Like PACE, PACE4 was able to process pro-vWF to its mature form, and efficient cleavage required both the P4 arginine and the P2 lysine. These data, taken together with previously published data showing that PACE4 cannot process pro-factor IX, demonstrate that PACE and PACE4 have overlapping but not identical substrate specificities. Further differences between PACE and PACE4 specificities were elucidated by monitoring inhibition of processing activity mediated by the serine protease inhibitor α_1-antitrypsin Pittsburgh mutant. Pro-vWF processing by PACE was inhibited by expression of the α_1-antitrypsin Pittsburgh mutant, whereas processing of pro-vWF by PACE4 was not affected. Processing of pro-vWF by the endogenous COS-1 cellular enzyme was also inhibited by the α_1-antitrypsin Pittsburgh mutant, suggesting the COS-1 cell endogenous processing enzyme is more closely related to PACE than to PACE4. The unique property of the α_1-antitrypsin Pittsburgh mutant to differentiate between these two enzymes provides an important tool to dissect the relative significance of these two processing enzymes.

Many growth factors, hormones, neuropeptides, and plasma proteins are synthesized as pro-proteins that require proteolytic removal of the propeptide to yield a biologically active protein. The discovery and functional analysis of a new family of cellular endoproteases in higher eukaryotes have unequivocally identified the subtilisin-like serine proteases as propeptide cleaving enzymes (Rehemtulla & Kaufman, 1992a; Steiner et al., 1992; Barr, 1991). These proteins include paired basic amino acid cleaving enzyme (PACE)[1] (also termed *furin*), which is involved in processing of many proteins secreted through the constitutive pathway, as well as PC2 and PC3 (also termed PC1). PC2 and PC3/PC1 are predominantly expressed in neuroendocrine tissues and play a role in the maturation of pro-proteins that are secreted through the regulated secretory pathway. Three recently described additional members of the family are PC4, which is predominantly expressed in the testis (Nakayama et al., 1992); PC5 (Lusson et al., 1993); and PACE4, which is expressed in many tissues including liver and endothelial cells (Kiefer et al., 1991). Although PC1, PC2/PC3, and PACE have been shown to possess propeptide cleaving activity, functional processing activities have not been described for the newly discovered enzymes PC4, PC5, and PACE4.

The five known members of the mammalian subtilisin-like protease family as well as the yeast enzyme Kex2 have conserved structural domains that include a signal peptide, a pro-sequence that regulates enzyme activity in PACE (Rehemtulla et al., 1992), and a catalytic domain which has the greatest degree of sequence conservation. The carboxy terminus diverges the most among the members of the family. PACE and PACE4 each contain a cysteine-rich region at or close to the carboxy terminus. PACE like its yeast homologue Kex2 contains a transmembrane domain that plays a role in the localization of the protein within the Golgi compartment (Rehemtulla et al., 1992; Redding et al., 1991; Misumi et al., 1991). In contrast, PC2, PC1/PC3, PC4, and PACE4 lack transmembrane domains while PC2 and PC3/PC1 contain carboxy-terminal glutamine-rich amphipathic helices which may play a role in membrane association and/or trafficking to storage granules (Smeekens et al., 1991; Seidah et al., 1991). Previous results from DNA transfection of mammalian cells demonstrated that pro-von Willebrand factor was processed by PACE (Van de Ven et al., 1990; Wise et al., 1990) but not by PC2 or PC3/PC1 (Rehemtulla & Kaufman, 1992b). Recently we demonstrated that pro-factor IX was processed to its mature form by PACE coexpression, whereas coexpression of PACE4 had no effect (Wasley et al., 1993). In this study, we demonstrate using wild-type and mutant vWF

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[1] Abbreviations: PACE, paired basic amino acid cleaving enzyme; vWF, von Willebrand factor; PC, propeptide convertase; CHO, Chinese hamster ovary; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; α1-AT$_P$, α_1-antitrypsin, Pittsburgh mutant.

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that PACE4 is a functional propeptidase with a substrate specificity overlapping but not identical to PACE. In addition, PACE4 and PACE exhibit different sensitivities to inhibition mediated by the Pittsburgh mutant of α_1-antitrypsin (α-AT$_P$).

MATERIALS AND METHODS

Plasmid DNA and Transfection. The PACE4 cDNA (Kiefer et al., 1991) was obtained on an *Xho*I–*Sal*I fragment and subcloned into an expression vector, pNOT; a derivative of pMT21 (Kaufman et al., 1991) into which additional cloning sites were introduced was kindly provided by M. Whitters of the Genetics Institute (Cambridge, MA). The PACE cDNA expression vector (Wise et al., 1990), the wild-type (Wise et al., 1990), and the mutant (Rehemtulla & Kaufman, 1992b) vWF expression vectors were described previously. The α1-AT and α1-AT$_P$ cDNAs (Bathurst et al., 1987) (kindly provided by I. Bathurst, Chiron Corp., Emeryville, CA) were inserted into pMT3 (Wise et al., 1990) as an *Eco*RI–*Sal*I fragment.

COS-1 cells were propagated and transfected by the diethylaminoethyl (DEAE)-dextran procedure as previously described (Wise et al., 1990). For cotransfection, 4 μg of each plasmid DNA was transfected per tissue culture plate (100 cm^2). Using this system, we have previously shown that at least four separate plasmid DNA molecules are expressed in each transfected cell (Davies et al., 1993), consistent with earlier observations (Wong, 1990). At 40 h post-transfection, analysis of vWF expressed in COS-1 cells was performed by labeling cells with [^{35}S]methionine (100 μCi/mL, 1000 Ci/mmol; Amersham, Arlington Heights, IL). Cells were incubated for 15 min at 37 °C in methionine-free minimal essential medium (Flow Labs, ICN, Costa Mesa, CA) containing 10% dialyzed heat-inactivated fetal bovine serum before addition of [^{35}S]methionine. After being labeled for the indicated periods of time, chase was performed where indicated by removing the radiolabel and adding complete medium containing excess unlabeled methionine. Cell extracts and conditioned medium were harvested, phenylmethanesulfonyl fluoride was added (0.1 mmol/L), and samples were analyzed by immunoprecipitation with the indicated antibodies and SDS–PAGE analysis as described previously (Wise et al., 1990). Autoradiography was performed using En^{3}hance (DuPont—New England Nuclear, Boston, MA), and band intensities were quantitated using an LKB Ultroscan-XL laser densitometer (Pharmacia, Uppsala, Sweden).

Immunoprecipitation of PACE and PACE4 was performed as described (Rehemtulla et al., 1992b) using a PACE-specific antiserum (Wise et al., 1990) or an anti-peptide antibody directed against residues 141–155 (QEVKRRVKRQVRSDY) of the predicted PACE4 amino acid sequence (Kiefer et al., 1991). The antibody was raised in rabbits against the peptide coupled to keyhole limpet hemocyanin and provided as a custom service by Hazelton Research Products Inc. (Denver, PA). Immunoprecipitation of vWF was performed using rabbit polyclonal anti-vWF (Diagnostica Stago, France).

RESULTS

PACE4 Is Expressed as a Cell-Associated Protein. To examine expression of PACE4, COS-1 cells were transfected with the PACE4 expression plasmid and metabolically labeled with [^{35}S]methionine at 48 h post-transfection. Immunoprecipitation of cell extracts and conditioned media after labeling, was performed using an anti-peptide antibody directed against residues 141–155 of the PACE4 sequence (Kiefer et al., 1991). Analysis of cell extracts from PACE4-transfected cells (Figure 1, lane 2) but not mock-transfected cells (Figure 1, lane 1) detected a 120-kDa immunoreactive species. In contrast to PACE which was secreted from cells and detected in the conditioned medium as a 90-kDa species (Rehemtulla & Kaufman, 1992b), PACE4 was not detected in the conditioned medium from PACE4-transfected cells (data not shown).

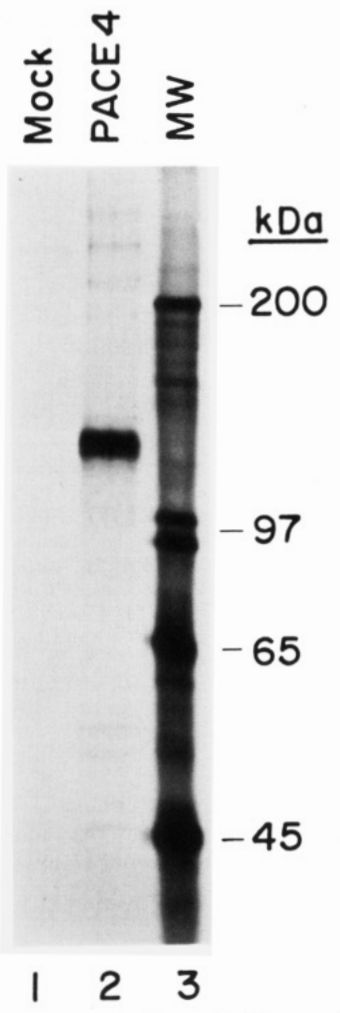

FIGURE 1: PACE4 is synthesized as a 120-kDa protein. COS-1 cells were transfected with the PACE4 expression vector, and 40 h post-transfection, the cells were labeled with [^{35}S]methionine for 1 h. Cell extracts were prepared, and a PACE4-specific antiserum was used for immunoprecipitation followed by SDS–PAGE and autoradiography as described under Materials and Methods. Lane 1, immunoprecipitation of mock-transfected cell extracts. Lane 2, immunoprecipitation of PACE4-transfected cell extracts.

PACE4 and PACE Have Similar Substrate Requirements for Processing of Pro-vWF. The substrate specificity of PACE4 was studied in a transient DNA transfection system. Transfection of the vWF expression vector into COS-1 cells followed by metabolic labeling with [^{35}S]methionine and immunoprecipitation using vWF-specific antiserum demonstrated that vWF was secreted as two species (Figure 2, lane 1) which represent pro-vWF and mature vWF (Wise et al., 1990; Rehemtulla & Kaufman, 1992b). A majority of the secreted vWF was in the unprocessed pro-vWF form due to inefficient propeptide cleavage by the endogenous COS-1 cell protease. Cotransfection of the vWF expression vector with the PACE4 expression vector resulted in complete processing of pro-vWF to its mature form (Figure 2, lane 11). Similarly, cotransfection of the vWF expression vector with the PACE expression vector also resulted in the secretion of completely

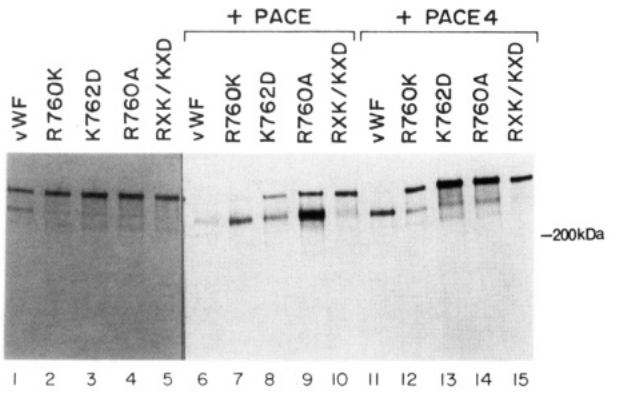

FIGURE 2: Processing of pro-vWF by PACE and PACE4 requires a P4 arginine as well as a P2 lysine. COS-1 cells were transfected with wild-type (lanes 1, 6, and 11) or the mutant vWF expression vectors R760K (lanes 2, 7, and 12), K762D (lanes 3, 8, and 13), R760A (lanes 4, 9, and 14), and RXK/KXD (lanes 5, 10, and 15) either in the absence (lanes 1–5) or in the presence of the expression vectors for the processing enzymes PACE (lanes 6–10) or PACE4 (lanes 11–15). At 40 h post-transfection, cells were metabolically pulse-labeled with [^{35}S]methionine for 1 h and chased for 5 h by addition of excess unlabeled methionine. Radiolabeled vWF was immunoprecipitated from the conditioned media samples as described under Materials and Methods, and the samples were analyzed by SDS–PAGE followed by autoradiography. Three independent transfection experiments provided results that were similar to those presented here.

processed mature vWF (Figure 2, lane 6). From previous amino-terminal sequence analysis, we demonstrated that PACE cleaved pro-vWF at the authentic site (Wise et al., 1990).

The amino acid sequence requirements for vWF processing mediated by PACE and PACE4 were compared utilizing mutants of vWF in which sequences around the propeptide cleavage site were substituted. The mutant R760K has a conservative substitution of arginine to lysine at four residues upstream of the cleavage site (P4 position), while R760A contains a nonconservative arginine to alanine substitution at the same position. The mutant K762D has a lysine to aspartic acid substitution at two residues upstream of the cleavage site (P2 position). Transient expression of wild-type vWF in COS-1 cells resulted in secretion of vWF of which 30% was processed to the mature form (Figure 2, lane 1). In contrast, processing of the R760K vWF mutant expressed in COS-1 cells was reduced to 10% (Figure 2, lane 2). The K762D and R760A (Figure 2, lanes 3 and 4) mutants were also processed inefficiently by the COS-1 cell enzyme (8% and 5%, respectively). The double-mutant RXK/KXD which has both the P4 arginine to lysine as well as the P2 lysine to aspartic acid substitutions was not detectably processed (Figure 2, lane 5).

The efficiency of propeptide cleavage of these mutants in the presence of PACE or PACE4 was measured in cotransfection experiments. Whereas wild-type vWF was processed to 100% in the presence of either PACE (Figure 2, lane 6) or PACE4 (Figure 2, lane 11), processing of mutant R760K vWF was reduced to 70% (Figure 2, lane 7) in the presence of PACE, and to 27% in the presence of PACE4 cotransfection. Processing of mutant R760A vWF was decreased to 80% and 14% in the presence of PACE and PACE4, respectively (Figure 2, lanes 9 and 14, respectively). Similarly, processing of mutant K762D vWF was decreased to 60% and 11% in the presence of PACE and PACE4, respectively (Figure 2, lanes 8 and 13, respectively). Both propeptide cleaving enzymes were unable to significantly process the double-mutant RXK/KXD vWF (Figure 2, lanes 10 and 15). The processing efficiencies for the mutant vWF molecules in the presence of

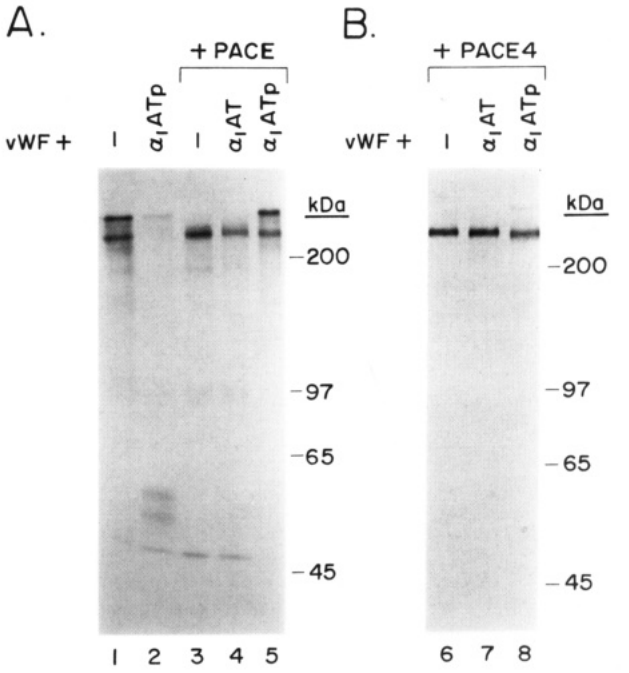

FIGURE 3: α1-AT$_P$ inhibits the processing activity of PACE but not PACE4. COS-1 cells were transfected with the vWF expression vector in the absence (lane 1) or in the presence of either the PACE (lanes 3–5) or the PACE4 (lanes 6–8) expression vectors as well as with wild-type α1-AT (lanes 4 and 7) or the α1-AT$_P$ mutant (lanes 2, 5, and 8) expression vectors. Cells were labeled with [^{35}S]-methionine, and samples of conditioned medium were analyzed by immunoprecipitation with anti-vWF antibody and SDS–PAGE as described under Materials and Methods. The polypeptide species migrating at 55–60 kDa in lane 2 represents nonspecifically adsorbed α1-AT$_P$ which is expressed and secreted at higher levels when only two plasmid DNAs are cotransfected.

PACE observed here are consistent with our earlier observations (Rehemtulla & Kaufman, 1992b) and demonstrate the reproducibility of this cotransfection assay system.

Unlike PACE, PACE4 Is Not Inhibited by α_1-Antitrypsin Pittsburgh. Wasley et al. (1993) have previously demonstrated that *in vivo* proteolytic processing of factor IX by PACE could be inhibited by the Pittsburgh mutant of α_1-antitrypsin in which the active-site methionine at residue 358 was substituted by arginine (α1-AT$_P$; Bathurst et al., 1987). Similarly, Misumi et al. (1990) also demonstrated inhibition of PACE-mediated processing of complement C3 by α1-AT$_P$. To examine the ability of α1-AT$_P$ to inhibit PACE and/or PACE4-mediated processing of pro-vWF, cotransfection of the PACE or PACE4 expression plasmid and the vWF expression plasmid was performed either in the absence or in the presence of the α1-AT$_P$ expression plasmid, or, as a control, a wild-type α1-AT expression plasmid. Expression of wild-type α1-AT had no effect on the processing of pro-vWF mediated by PACE (Figure 3, lane 4) or PACE4 (Figure 3, lane 7) while expression of the mutant α1-AT$_P$ significantly inhibited PACE processing activity (Figure 3, lane 5). Processing of pro-vWF by the endogenous COS-1 cell propeptide cleaving enzyme was also inhibited by α1-AT$_P$ expression (Figure 3, lane 2). In contrast, mutant α1-AT$_P$ (Figure 3, lane 8) expression only slightly inhibited the PACE4-mediated processing of pro-vWF (Figure 3, lane 8).

To determine whether the differential sensitivity to inhibition of propeptide processing was due to differences in the expression level of the propeptidase or the inhibitor, the expression of α1-AT$_P$, PACE, and PACE4 was evaluated in the cotransfected cells. Analysis of total [^{35}S]methionine-radiolabeled conditioned media revealed the presence of a

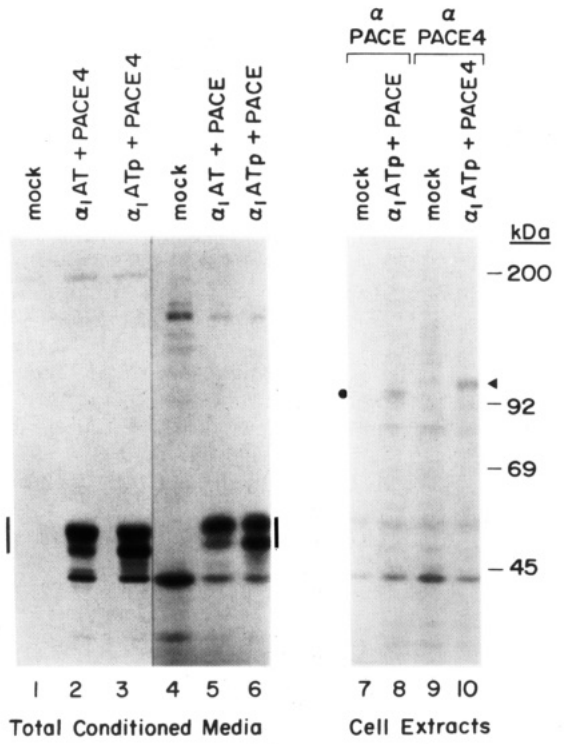

FIGURE 4: Expression of PACE, PACE4, α1-AT, and α1-AT$_P$ in cotransfected cells. COS-1 cells were transfected with the expression vectors for α1-AT (lanes 2 and 5) or α1-AT$_P$ (lanes 3, 6, 8, and 10) in the presence of PACE (lanes 5, 6, and 8) or PACE4 (lanes 2, 3, and 10). α1-AT and α1-AT$_P$ were detected by analysis of total conditioned media prepared from cells metabolically labeled for 20 min with [^{35}S]methionine. PACE (lane 8) and PACE4 (lane 10) were detected by immunoprecipitation using specific antibodies as described under Materials and Methods. Lanes 1 and 4 represent total radiolabeled conditioned medium prepared from mock-transfected cells as a control, and lanes 7 and 9 represent control immunoprecipitations of cell extracts from mock-transfected cells using the PACE- and the PACE4-specific antiserum, respectively. The filled circle indicates that the anti-PACE immunoreactive band, and the filled arrowhead indicates the anti-PACE4 immunoreactive band. Bars indicate a 55- and 60-kDa doublet present in conditioned media of cells expressing α1-AT or α1-AT$_P$ but not mock-transfected cells.

doublet migrating at 55–60 kDa from cells cotransfected with α1-AT$_P$ or with α1-AT in the presence of PACE4 (Figure 4, lanes 2 and 3) or PACE (Figure 4, lanes 5 and 6) but not mock-transfected cells (Figure 4, lanes 1 and 4). This size is that expected for α1-AT (Rosenberg et al., 1984). The amounts of wild-type and Pittsburgh mutant α1-AT were similar in the conditioned medium. This shows that the expression levels of the wild-type and mutant α1-AT in the presence of PACE or PACE4 were not significantly different. Quantitative immunoprecipitation of cell extracts prepared from α1-AT$_P$ and PACE cotransfected cells with an anti-PACE antibody detected the presence of a 100-kDa species (Figure 4, lane 8) not present in mock-transfected cells (Figure 4, lane 7). In addition, a similar amount of PACE4 immunoreactive material migrating at 120 kDa was detected in cells cotransfected with α1-AT$_P$ and PACE4 (Figure 4, lane 10) but not in mock-transfected cells (Figure 4, lane 9). These results show that the expression level of α1-AT$_P$ was comparable to that of α1-AT and the expression level of PACE was comparable to PACE4 in the cotransfected cells.

DISCUSSION

PACE and PACE4 are both expressed in a wide variety of tissues including liver, kidney, and endothelial cells (Roebroek et al., 1986; Kiefer et al., 1991). In addition, the catalytic domain of PACE4 has the greatest homology to PACE compared to other members of the mammalian subtilisin-like proteinases. In this study, we show that PACE4 has a propeptide processing activity and in addition, using anti-peptide antibodies against sequences derived from the deduced sequence, we have identified PACE4 as a 120-kDa protein that is predominantly cell-associated. COS-1 cells were cotransfected with expression vectors for PACE4 and vWF. vWF expressed in COS-1 cells was inefficiently processed by the endogenous COS-1 cell enzyme and resulted in secretion of vWF of which 70% was in the pro form. Coexpression of vWF with PACE4 yielded secretion of completely processed mature vWF. This is the first demonstration that PACE4 has propeptide cleaving activity.

We have previously shown that efficient vWF processing by PACE required the presence of a P4 arginine as well as a basic residue at the P2 position (Rehemtulla & Kaufman, 1992b), consistent with results using a different substrate, pro-renin (Hosaka et al., 1991). The substrate sequence requirements for PACE4 processing were studied by cotransfection of the PACE4 expression vector with the expression vectors encoding vWF mutants. The results demonstrated that efficient PACE4 processing of pro-vWF required a P4 arginine as well as a P2 basic residue. Substitution of the P4 or the P2 residues had a more pronounced effect on PACE4 processing compared to PACE. Since the synthesis of PACE was similar to PACE4 in the cotransfected cells, the reduced efficiencies for PACE4 cleavage of mutant vWF likely do not result from a lower level of expression. The differences in the ability of PACE and PACE4 to cleave mutant vWFs with P4 and P2 substitution may reflect different efficiencies for these enzymes to cleave vWF or that the two enzymes may have different sequence requirements. Purification of the two proteases is in progress to enable detailed analysis of substrate specificities.

In a separate transfection study using Chinese hamster ovary (CHO) cells, we have demonstrated that PACE can efficiently cleave pro-factor IX to yield mature factor IX (Wasley et al., 1993). In contrast, PACE4 was not able to process pro-factor IX under identical conditions in CHO cells (Wasley et al., 1993). Additional experiments have shown that PACE4 can process pro-vWF in the CHO cell transfection system (A. Rehemtulla, unpublished experiments), indicating that the differences in the ability of PACE and PACE4 to recognize pro-factor IX and pro-vWF do not result from differences between the CHO and COS-1 cell assay systems. This is interesting since the cleavage sites for both factor IX (**RPKR**) and vWF (**RSKR**) possess a P4 arginine in addition to the paired basic amino acid residues. The inability of PACE4 to cleave factor IX suggests that amino acid requirements other than the RXKR sequence may be involved in substrate recognition. These sequences could involve conformational features in addition to differences in the primary sequence. The possibility also exists that differences between the two enzyme activities were due to the fact that they were localized to different subcellular compartments.

Differences between PACE and PACE4 were also evident when the two enzymes were functionally analyzed in the presence of α1-AT$_P$ (Owen et al., 1983). PACE-mediated processing of pro-vWF was significantly diminished by coexpression of α1-AT$_P$, whereas coexpression of wild-type α1-AT had no effect. The different abilities to inhibit PACE processing were not due to differences in expression levels since analysis of secreted proteins revealed similar amounts

of α1-AT$_P$ and α1-AT in the respective conditioned media. The inhibitory activity of α1-AT$_P$ was specific for PACE since under similar conditions α1-AT$_P$ expression only slightly inhibited processing of pro-vWF mediated by PACE4. The inhibition of PACE compared to PACE4 was not due to different expression levels of the propeptide cleaving enzymes. Interestingly, processing by the endogenous COS-1 cell enzyme was also inhibited by α1-AT$_P$, suggesting that the endogenous COS-1 cell enzyme is more related to PACE than to PACE4. Misumi et al. (1990) also demonstrated the ability of α1-AT$_P$ to inhibit processing by PACE; however, the inhibition of PACE mediated by α1-AT$_P$ observed in the *in vivo* transfection system differs from the observations of Molloy et al. (1992) where inhibition of PACE activity by α1-AT$_P$ was not detected *in vitro*. The difference may be due to the conditions of the *in vitro* assay which utilized a synthetic peptide substrate and a truncated, soluble form of PACE. At present, we do not know whether the α1-AT$_P$ mutant directly inhibits PACE activity in this *in vivo* system. With this caveat, the inability of α1-AT$_P$ efficiently inhibit the processing activity of PACE4 compared to PACE suggests that PACE and PACE4 must exhibit conformational differences at or around the catalytic site such that access by the protease inhibitor is blocked. Such structural differences may also be responsible for their differential ability to process pro-factor IX. Analysis of protein structure using molecular modeling may provide insight into structural differences between these two proteases that can lead to differences in substrate recognition.

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