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Biochemistry

Expression of a human proprotein processing enzyme: Correct cleavage of the von Willebrand factor precursor at a paired basic amino acid site

(paired basic amino acid cleaving enzyme/furin/propeptide cleavage/endopeptidase/COS cell expression)

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ABSTRACT **Intracellular proteolytic processing of precursor polypeptides is an essential step in the maturation of many proteins, including plasma proteins, hormones, neuropeptides, and growth factors. Most frequently, propeptide cleavage occurs after paired basic amino acid residues. To date, no mammalian propeptide processing enzyme with such specificity has been purified or cloned and functionally characterized. We report the isolation and functional expression of a cDNA encoding a propeptide-cleaving enzyme from a human liver cell line. The encoded protein, called PACE (paired basic amino acid cleaving enzyme), has structural homology to the well-characterized subtilisin-like protease Kex2 from yeast. The functional specificity of PACE for mediating propeptide cleavage at paired basic amino acid residues was demonstrated by the enhancement of propeptide processing of human von Willebrand factor when coexpressed with PACE in COS-1 cells.**

With few exceptions, proteins that transit the secretory apparatus in eukaryotes are synthesized as larger precursor polypeptides. In addition to signal peptide cleavage upon translocation into the endoplasmic reticulum (1, 2), many polypeptides require further proteolytic processing for their full maturation prior to secretion and, in many cases, for their biological activity. Frequently, cleavage of these precursors occurs at sites marked by paired basic amino acid residues—primarily, Lys-Arg and Arg-Arg (for reviews, see refs. 3 and 4). These cleavages remove propeptides that function in a variety of essential roles in the maturation of the precursor proteins including (*i*) correct folding and disulfide bond formation (5, 6), (*ii*) γ-carboxylation of glutamic acid residues (7, 8), (*iii*) directing intracellular targeting (9), and (*iv*) regulating the coordinate synthesis of multiple mature peptides from a single precursor polypeptide, typified by proopiomelanocortin (10, 11). In addition to these natural cellular products, several viral polyproteins require cleavage at paired basic amino acid residues. For example, the retroviral envelope protein precursors, including that of the human immunodeficiency virus, require cleavage at a paired basic amino acid site for infectivity (12, 13).

Although several candidate endoproteolytic enzymes have been proposed to be involved in the propeptide processing reactions (14–17), the enzymes responsible for these cleavages in mammalian cells are surprisingly poorly characterized at the molecular level. In contrast, the yeast enzyme Kex2, a membrane-bound, Ca^{2+}-dependent serine protease, is well characterized and is considered to be a prototypic proprotein endopeptidase (18–20). Kex2 functions late in the secretory pathway of *Saccharomyces cerevisiae* to cleave the polypeptide chains of prepro-killer toxin and prepro-α-factor at the paired amino acid sequences Lys-Arg and Arg-Arg. In addition to these natural functions, the Kex2 protease can mediate the processing of proinsulin and proalbumin expressed in yeast (21, 22) and can properly cleave the propeptide of proalbumin *in vitro* (23). Finally, Kex2 can function in transfected murine cells to process proopiomelanocortin prohormone to product peptides normally found *in vivo* (11).

Recently, two distinct human sequences were identified based on homology to the yeast Kex2 protease. A human insulinoma cDNA, designated PC2, that encodes a putative subtilisin-like protease has been implicated in the endoproteolytic processing of prohormones (24); however, no functional activity has been reported. Fuller *et al.* (20) reported significant homology between Kex2 and furin, the predicted product of the human *FUR* gene, a transcription unit found immediately upstream of the *FES/FPS* oncogene (25, 26). Subsequent cDNA cloning (ref. 27; GenBank data base accession no. X17094) confirmed the overall homology of the *FUR* gene product to Kex2 and the subtilisin protease family. Here we report the isolation and functional expression of a cDNA encoding the *FUR* gene product. The cDNA, when transfected into mammalian cells directs the synthesis of a 90-kDa intracellular protein with an activity that increases the efficiency of processing of pro-von Willebrand factor (vWF) at its natural cleavage site. We propose the acronym PACE (paired basic amino acid cleaving enzyme) for this human gene product that may be a prototype for mammalian propeptide cleaving enzymes.

MATERIALS AND METHODS

Molecular Cloning of PACE cDNAs. We constructed a human liver cell line (HEPG2) cDNA library in the yeast expression vector pAB23BXN, a derivative of pAB23BX (28), into which a synthetic polylinker was inserted for unidirectional cDNA cloning as described (29). Oligonucleotide probes, based on the *FUR* DNA sequence (25, 26), were used to identify a 3.3-kilobase (kb) cDNA clone from the library. For isolation of the 5′ end of the PACE cDNA, a second cDNA library from HEPG2 poly(A)$^+$ mRNA was constructed in bacteriophage Lambda ZAP II (Stratagene), using PACE-specific internally primed message. The longest clone isolated from this library was used to construct a composite cDNA for PACE of 4.4 kb, which contained 388 base pairs of 5′ untranslated region, an open reading frame corresponding to 794 amino acids identical to the *FUR* gene product (27), and 1597 bases of 3′ untranslated region.

Expression Vector Construction. PACE cDNA was inserted into the cloning site (*Eco*RI/*Sal* I) of the expression vector

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Abbreviations: vWF, von Willebrand factor; PACE, paired basic amino acid cleaving enzyme; HA, influenza hemagglutinin.

pMT3. This vector is a derivative of pMT2 (30) with a deletion of the dihydrofolate reductase (DHFR) coding region on the 3′ side of the cloning site. The 2.47-kb (*Eco*RI/*Sal* I) PACE cDNA fragment included the 794-codon PACE coding sequence and 74 bases of 3′ untranslated sequence before a *Sal* I site. At the 5′ end, the sequence immediately preceding the ATG was modified, by polymerase chain reaction (31), to conform to the consensus translation start site by using an *Eco*RI-containing primer. For expression of pro-vWF, the vector pMT2-vWF (32) was used.

COS Cell Transfection and Culture. Plasmid DNA was introduced for transient expression in COS-1 cells by a calcium phosphate transfection protocol (33). Cells were transfected with 40 μg of plasmid or, in the case of cotransfections, an equal molar ratio of plasmids totaling 60 μg per 10-cm dish in 10 ml of medium.

Labeling, Immunoprecipitation, and Gel Electrophoresis. COS-1 cell products were radiolabeled 48 hr after transfection with [^{35}S]methionine and [^{35}S]cysteine in medium lacking cysteine and methionine. Cells were lysed at the indicated times in Nonidet P-40 lysis buffer (34). Cell extracts and conditioned medium samples were treated with protease inhibitors, immunoprecipitated as described (6), and analyzed on reduced SDS/polyacrylamide gels by fluorography in EN^{3}HANCE (DuPont). N-glycanase (Genzyme) digestion of immunoprecipitated material was performed as in ref. 34.

Anti-PACE Antibodies. Rabbit anti-PACE antiserum was generated against the predicted catalytic domain of PACE by expression of amino acids 146–372 of PACE as a superoxide dismutase (SOD) fusion protein in *Escherichia coli* using the SOD fusion vector pTAC7 (35). The induced fusion protein was purified by preparative PAGE and used to immunize rabbits in complete Freund's adjuvant.

RESULTS

Molecular Cloning and Structure of PACE. PACE cDNA was isolated from a human liver cell line cDNA library. The large open reading frame (794 amino acids) encodes a precursor protein with a calculated molecular mass of 86.7 kDa. Comparison of the amino acid sequences of PACE and Kex2, as well as that of the PC2-encoded protein (Fig. 1), reveals a striking similarity. This is particularly evident in a region of ≈250 residues that includes a putative catalytic domain homologous to the family of subtilisin-related serine proteases (for compilation, see ref. 36). PACE, Kex2, and PC2 exhibit considerably more sequence similarity to one another than to other subtilisin-related proteases, sharing a number of identical residues that distinguish them from other members of this family. In addition to five invariant cysteine residues, stretches of especially high similarity are clustered around regions that align with residues of subtilisin thought to be involved in catalysis. Asp-153, His-194, and Ser-368 correspond to residues in subtilisin that constitute the "charge relay" system during catalysis (37) and are invariant among all members of this protease family. Significant similarity extends beyond the subtilisin-like regions among these three sequences (Fig. 1*B*). Both PACE and Kex2 contain potential hydrophobic transmembrane domains. Between the subtilisin-like regions and putative transmembrane domains, PACE contains a cysteine-rich region, whereas Kex2 possesses a region rich in serine and threonine. PC2 lacks either type of region and also appears to lack a transmembrane domain.

Expression of PACE cDNA in COS-1 Cells. PACE cDNA was inserted into the simian virus 40 (SV40)-based expression vector pMT3 to generate the plasmid pMT3-PACE. This was transfected into SV40-transformed monkey kidney cells (COS-1). To monitor PACE synthesis, the transfected COS-1 cells were pulse-labeled for 30 min with ^{35}S-labeled amino acids and cell extracts were prepared for immunoprecipita-

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PACE  MELRPWL---LWVVAATGTLVLLAADAQGQKVFTNTWAVRIPGGPAVANSVARKHGFLNL   57
KEX2  MKVRKYITLCFWWAFSTSALVSSQQIPLKDHTSRQYFAVESNETLSRLEEMHPNWKYEHD  60
PC2   MKG---GCVSQWKAAAGFLFCVMVFASAERPVFTNHFLVELHKG-GEDKARQVAAEHGFG   56

PACE  GQIFGDYYHFWHR--GVTKRS-------------LSPHR--PRHSRLQREPQVQWLEQQV  100
KEX2  VRGLPNHYVFSKELLKLGKRSSLEELQGDNNDHILSVHDLFPRNDLFKRLPVPAPPMDSS  120
PC2   VRKLP----FAEGLYHFYHNG----LAKAKRRRSLHHKQQLERDPRVK-MALQQEGFDRK  107

PACE  AKR-RTKRDVYQEPTDPKFPQQWYL------SGVTQRDLNVKAAWAQGYTGHGIVVSILD  153
KEX2  LLP-VKEAEDKLSINDPLFERQWHLVNP----SFPGSDINVLDLWYNNITGAGVVAAIVD  175
PC2   KRGYRDINEIDINMNDPLFTKQWYLINTGQADGTPGLDLNVAEAWELGYTGKGVTIGIMD  167

PACE  DGIEKNHPDLAGNYDPGASFDVNDQDPDPQPRYTQMNDNRHGTRCAGEVAAVANNGVCGV  213
KEX2  DGLDYENEDLKDNFCAEGSWDFNDNTNLPKPRLSDDY---HGTRCAGEIAAKKGNNFCGV  232
PC2   DGIDYLHPDLASNYNAEASYDFSSNDPYPYPRYTDDWFNSHGTRCAGEVSAAANNNICGV  227

PACE  GVAYNARIGGVRMLDGEVT-DAVEARSLGLNPNHIHIYSASWGPEDDGKTVDGPARLAEE  272
KEX2  GVGYNAKISGIRILSGDITTE-DEAASLIYGLDVNDIYSCSWGPADDGRHLQGPSDLVKK  291
PC2   GVAYNSKVAGIRMLDQPFMTDIIEASSISHMPQLIDIYSASWGPTDNGKTVDGPRDVTLQ  287

PACE  AFFRGVSQGRGGLGSIFVWASGNGGREHDSCNCDGYTNSIYTLSISSATQFGNVPWYSEA  332
KEX2  ALVKGVTEGRDSKGAIYVFASGNGGTRGDNCNYDGYTNSIYSITIGAIDHKDLHPPYSEG  351
PC2   AMADGVNKGRGGKGSIYVWASGDGGSY-DDCNCDGYASSMWTISINSAINDGRTALYDES  346

PACE  CSSTLATTYSSG--NQNEKQIVTTDLRQKCTESHTGTSASAPLAAGIIALTLEANKNLTW  390
KEX2  CSAVMAVTYSSG----SGEYIHSSDINGRCSNSHGGTSAAAPLAAGVYTLLLEANPNLTW  407
PC2   CSSTLASTFSNGRKRNPEAGVATTDLYGNCTLRHSGTSAAAPEAAGVFALALEANLGLTW  406

PACE  RDMQHLVVQTSKPAHLNAN--DWATNGVGRKVSHSYGYGLLDAGAMVALAQNWTTVAPQR  448
KEX2  RDVQYLSILSAVGLEKNADG-DWRDSAMGKKYSHRYGFGKIDAHKLIEMSKTWENVNAQT  466
PC2   RDMQHLTVLTSKRNQLHDEVHQWRRNGVGLEFNHLFGYGVLDAGAMVKMAKDWKTV--PE  464

PACE  KCIIDIL----TEPKDIGKRLEVRKTVTACLGEPNHITRLEHAQARLTLSYNRRGDLAIH  504
KEX2  WFYLPTLYVSQSTNSTEETLESVITISEKSLQDANF-KRIEHVTVTVDIDTEIRGTTTVD  525
PC2   RFHCVGGSVQDPEKIPSTGKLVLTLTTDACEGKENFVRYLEHVQAVITVNATRRGDLNIN  524

PACE  LVSPMGTRSTLLAARPHDYSAD-GFNDWAFMTTHSWDEDPSGEWVLEIENTSEANNYGTL  563
KEX2  LISPAGIISNLGVVRPRDVSSE-GFKDWTFMSVAHWGENGVGDWKIKVKTT-ENGHRIDF  583
PC2   MTSPMGTKSILLSRRPRDDDSKVGFDKWPFMTTHTWGEDARGTWTLELGFVGSAPQKGVL  584

PACE  TKFTLVLYGTAPEGLPVPPESSGCKTLTSSQACVVCEEGFSLHQKSCVQHCPPGFAPQVL  623
KEX2  HSWRLKLFGESIDSSKTETFVFGNDKEEVEPAATESTVSQYSASSTSISISATSTSSISI  643
PC2   KEWTLMLHGTQSAPYIDQVVRDYQSKLAMSKKEELEEELDEAVERSLKSILNKN        638

PACE  DTHYSTENDVETIRASVCAPCHASCATCQGPALTDCLSCPSHASLDPVEQTCSRQSQSSR  683
KEX2  GVETSAIPQTTTASTDPDSDPNTPKKLSSPRQAMHYFLTIFLIGATFLVLYFMFFMKSRR  703

PACE  ESPPQQQPPRLPPEVEAGQRLRAGLLPSHLPEVVAGLSCAFIVLVFVTVFLVLQLRSGFS  743
KEX2  RIRRSRAETYEFDIIDTDSEYDSTLDNGTSGITEPEEVEDFDFDLSDEDHLASLSSSENG  763

PACE  FRGVKVYTMDRGLISYKGLPPEAWQEECPSDSEEDEGRGERTAFIKDQSAL          794
KEX2  DAEHTIDSVLTNENPFSDPIKQKFPNDANAESASNKLQELQPDVPPSSGRS          814
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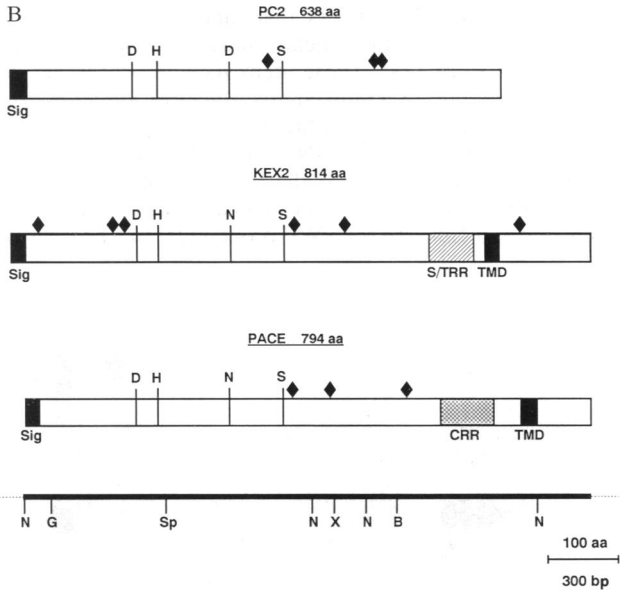

FIG. 1. Sequence and organizational similarity of PC2, Kex2, and PACE. (*A*) Amino acid sequences of PACE, Kex2, and PC2 were aligned with the aid of the FASTA algorithm (51). Identical residues are shown by shaded boxes. Asterisks indicate likely active site residues based on those found in subtilisin. (*B*) The organization of PC2, Kex2, and PACE proteins is shown schematically. Active site aspartate, histidine, asparagine, and serine residues are shown. ◆, Potential glycosylation sites; ■ putative signal peptides (Sig) and transmembrane domains (TMD); ▩ cysteine-rich (CRR) and serine/threonine-rich (S/TRR) regions. The corresponding PACE cDNA region is represented below, with the positions of several restriction sites shown. N, *Nco* I; G, *Bgl* II; Sp, *Sph* I; X, *Xho* I; B, *Bam* HI. aa, Amino acids; bp, base pairs.

tion with anti-PACE antiserum. The immunoprecipitates were then analyzed by SDS/PAGE. In extracts from pMT3-PACE transfected cells, immunoreactive species were detected that migrated primarily as a doublet of ≈90 kDa (Fig. 2*A*, CE, lanes 3 and 4). In control lysates from COS-1 cells, immunoreactive protein was not detected (Fig. 2*A*, CE, lanes 1 and 2). Treatment of these PACE immunoprecipitates with N-glycanase indicated the presence of asparagine-linked oligosaccharides (data not shown). However, these digestions did not fully reduce the complexity of the bands observed in Fig. 2*A*, suggesting that differential glycosylation is not the only source of the observed heterogeneity in the expressed PACE. Additional, more extensive pulse–chase experiments (R.J.W., unpublished data) demonstrated that the PACE translation product does not accumulate to high levels inside the cell when compared with another integral membrane glycoprotein (influenza hemagglutinin) when synthesized at similar levels. Secreted products were analyzed from conditioned medium following a 12-hr chase period in medium containing an excess of unlabeled amino acids. Immunoprecipitation of the conditioned medium from pMT3-PACE transfected cells detected an immunoreactive protein migrating at 75 kDa (Fig. 2*A*, CM, lanes 3 and 4). The relative quantity of the 75-kDa PACE protein observed in the conditioned medium was 5- to 10-fold less than that remaining inside the cells at the 12-hr chase period (data not shown). This secreted species may represent a truncated molecule that possibly results from PACE autoproteolysis.

PACE Expression Enhances the Processing of vWF. To test the function of PACE as a putative propeptide processing enzyme, the effect of PACE expression on the processing of vWF was examined by coexpression of the two molecules in COS-1 cells. vWF is a multimeric plasma protein synthesized in endothelial cells as a large precursor (prepro-vWF). In the endoplasmic reticulum, pro-vWF forms carboxyl-terminal-linked disulfide-bonded dimers that, upon transport to the Golgi and post-Golgi compartments, undergo a complex series of processing steps (38). These steps include processing of N-linked carbohydrate, O-linked glycosylation, sulfation, assembly of disulfide-linked multimers, and propeptide cleavage. Transfection of a vWF cDNA expression vector (pMT2-vWF) into COS-1 cells directs the synthesis of prepro-vWF (31). Although COS-1 cells do possess a protease capable of recognizing and cleaving the vWF propeptide, this process is inefficient and ≈50% of the secreted protein from a typical expression experiment is uncleaved pro-vWF (6).

If PACE can recognize and cleave the vWF propeptide, then coexpression of PACE with pro-vWF may result in greater conversion of pro-vWF to the mature form. To test this, COS-1 cells were transfected with either pMT3-PACE or pMT2-vWF or cotransfected with both plasmids. Transfected cells were labeled with ^{35}S-amino acids and samples were prepared for analysis. Immunoprecipitation of cell extracts from 30-min pulse-labeled cells with an anti-vWF antibody detected only single chain pro-vWF precursor in COS-1 cells transfected with pMT2-vWF alone (Fig. 2*B*, CE, lane 2). An intracellular mature form of vWF is not detected even after longer chase periods since the cleavage of the propeptide occurs immediately prior to, or upon secretion from, the cell (6). In the conditioned medium, both cleaved (mature) and uncleaved (pro-vWF) forms are readily detected in nearly equal amounts (Fig. 2*B*, CM, lane 2). In contrast, inside the COS-1 cells that were cotransfected with pMT2-vWF and pMT3-PACE (VWF+PACE), there was a significant amount of propeptide cleavage at the 30-min pulse time point detected by the appearance of the 100-kDa propeptide and the 225-kDa mature subunit (Fig. 2*B*, CE, lane 4). In the conditioned medium, after a 12-hr chase period, the secreted vWF was completely processed (Fig. 2*B*, CM, lane 4). Amino-terminal analysis of [^{35}S]methionine-labeled vWF secreted in the presence of PACE showed a peak of radioactivity at cycle 8 (data not shown) consistent with cleavage at the authentic site within the pro-vWF precursor (39).

The vWF propeptide is detected intracellularly only in the presence of PACE expression (Fig. 2 *B* and *C*). Although the anti-vWF antibody does not recognize the propeptide, the propeptide is coprecipitated due to disulfide bonding that occurs between the propeptide and the mature portion of the vWF molecule as an intermediate in the process of multimer assembly (6). The secreted vWF in the conditioned medium contains no disulfide bonds between the propeptide and mature subunit so the propeptide no longer coprecipitates with the anti-vWF antibody (Fig. 2*B*, CM, lanes 2 and 4). The identity of the vWF propeptide was confirmed by immunoprecipitation of the same cell extracts and conditioned media with a monoclonal antibody directed against the propeptide of vWF, also known as vWF antigen II (40) (Fig. 2*C*). From extracts of cells transfected with pMT2-vWF alone, the vWF antigen II antibody (anti-AgII) precipitated the unprocessed

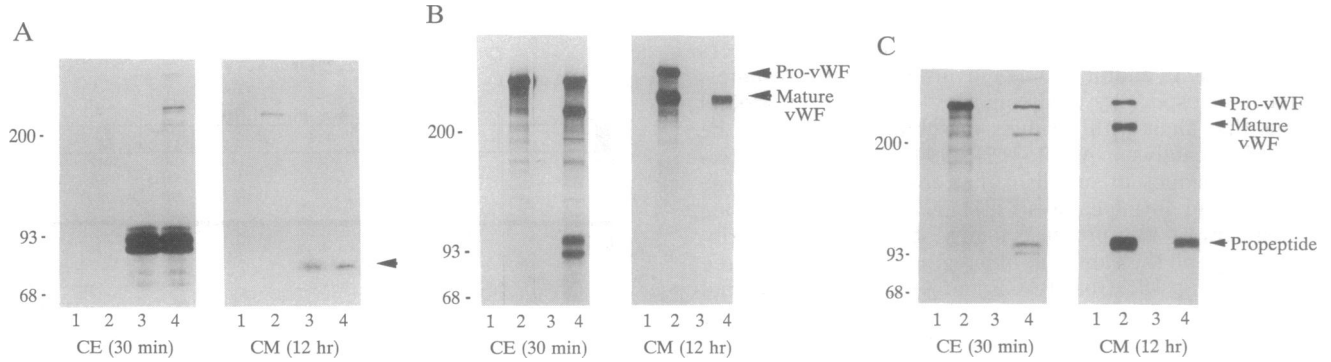

FIG. 2. Expression of PACE cDNA in COS-1 cells and its coexpression with pro-vWF. SDS/PAGE of radiolabeled protein products immunoprecipitated with excess antibody. Each lane represents an equivalent portion of the total labeled cell extract (CE) and conditioned medium (CM). COS-1 cells were transfected with the following: lanes 1, pMT2; lanes 2, pMT2-vWF; lanes 3, pMT3-PACE; lanes 4, vWF-pMT2 + PACE-pMT3. (*A*) Immunoprecipitation performed with anti-PACE antiserum. Cell extract after 30-min pulse and conditioned medium after 12-hr chase are shown. Positions of molecular mass markers (kDa) are indicated on the left. Arrow on the right shows the band of immunoreactive protein seen in the medium. (*B*) Samples immunoprecipitated with an anti-vWF polyclonal antibody that specifically recognizes the mature portion of vWF. The migration of the pro-vWF and the mature vWF subunit from the conditioned medium is indicated on the right. Arrow in the center indicates the intracellular cleaved propeptide that appears as a doublet due to glycosylation. (Note that lane 2 of CE has been replaced by a shorter exposure from the same gel.) (*C*) Equivalent portions of CE and CM immunoprecipitated with a monoclonal antibody specific for the propeptide of vWF (anti-vWAgII). Each secreted vWF species is indicated by arrows on the right.

pro-vWF due to the presence of the uncleaved propeptide within the molecule (Fig. 2*C*, CE, lane 2). Immunoprecipitation of vWF+PACE cotransfected cell extracts with the anti-AgII antibody detected the vWF propeptide as a doublet at 100 kDa (Fig. 2*C*, CE, lane 4). Since pulse-chase experiments (6) and digestion with N-glycanase (R.J.W., unpublished data) indicate that the lower band of the propeptide doublet is due to incomplete N-linked glycosylation, it appears that propeptide cleavage by PACE may begin in the endoplasmic reticulum of transfected COS-1 cells. The detection of activity in this compartment may be due to the high levels of expression obtained in COS-1 cells. In the conditioned medium of the pMT2-vWF transfected cells, the propeptide specific antibody precipitated the free propeptide and the multimers of vWF that contain a mixture of mature vWF and pro-vWF due to the incomplete processing in COS-1 cells (Fig. 2*C*, CM, lane 2). However, in the conditioned medium from vWF+PACE cotransfected cells, only free propeptide is detected with the anti-AgII antibody since all of the pro-vWF is processed to the mature form and the vWF multimers in the medium contain no propeptide (Fig. 2*C*, CM, lane 4). Formation of vWF multimers, confirmed by nonreducing agarose gel electrophoresis (6), was comparable for both pMT2-vWF transfected cells and those cotransfected with pMT3-PACE (R.J.W. and G. Morris, unpublished data).

To further test the recognition specificity of PACE, cotransfection experiments were performed with propeptide cleavage mutants that are not detectably cleaved upon expression in COS-1 cells (6). One mutant contains a nonconservative substitution, Lys-Arg/Ser (KRS) to Asp-Glu/Ser (DES), as the propeptide cleavage site. In contrast to wild-type pro-vWF (Fig. 3*A*, lanes 1 and 3) this mutant, designated vWF-DES, is secreted as an uncleaved pro-vWF species from COS-1 cells when transfected alone or when cotransfected with PACE (lanes 2 and 4). Thus, cleavage mediated by both the endogenous COS-1 processing enzyme and PACE is inhibited by mutation at the Lys-Arg paired basic residues. This result further supports the amino-terminal sequence analysis, suggesting that PACE cleavage occurs specifically at the natural propeptide cleavage site. A second cleavage mutant, designated vWF-KKS, contained a conservative substitution of Lys-Lys for Lys-Arg at the propeptide cleavage site. Like the mutant vWF-DES, this mutant is not detectably cleaved by COS-1 cells (Fig. 3*B*, lane 3). Coexpression of the vWF-KKS mutant with PACE resulted in significant propeptide cleavage, although a portion of the secreted vWF remained uncleaved (lane 6). This demonstrates that a conservative amino acid substitution (Lys-Arg to Lys-Lys) at the dibasic propeptide cleavage site is recognized by PACE. However, by comparison of the cleavage of the wild-type vWF (lane 5) to that of the KKS mutant (lane 6), it appears that the Lys-Lys pair may be a poorer substrate for PACE cleavage than the Lys-Arg pair present in wild-type vWF. Although further studies are required to define the specificities of PACE, there is a recognition preference for Lys-Arg and/or Arg-Arg, compared to Lys-Lys, for propeptide cleavage mediated by the yeast enzyme Kex2 (41) or by the endogenous processing proteases within a mouse pituitary and a rat insulinoma cell line (42, 43).

Since we observed variable levels of vWF expression in the cotransfection experiments (see Figs. 2*B* and 3*A*), an additional control experiment was performed to demonstrate that the enhanced vWF propeptide cleavage was specifically due to PACE activity and not secondary to the reduction in the rate and quantity of vWF secreted per cell as a result of competition for expression from the cotransfected plasmid. COS-1 cells were cotransfected with pMT2-vWF and an expression vector, pMT2-HA, that directs the synthesis of influenza hemagglutinin (HA), an integral membrane glycoprotein that is not secreted. In cell extracts of HA+vWF cotransfected cells, a high level of HA expression was confirmed by specific immunoprecipitation (data not shown). In the conditioned medium (Fig. 3*B*, lane 4), vWF processing was not altered while vWF synthesis and secretion were decreased by ≈50% (equivalent to that observed in PACE coexpression). Thus, under these control cotransfection conditions, a reduced rate of vWF expression does not enhance the efficiency of vWF propeptide cleavage. This indicates that the enhanced propeptide cleavage observed in the presence of PACE cotransfection is due to PACE expression.

DISCUSSION

Since the discovery of the proteolytic processing of proinsulin and proglucagon (5, 44, 45), the identity of the enzymes responsible for endoproteolytic processing in the mammalian secretory pathway has remained an enigma of cell biology. The purification of a proprotein cleavage enzyme has been hampered by the low level of activity in mammalian tissue, their membrane association, and by the susceptibility of

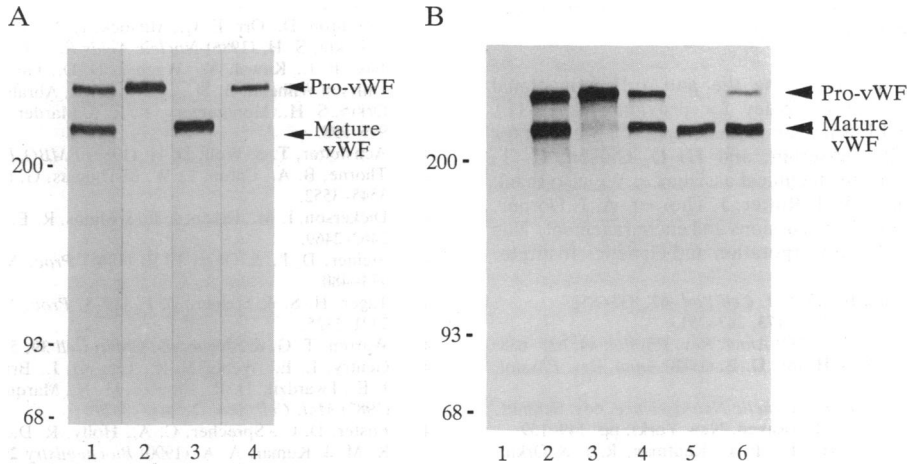

FIG. 3. Coexpression of PACE with propeptide cleavage site mutants of vWF and the effect of coexpression of vWF with a control expression vector construct. Each lane is loaded with the products secreted into the conditioned medium (12-hr chase) and immunoprecipitated with the anti-vWF antibody. (*A*) Lanes: 1, COS-1 transfected with pMT2-vWF alone; 2, cells transfected with pMT2-vWF-DES alone; 3, pMT2-vWF and pMT3-PACE cotransfection; 4, pMT2-vWF-DES + pMT3-PACE cotransfection. (*B*) Immunoprecipitated conditioned medium from the following: lane 1, COS-1 transfected with pMT2; lane 2, cells transfected with pMT2-vWF alone; lane 3, cells transfected with pMT2-vWF-KKS propeptide cleavage mutant; lane 4, pMT2-vWF + pMT2-HA cotransfection; lane 5, pMT2-vWF + pMT3-PACE cotransfection; lane 6, pMT2-vWF-KKS + pMT3-PACE cotransfection.

assay substrates to nonspecific cleavage *in vitro* by contaminating proteases such as those released from lysosomes. We have, therefore, approached this problem through *in vivo* reconstitution of a specific, processing pathway in mammalian cells by introducing a cDNA encoding a putative propeptide processing enzyme, PACE. We have demonstrated that expression of the PACE cDNA can mediate and enhance correct processing of the propeptide of vWF. Thus, PACE is a likely candidate for a prototypical mammalian propeptide processing enzyme.

Results from gene transfer experiments, in which precursor polypeptides were introduced into cells that have only a constitutive secretory pathway, have shown that the propeptide of the heterologous proprotein is usually cleaved correctly but inefficiently (for examples, see refs. 6 and 46–49). The inefficiency of cleavage may represent a saturation of the propeptide cleaving capacity of these cells. Alternatively, a portion of the expressed proprotein may bypass the compartment containing the endogenous protease, or its rate of transit through the compartment may be too rapid for complete cleavage to occur. Our results show that expression of PACE in COS-1 cells can improve the efficiency of processing of a proprotein expressed at a high level. The establishment of cell lines that express PACE should allow the production of more completely processed protein products.

Stable expression of PACE in mammalian cell lines will provide a model system to further study the biochemistry and substrate specificity of this prototypic processing enzyme. For example, it has been shown that PACE can cleave efficiently the murine type β nerve growth factor precursor (50). It will be important to determine the specificity of PACE for cleavage of other protein precursors including prohormones with multiple dibasic processing sites, such as proinsulin and proopiomelanocortin, and also for the cleavages required by viruses, such as human immunodeficiency virus and the hepatitis C virus. We have shown that PACE expressed in COS-1 cells can mediate propeptide cleavage at the paired basic amino acid recognition site in pro-vWF. Although pro-vWF contains 28 paired basic residues throughout its structure, PACE recognized and cleaved only at dibasic residues located at the natural propeptide cleavage site. The other paired basic residues may simply be inaccessible to PACE or, alternatively, other determinants, such as arginine at the −4 position, may play a role in cleavage recognition. The availability of PACE cDNAs and their expressed products will allow exploration of this and other fundamental issues of proprotein processing.

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