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Regulation of PACE propeptide-processing activity: Requirement for a post-endoplasmic reticulum compartment and autoproteolytic activation

(furin/von Willebrand factor/serine protease)

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ABSTRACT **PACE (paired basic amino acid cleaving enzyme) is a subtilisin-like serine protease involved in processing of propeptides in the constitutive secretory pathway. We here demonstrate that the transmembrane and cytoplasmic domains of PACE are required for retention in the secretory pathway but not for propeptide-cleaving activity. Addition of the endoplasmic reticulum retention signal Lys-Asp-Glu-Leu (KDEL) to the carboxyl terminus of the truncated molecule resulted in intracellular retention of the protein and loss of activity, indicating that the endoplasmic reticulum is an inappropriate environment for propeptide processing. In addition, mutation of a consensus PACE cleavage site within the amino-terminal region prevented processing of PACE to a mature form and destroyed activity. These data indicate that PACE is synthesized as a proprotein which requires autoproteolytic removal of an 81-residue pro sequence for optimal activity. A mutant form of PACE that lacked the pro sequence was nonfunctional, and addition of a pro sequence from a homologous subtilisin-like serine protease, PC2, did not restore activity. By analogy to the bacterial subtilisin family, the propeptide of PACE may guide the folding of PACE into an active enzyme.**

Propeptide cleavage of neuropeptides, polypeptide hormones, growth factors, and plasma proteins often occurs after paired basic amino acid residues (Arg-Arg or Lys-Arg). Some of the enzymes responsible for this process have recently been identified and are homologous to the yeast propeptidase Kex2 and bacterial subtilisins (1). Four members of the family have been identified and some have been functionally characterized. The first of these was initially identified as a protein coding sequence present upstream of the *fes/feps* protooncogene and hence named furin (2); this enzyme was later named PACE (paired basic amino acid cleaving enzyme) because of its ability to cleave after paired basic amino acid residues (3). PACE is able to process substrates such as pro-nerve growth factor (NGF) (4) and pro-von Willebrand factor (vWF) (3), which contain the sequence Arg-Xaa-(Lys/Arg)-Arg. Two additional members of this subtilisin-like serine protease family, prohormone convertase 2 (PC2) and PC1/PC3 (5–7), are selectively expressed in neuroendocrine tissues and thus may predominantly be involved in processing of neuroendocrine precursors such as proopiomelanocortin and proinsulin (8, 9). Both PC2 and PC1/PC3 contain an amphipathic α-helical sequence at their carboxyl terminus. A similar sequence in carboxypeptidase E facilitates membrane association (10). The amphipathic helix may play a role in localizing PC2 and PC1/PC3 to secretory granules. The fourth member of the family, PACE4 (11), appears to be ubiquitously expressed and contains neither a transmembrane domain nor an amphipathic helix.

Unlike PC2 and PC1/PC3, PACE is expressed ubiquitously and, like its yeast homologue Kex2, contains a predicted transmembrane domain at its carboxyl terminus. Subcellular fractionation as well as immunofluorescence studies have localized Kex2 to the Golgi compartment (12). Here we demonstrate that the PACE transmembrane and cytoplasmic domains are required for intracellular localization of the enzyme but not for propeptide-cleaving activity. Addition of the endoplasmic reticulum (ER) retention signal KDEL resulted in intracellular retention of soluble PACE and loss of activity. This suggests that the PACE localized to the ER by the KDEL retention signal is in an inappropriate environment for PACE activity. We also demonstrate here that PACE is synthesized as a proprotein that requires autoproteolytic removal of the propeptide for optimal activity. Deletion of the PACE propeptide destroyed processing activity. The results suggest that the propeptide may play a role in protein folding analogous to the propeptide of prosubtilisin (13). Failure to complement the deletion of the propeptide with a heterologous propeptide sequence of PC2 suggests that individual members of the family exhibit specific requirements for proper folding and activity.

MATERIALS AND METHODS

Recombinant Plasmids and Transfections. PACE cDNA in the expression plasmid pMT3 and vWF cDNA in the expression plasmid pMT2 have been described (3). COS-1 monkey kidney cells were cultured and transfected as described (14). Cotransfections were performed using equal amounts of the respective plasmids. PC2 and PC3 cDNAs in the expression vector pMT3SV2 (14) were kindly provided by S. Smeekens (Howard Hughes Medical Institute, University of Chicago, IL).

Analysis of Expressed Proteins. Forty hours after transfection, cells were radiolabeled with [^{35}S]methionine (250 μCi/ml; >8000 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq) in methionine-free medium containing 2% dialyzed fetal bovine serum for 1 hr and a chase was performed in complete medium containing 10% fetal bovine serum for 4 hr. Conditioned media samples were harvested and soybean trypsin inhibitor (1 mg/ml), phenylmethylsulfonyl fluoride (1 mM), and aprotinin (0.2 mg/ml) were added. Immunoprecipitation with anti-vWF-specific antiserum (Diagnostica Stago, France) was performed as described (3). Immunoprecipitated material was resolved by electrophoresis in SDS/8% polyacrylamide gels in the presence of reducing agent and visualized by fluorography with EN^{3}HANCE (DuPont).

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Abbreviations: ER, endoplasmic reticulum; PC, prohormone convertase; vWF, von Willebrand factor.

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Western blot analysis was performed by electroblotting SDS/PAGE-resolved proteins onto nitrocellulose membranes. PACE-specific bands were identified with a rabbit anti-PACE antiserum (kindly provided by P. Barr, Chiron) (3).

Mutagenesis. Mutagenesis of the PACE cDNA was performed in pBluescript (Stratagene). The uracil incorporation method (Bio-Rad) was used to modify the coding sequence and the mutated fragment was reintroduced into pMT3. Oligonucleotides used for mutagenesis were as follows: PACE KDEL, 5′-GCCCTCACACCTGCCTGAGAAAGATGAGTTGTGATGAGCCCACTGCCCA-3′; PACE KDEV, 5′-CACCTGCCTGAGAAAGATGAGGTTTGATGAGCCCAC-3′; PACE KTDR, 5′-ACACGTCCCGATCGGTTTTTCGCTTTGCC-3′; PACE Δpro, 5′-GCTCAGGGCCAGAAGGTCGACGTGTACCAGGAG-3′. PACE Δpro has residues 29–108 deleted (3). The deletion also introduces a *Sal* I site. PACE PC2pro was constructed by using Vent DNA polymerase (New England Biolabs) and the oligonucleotides 5′-GCGACCGGTCGACACGAATCATTTT-3′ and 5′-CTCATTGATGTCGACGTAACCTCGCTT-3′ to amplify the PC2 propeptide sequence. The amplified fragment was digested with *Sal* I and ligated to *Sal* I-digested PACE Δpro. The DNA sequences of all mutagenized fragments were confirmed by dideoxynucleotide sequencing with the Sequenase system (United States Biochemical).

RESULTS

Transmembrane Domain of PACE Is Not Required for Propeptide-Cleaving Activity. PACE is unique among the known mammalian propeptide-cleaving enzymes in that it, like its yeast homologue Kex2, contains a predicted transmembrane domain at its carboxyl terminus. The transmembrane domain of Kex2 localizes the enzyme to the Golgi apparatus (12, 15). To investigate the role of the PACE transmembrane domain and cytoplasmic tail, we constructed a mutant form of the protein (PACE SOL) that lacks residues 716–794 (3), which encompass the predicted transmembrane domain and cytoplasmic tail. Western blot analysis of extracts of COS-1 cells transfected with the wild-type PACE expression vector revealed a doublet at ≈100 kDa that was recognized by anti-PACE antiserum (Fig. 1*A*, lane 5). PACE was not detected in the conditioned medium of these cells by Western blot analysis (data not shown). However, the anti-PACE antiserum immunoprecipitated low levels of a 90-kDa polypeptide from the conditioned medium after metabolic labeling of the transfected cells (Fig. 1*B*, lane 1). Western blot analysis of extracts prepared from cells transfected with an expression vector encoding PACE SOL revealed the presence of a 97-kDa doublet. In contrast to wild-type PACE, expression of PACE SOL resulted in secretion of large amounts of immunoreactive material into the medium (Fig. 1*B*, lane 2). The majority migrated at 91 kDa and a small amount comigrated with secreted wild-type PACE at 90 kDa. These results show that the transmembrane domain is required for retention of PACE within the secretory pathway.

As described previously (3, 16), cotransfection of the PACE expression construct with a pro-vWF expression construct resulted in enhanced processing of pro-vWF. vWF expressed and secreted from COS-1 cells appeared as a doublet with the upper band representing pro-vWF and the lower band representing mature processed vWF (Fig. 2). Cotransfection of pro-vWF with wild-type PACE or PACE SOL resulted in enhanced processing of pro-vWF (Fig. 2). The enhanced processing by PACE SOL demonstrates that residues 716–794, which code for a predicted transmembrane domain and cytoplasmic tail, are not required for proteolytic activity.

PACE Retained in the ER by the KDEL Receptor Is Not Active. The observation that PACE SOL was active suggested that localization of the enzyme to membranes of the secretory apparatus was not essential. To determine whether processing activity could occur in the ER or required transit to the Golgi complex, two additional mutants were constructed. PACE KDEL had the tetrapeptide sequence KDEL added to the carboxyl terminus of PACE SOL (after residue 715). PACE KDEV had the carboxyl-terminal leucine of PACE KDEL mutated to valine. The presence of the KDEL sequence directs retention in the ER, and mutation of the carboxyl-terminal leucine to valine disrupts the retention. Transfection of PACE KDEL and KDEV expression constructs into COS-1 cells followed by Western blot analysis of

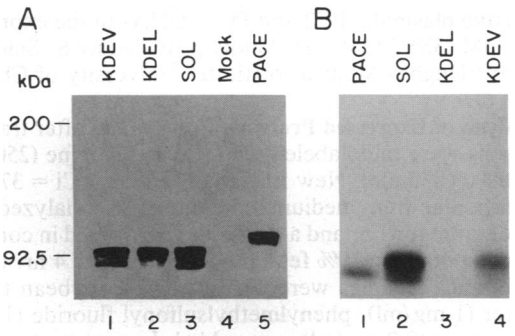

FIG. 1. (*A*) Western blot analysis of PACE and PACE mutants. COS-1 cells were transfected with expression constructs encoding wild-type PACE or mutants of PACE, and cell extracts were subjected to Western blot analysis. Lanes: 1, PACE KDEV; 2, PACE KDEL; 3, PACE SOL; 4, no DNA; 5, wild-type PACE. (*B*) Immunoprecipitations of conditioned media prepared from metabolically labeled COS-1 cells transfected with the indicated expression constructs. Lanes 1, wild-type PACE; 2, PACE SOL; 3, PACE KDEL; 4, PACE KDEV. Molecular size markers shown in *A* also represent approximate mobility of proteins in *B*.

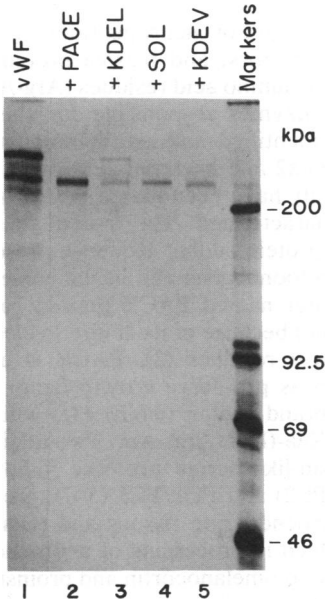

FIG. 2. Functional analysis of PACE and PACE mutants. Expression vectors for wild-type PACE or mutants of PACE were transfected into COS-1 cells in the presence of the pro-vWF expression vector. At 48 hr posttransfection, cells were metabolically labeled and conditioned media were collected over 5 hr. vWF was immunoprecipitated from the conditioned media and analyzed by SDS/PAGE and autoradiography. Lanes: 1, pro-vWF alone; 2, pro-vWF cotransfected with wild-type PACE; 3, pro-vWF cotransfected with PACE KDEL; 4, pro-vWF cotransfected with PACE SOL; 5, pro-vWF cotransfected with PACE KDEV.

cell extracts with the PACE-specific antiserum revealed that intracellular forms of both mutants comigrated with PACE SOL (Fig. 1*A*, lane 1). However, while PACE SOL and PACE KDEV appeared as a pair of bands of equal intensity, PACE KDEL exhibited less of the lower molecular weight species (Fig. 1*A*, lane 2). Immunoprecipitation of conditioned medium from [^{35}S]methionine-labeled COS-1 cells expressing the mutants did not detect PACE KDEL secreted into the medium (Fig. 1*B*, lane 3). In contrast, PACE KDEV was detected in the medium and comigrated with PACE SOL (Fig. 1*B*, lane 4). These results indicate that PACE KDEL was retained intracellularly, probably in the ER, due to the presence of the KDEL sequence.

To investigate whether the ER-retained PACE was active, cotransfection experiments with pro-vWF were performed. Analysis of pro-vWF secreted from transfected cells revealed that PACE KDEL was not capable of processing vWF. In contrast, PACE KDEV efficiently processed pro-vWF to the mature species (Fig. 2, lanes 3 and 5). This suggests that transport out of the ER is required for PACE activity.

PACE Is Synthesized as a Proprotein and Requires Proteolytic Removal of the Propeptide for Activity. As shown in Fig. 1, wild-type as well as truncated mutants of PACE migrated as a closely spaced doublet. Since PACE contains a canonical PACE cleavage site (-Arg^{104}ThrLysArg^{107}-) at its amino terminus, it was proposed (3, 16) that the doublet observed in cell extracts may result from proteolysis at this site. To test this hypothesis, the arginine at 104 and lysine at 106 were mutated to lysine and aspartic acid, respectively (PACE KTDR). Analogous substitutions of the cleavage site in pro-vWF were previously shown to inhibit processing by PACE (16). Expression of the mutant (PACE KTDR) in COS-1 cells indicated that unlike wild-type PACE, PACE KTDR appeared to consist of predominantly the higher molecular weight species of the intracellular doublet as determined by Western blot analysis of cell extracts (Fig. 3). This suggested that PACE was synthesized as a 100-kDa proprotein migrating as the higher molecular weight band of the doublet and was cleaved at Arg^{107} to yield a smaller mature protein. To determine whether this cleavage was required for activity, the ability of PACE KTDR to cleave pro-vWF was examined by cotransfection experiments. Cotransfection of wild-type PACE with pro-vWF resulted in secretion of only mature, processed vWF (Fig. 4). In contrast, cotransfection of pro-vWF with PACE KTDR resulted in secretion of predominantly pro-vWF, indicating the loss of processing activity.

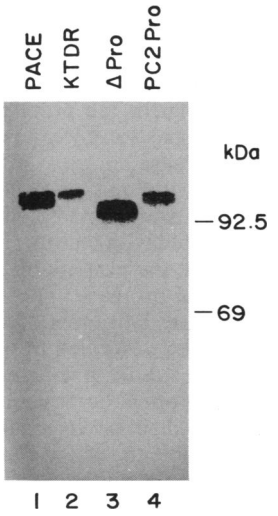

FIG. 3. Western blot analysis of PACE and PACE propeptide mutants. COS-1 cells were transfected with expression vectors encoding PACE and various PACE mutants and cell extracts were analyzed 48 hr posttransfection. Lanes: 1, wild-type PACE; 2, PACE KTDR; 3, PACE Δpro; 4, PACE PC2pro.

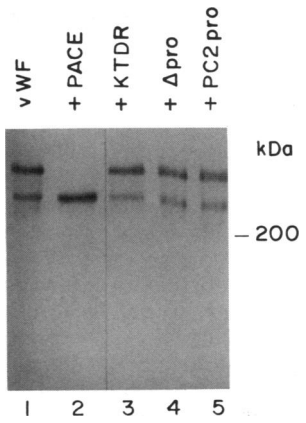

FIG. 4. Functional analysis of PACE and PACE propeptide mutants. COS-1 cells were transfected and conditioned media from metabolically labeled cells were prepared. Immunoprecipitations were performed using a vWF-specific antiserum and analyzed by SDS/PAGE and autoradiography. Cells were transfected with a pro-vWF expression construct as well as the indicated PACE constructs. Lanes: 1, pro-vWF alone; 2, wild-type PACE; 3, PACE KTDR; 4, PACE Δpro; 5, PACE PC2pro.

Since failure to proteolytically remove the propeptide resulted in loss of activity, we constructed a mutant of PACE that had a deletion of sequences coding for the propeptide (residues 29–108; PACE Δpro). Expression constructs coding for PACE Δpro and pro-vWF were cotransfected into COS-1 cells and the secreted vWF was analyzed by [^{35}S]methionine labeling and immunoprecipitation of conditioned medium. The results revealed that PACE Δpro was not active in processing pro-vWF (Fig. 4, lane 3). Western blot analysis detected a polypeptide expressed from PACE Δpro within the cell extracts that comigrated with the lower band of the doublet in wild-type PACE (Fig. 3).

To investigate the ability of a heterologous propeptide sequence to complement the Δpro mutation, a hybrid was constructed where nucleotide sequences coding for residues 29–112 of the homologous PC2 protein were inserted in PACE Δpro to yield the mutant PACE PC2pro. Expression of the PACE PC2pro mutant in COS-1 cells revealed a 100-kDa band that comigrated predominantly with the upper band of the doublet in wild-type PACE (Fig. 3). This is the appropriate molecular mass for the protein containing the propeptide of PC2 and the mature sequence of PACE. An additional band migrating faster than the major band was also detected, indicating that some cleavage of PACE PC2pro propeptide may have occurred, possibly due to the endogenous COS-1 cell processing enzyme. Further cotransfection experiments with pro-vWF demonstrated that the chimeric protein was unable to process vWF (Fig. 4, lane 5).

Immunoprecipitation of conditioned media prepared from metabolically labeled COS-1 cells expressing the KTDR, Δpro, and PC2pro mutants of PACE revealed that unlike wild-type PACE, the mutants were not secreted as a 90-kDa form (data not shown). The lack of the secreted 90-kDa form of PACE in cells expressing nonfunctional mutants of PACE (e.g., PACE KDEL, Fig. 1*B*, lane 3, and PACE SA, ref. 16) is consistent with a requirement for autoproteolytic processing at dibasic (Lys-Arg, Arg-Arg) residues at the carboxyl terminus to remove the transmembrane domain for secretion.

DISCUSSION

Like the yeast prohormone-processing enzyme Kex2, PACE contains a predicted hydrophobic transmembrane domain

that divides the protein into a 716-residue lumenal proteolytic domain and a short cytoplasmic tail. Recent studies of Kex2 expression in yeast cells suggested that the enzyme was localized to the Golgi apparatus (12, 17) and both the carboxyl-terminal cytoplasmic tail and a functional clathrin heavy chain were required for intracellular localization (15). To study the role of the transmembrane domain and the cytoplasmic tail in intracellular localization and the functional significance of membrane association, we have constructed a truncated soluble form of PACE that has 78 residues deleted from the carboxyl terminus (PACE SOL). Transient expression of this mutant in COS-1 cells demonstrated that the protein was efficiently secreted into the medium. Thus, the transmembrane domain and cytoplasmic tail of PACE are required for localization within the secretory pathway. Interestingly, cotransfection experiments with pro-vWF showed that the soluble mutant was functional intracellularly, since it was able to efficiently process pro-vWF. This demonstrates that the transmembrane domain and cytoplasmic tail of PACE are not required for functional activity but play a role in localizing the enzyme in the Golgi apparatus. As indicated by Western blot analysis of cell extracts, the intracellular steady-state levels of the soluble mutant were high, suggesting a slow rate of transit through the secretory pathway. This high intracellular level of PACE SOL may facilitate efficient processing of pro-vWF despite the eventual secretion of PACE SOL. Mixing of conditioned media from cells transfected with PACE SOL and pro-vWF did not result in enhanced processing (data not shown), indicating that PACE SOL does not cleave pro-vWF in the conditioned medium. However, recent purification of PACE SOL from the conditioned medium demonstrated the ability of PACE SOL to cleave a synthetic substrate in an *in vitro* assay (Chris Rhodes, A.R. and R.J.K., unpublished observation).

An earlier unexpected observation was that cells transfected with PACE secreted low levels of a 90-kDa form of PACE (3). In contrast, a secreted form of PACE was not detected from cells transfected with inactive mutants of PACE such as PACE KDEL (Fig. 1*B*, lane 3) and PACE SA (16), an active-site serine-to-alanine mutant of PACE (data not shown). Cells transfected with the PACE SOL expression construct secreted a protein that comigrated with secreted wild-type PACE, suggesting that the 90-kDa band may be generated through autocatalytic cleavage after paired basic amino acid residues that reside at the carboxyl-terminal side of the catalytic domain. Interestingly, the mammalian homologues PC2 and PC3 and the yeast homologue Kex2 also contain paired basic residues at similar locations, suggesting that autocatalytic cleavage may be a common mechanism by which proteins of this family are turned over.

Since various reports suggested that PACE was localized to the Golgi apparatus, we wanted to examine whether the environment of the ER was favorable for PACE activity. Addition of the KDEL sequence for ER retention onto PACE SOL prevented secretion of the protein and destroyed pro-vWF-processing activity. Mutation of the terminal leucine residue of PACE KDEL to valine in PACE KDEV resulted in secretion of PACE and restoration of activity. This rules out the possibility that the addition of four carboxyl-terminal residues to PACE SOL altered protein conformation and resulted in loss of activity and suggests that the ER is an unfavorable environment for propeptide-processing activity. It is possible that the proprotein fails to achieve an appropriate conformation in the ER for activity. However, it is not possible to rule out that binding of PACE KDEL to the KDEL receptor results in loss of function either through steric constraints or by sequestering the enzyme in an unfavorable intracellular location. These data conflict with a recent study (17) which attached an HDEL sequence to a truncated mutant of Kex2 and demonstrated that the mutant

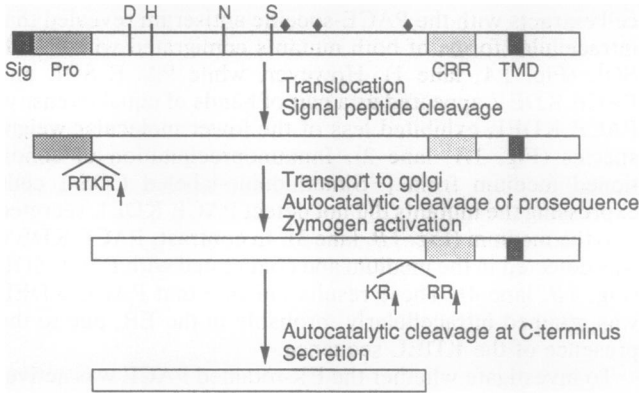

FIG. 5. Intracellular maturation and regulation of PACE. Sig, signal peptide; Pro, propeptide sequence; CRR, cysteine-rich region; TMD, transmembrane domain; D, H, N, and S, aspartic, histidine, asparagine and serine residues involved in catalytic activity; diamonds, potential N-linked glycosylation sites. Cleavage sites are indicated with arrows after paired basic residues.

retained activity. The conflicting data could be due to biochemical differences between Kex2 and PACE as well as differences in the mammalian cell and the yeast cell system.

The appearance of PACE as well as PACE SOL as a doublet inside the cell indicated that, like Kex2 (18), PACE is synthesized as a zymogen and removal of a propeptide sequence by cleavage after paired basic amino acid residues activates the protease. Examination of the PACE sequence revealed a dibasic consensus cleavage sequence with an arginine residue in the −4 position (RTKR). This sequence is a typical PACE cleavage site (16). To examine the functional significance of this putative cleavage site, a mutant PACE was constructed where the RTKR sequence was mutated to KTDR. Western blot analysis of PACE KTDR showed that the mutant consisted predominantly of the upper band of the doublet present in wild-type PACE. Cotransfection experiments revealed that PACE KTDR was not able to process pro-vWF. These data indicate that PACE is synthesized as a proprotein and requires removal of the propeptide for maturation into a functional protease. Not only PACE but also PC2, PC3, and PACE4 contain the RXKR sequence at homologous positions. Expression analysis of PC2 in COS-1 cells also identified two major species, and the higher molecular weight form was processed to the lower molecular weight when PACE was coexpressed (data not shown). This suggests that PC2 and possibly PC3 and PACE4 require cleavage by PACE for activation.

When sequences coding for the propeptide were deleted and the mutant (PACE Δpro) was coexpressed with pro-vWF in COS-1 cells, no increase in pro-vWF processing was observed, indicating that the mutant was inactive. Western blot analysis of PACE Δpro-transfected cells detected a polypeptide that comigrated with mature, propeptide-deleted PACE. This suggests that the propeptide is required for the generation of a functional protease. The observation that a functional protease requires a propeptide that is autoproteolytically removed has been described by Ikemura *et al.* (19, 20) in studies of bacterial subtilisin. Zhu *et al.* (13) have also demonstrated that the propeptide is required to guide the refolding of unfolded subtilisin *in vitro*. To examine whether the PC2 propeptide was able to restore functional activity of PACE Δpro, a hybrid protein was constructed where the propeptide sequence of PC2 was inserted in PACE Δpro. When expressed in COS-1 cells, the resulting mutant PACE PC2pro was not able to process pro-vWF. The polypeptide expressed from PACE PC2pro comigrated with pro-PACE and appeared as a doublet. The doublet may have resulted from cleavage of the PC2 propeptide by the endogenous

COS-1 cell enzyme. The inability of PACE PC2pro to enhance processing of pro-vWF could be due to a failure of the hybrid to obtain an appropriate conformation or due to retention in an inappropriate intracellular environment.

This work was undertaken to study the factors regulating PACE activity. The results demonstrate that PACE is synthesized as a proprotein and that autoproteolytic removal of the propeptide is required for proteolytic activity (Fig. 5). Data presented here also suggest that the propeptide plays a role in protein folding and that this function is protein-specific, since propeptide sequences from a homologous protein did not complement the Δpro mutation. These folding events probably occur in the ER, where the enzyme is not active; upon transit to the Golgi compartment, the propeptide is removed, resulting in mature functional PACE. After activation by proteolytic removal of the propeptide, PACE may be further processed to yield a secreted form. A balance of proteolytic activation and inactivation may regulate intracellular PACE activity.

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