

Proteolytic maturation of protein C upon engineering the mouse mammary gland to express furin

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Communicated by George E. Seidel, Jr., Colorado State University, Fort Collins, CO, July 7, 1995

ABSTRACT Endoproteolytic processing of the human protein C (HPC) precursor to its mature form involves cleavage of the propeptide after amino acids Lys⁻²-Arg⁻¹ and removal of a Lys¹⁵⁶-Arg¹⁵⁷ dipeptide connecting the light and heavy chains. This processing was inefficient in the mammary gland of transgenic mice and pigs. We hypothesized that the protein processing capacity of specific animal organs may be improved by the coexpression of selected processing enzymes. We tested this by targeting expression of the human proprotein processing enzyme, named paired basic amino acid cleaving enzyme (PACE)/furin, or an enzymatically inactive mutant, PACEM, to the mouse mammary gland. In contrast to mice expressing HPC alone, or to HPC/PACEM bigenic mice, coexpression of PACE with HPC resulted in efficient conversion of the precursor to mature protein, with cleavage at the appropriate sites. These results suggest the involvement of PACE in the processing of HPC *in vivo* and represent an example of the engineering of animal organs into bioreactors with enhanced protein processing capacity.

Human protein C (HPC) is a complex plasma glycoprotein that functions as an anticoagulant by proteolytically inactivating coagulation factors V_a and VIII_a (Fig. 1A) (1, 3). Congenital HPC deficiency has been associated with thrombotic disease (4, 5) and HPC was effective in replacement and other therapies (6, 7). However, the plasma concentration of 4 μg/ml limits the availability of HPC from this source, while mammalian cell lines secrete only low levels of recombinant HPC (rHPC) (8, 9). This laboratory has expressed rHPC in the mammary glands of mice (10) and swine (11) and has found that high-level expression resulted in the secretion of pro-, single-chain, and mature HPC (12), indicating that the endogenous processing enzymes may be saturated.

The subtilisin-like serine protease furin, also known as paired basic amino acid cleaving enzyme (PACE), has been proposed as a candidate for processing the precursors of several proteins. PACE processes pro-nerve growth factor β (13), proinsulin (14), proalbumin (14, 15), pro-complement C3 (15), pro-von Willebrand factor (16), and pro-factor IX (17) in cultured cells. The sequence and structural homology of human factor IX (HFIX) and HPC suggested that PACE may be involved in the processing of HPC (18). The improved processing of rHPC in baby hamster kidney cells by a yeast subtilisin-like endoprotease, Kex2p (EC 3.4.21.61) (19), prompted us to test the hypothesis that the protein processing capacity of animal organs may be improved by the expression of selected enzyme(s). We therefore generated mice bigenic for HPC and PACE to investigate whether human PACE, which shares 50% sequence homology with Kex2p in the catalytic domain (20), may improve the processing of the HPC precursor in the mammary gland.

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MATERIALS AND METHODS

Generation of Transgenic Animals. The whey acidic protein (WAP)-HPC construct and six lines of HPC mice have been described (12). In this report, HPC line 6.4 was used as a control. The 4.1-kb *Sau3A-Kpn I* WAP promoter and 1.6-kb *BamHI-EcoRI* fragment of 3' WAP untranslated sequences were cloned into pUC19 to generate plasmid pHL215. A 2.47-kb *EcoRI-Sal I* fragment of the human PACE coding region and 74 bp of 3' untranslated sequence were excised from a PACE cDNA clone (16). Fragments of PACE or the Ser²⁶¹Ala PACE mutant (PACEM) (16) were inserted into *Sma I*-digested pHL215 to generate pHL252 and pHL255. The transgenes were released by *Not I/HindIII* digestion and the WAP-HPC and WAP-PACE fragments were coinjected at 2 μg/ml in a 2:1 molar ratio into CD-1 mouse embryos (Charles River Breeding Laboratories; ref. 21). Polymerase chain reaction (PCR) of tail DNA was performed to detect transgenes by using primers (5' → 3') CAGTCACTGCGCTGACACCGGTAC and AUGGACCGCTTCGTCACTCCTCGA defining a 260-bp sequence at the 5' end of PACE and primers defining HPC and mouse WAP, as described (12). F₀ mice were bred with wild-type CD-1 to generate F₁ mice hemizygous for HPC-PACE or HPC-PACEM. Lines were established by the noncontinuous breeding of selected F₁ transgenic mice to wild-type mice. Positive F₀ and F₁ mice were additionally screened by Southern blot analysis (data not shown).

RNA Isolation and Northern Blot Analysis. RNA was isolated from tissues of primiparous females of the F₂ or F₃ generations and from control mice taken on day 10 of lactation, by using RNazol (Molecular Research Center, Cincinnati; ref. 22). Total RNA (15 μg) was fractionated on formaldehyde/1.2% agarose gels and transferred to GeneScreen-Plus membranes (DuPont/NEN). The 0.5-kb *BamHI-Nhe I* fragment from the HPC 3' region, the 0.85-kb *BamHI-Sal I* PACE cDNA fragment, the 0.75-kb *BamHI-Sph I* 18S rRNA fragment from plasmid pN29111 (ATCC 63178), and the 2.5-kb human PACE cDNA used to detect endogenous mouse PACE were radiolabeled by random-primer labeling. Blots were hybridized essentially as described (2). RNA was analyzed from a minimum of two animals in each experiment.

Detection of rHPC in Mouse Milk. Continuously bred primiparous and multiparous mice were milked mainly between days 5 and 15 of lactation. Females were separated from pups for 1–2 h and administered 0.6 international unit of oxytocin (Sigma) *i.p.*, and milk samples of 0.05–0.70 ml were collected. Milk was diluted with 2 vol of phosphate-buffered saline (pH 7.4) containing 50 mM EDTA and centrifuged twice at 11,600 × *g* for 15 min at 4°C. EDTA solubilized the casein micelles, leading to improved recovery of micelle-associated rHPC. rHPC was quantified by a sandwich ELISA using a sheep

Abbreviations: HPC, human protein C; r, recombinant; PACE, paired basic amino acid cleaving enzyme; PACEM, Ser²⁶¹Ala PACE mutant; WAP, whey acidic protein; HFIX, human factor IX.

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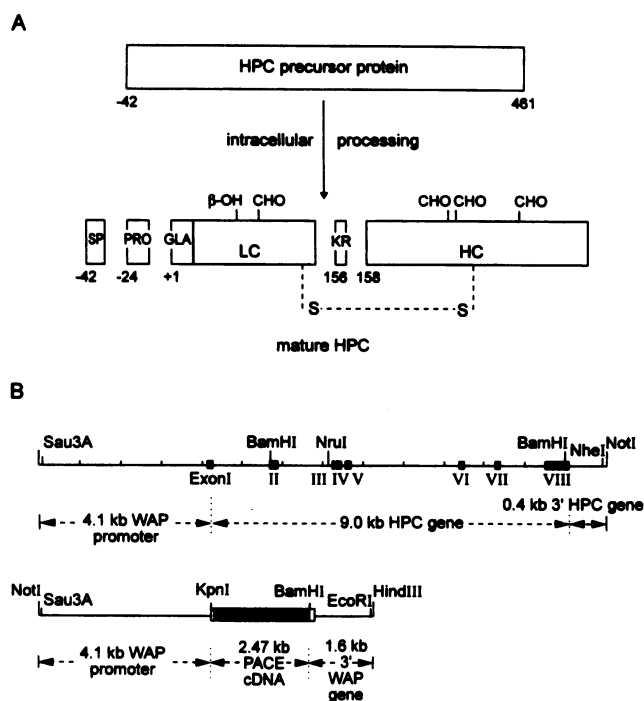


FIG. 1. (A) Posttranslational modifications of HPC. An 18-residue signal peptide (SP) is cleaved from the 461-amino acid HPC polypeptide; then core glycosylation (-CHO) at four N-linked sites and vitamin K-dependent γ -carboxylation of nine glutamic acid residues (GLA) near the N terminus occur in the endoplasmic reticulum (1). Further processing of oligosaccharides, endoproteolytic cleavage of a 24-amino acid propeptide (PRO) and the Lys¹⁵⁶-Arg¹⁵⁷ (KR) dipeptide, and β -hydroxylation of Asp⁷¹ (β -OH) occur in the Golgi, resulting in secretion of the mature protein that circulates in plasma as a light chain disulfide-linked to a heavy chain. (B) The WAP-HPC and WAP-PACE transgenes. HPC exons and PACE coding regions are depicted by solid boxes, untranslated WAP exons are depicted by open boxes, 5' and 3' flanking and intron sequences are depicted by lines. The WAP promoter and PACE cDNA junction is **GGTACC***aCACC***ATG**, and the PACE cDNA and 3' WAP gene junction is *TTTATCTG***ggG**-**GATCCC**. WAP sequences are shown in boldface type, linker is in lowercase type, and PACE is in italic type. Both constructs contain 5' flanking WAP sequences from nt -4098 to +25 (2).

anti-HPC polyclonal antibody (Affinity Biologicals, Yarker, ON, Canada) to capture rHPC from milk and a rabbit anti-HPC antibody (Sigma) for detection, as described (12). The change in A_{650} was read for 10 min in a Thermomax plate reader (Molecular Devices). Plasma-derived HPC at 2–128 ng/ml in control milk was used as a standard. Standard curves were analyzed by using the four-parameter curve fit, $r = 0.998$ to 1.0. Milk proteins were analyzed by SDS/PAGE in 8–16% (Novex, San Diego) or 10% gels and silver-stained or analyzed on a Western blot with the anti-HPC 8861 monoclonal antibody against a heavy chain epitope (12) or the sheep antibody and developed by ECL (Amersham).

Purification of rHPC. Pooled whole milk, 1.5–2 ml, collected between days 7 and 15 of lactation from several F₂ and F₃ animals of line C5.2 was thawed, diluted with 20 ml of 50 mM Tris/0.15 M NaCl/2 mM EDTA/2 mM benzamidine, pH 7.2, and centrifuged at 30,000 $\times g$ for 15 min at 4°C. The aqueous phase was filtered through a 0.45- μ m (pore size) Millex-HA (Millipore) membrane. The filtrate was loaded at 17 cm/h onto a 1.5 cm \times 2.7 cm column of 8861 monoclonal antibody immobilized on Sepharose CL-4B resin (Pharmacia) and equilibrated with 50 mM Tris/0.15 M NaCl/2 mM EDTA/2 mM benzamidine, pH 7.2. The column was washed with 5 mM ammonium acetate (pH 5.0); rHPC was eluted with

0.5 M ammonium acetate (pH 3.0) and immediately neutralized with 3 M Tris.

RESULTS

Generation of Bigenic Mice. Mice containing the HPC gene and PACE or PACEM cDNAs were produced by coinjection of two DNA fragments (Fig. 1B). The mouse WAP promoter was chosen because milligram per milliliter amounts of rHPC were secreted in milk by using the WAP-HPC gene construct (12). The intronless WAP-PACE cDNA construct was chosen to express low amounts of PACE, as inefficient expression of intronless transgenes has been documented (23).

Transgenic mice were identified by the detection of HPC- and PACE-specific PCR products of 502 bp and 260 bp, respectively (Fig. 2). Four of 13 mice carried both transgenes and all founders transmitted them. C1.2 δ showed a transmission frequency of 46% (11/24), while the C2.2, C4.1, and C5.2 δ were mosaic with frequencies of 6% (1/16), 7% (2/30), and 19% (3/16), respectively. In the HPC-PACEM experiment, 3 of 7 mice were transgenic, with 2 founders carrying both transgenes. Founder M2.3 δ transmitted the transgenes to 39% (5/13) of its progeny, but M2.1 δ was a somatic mosaic and did not transmit (0/16). M1.1 δ carried only PACE and did not transmit the transgene. Lines were established from HPC-PACE F₁ mice C1.2.20 δ , C2.2.8 δ , C4.1.6 δ , and C5.2.4 δ and from HPC-PACEM mice M2.3.10 δ and M2.3.4 δ . Breeding of F₁ mice resulted in Mendelian transmission of transgenes, suggesting integration at a single site.

Mammary Gland Expression of HPC and PACE. Northern blot analyses revealed both HPC and PACE transcripts in the lactating mammary glands of bigenic mice (Fig. 3). The major species of HPC mRNA was 1725–1775 nt long, with precursor RNAs of 2250, 2400, and 4800 nt and a minor species 200 nt shorter, which may represent an alternative 3' polyadenylation site (18). Human PACE mRNA of the expected size of 2700 nt was detected, while endogenous PACE transcripts were not observed upon prolonged exposure of autoradiograms. However, upon low-stringency hybridization endogenous mouse PACE transcripts were detected at levels at least one order of magnitude lower (data not shown). The amount of rHPC secreted into the milk of F₀ females as determined by ELISA (1–75 μ g/ml) was lower than in later generations (0.31–1.63 mg/ml; Table 1 and data not shown). This may be due to mosaicism of the founders. rHPC in the milk of HPC-PACEM mice ranged from 0.17 to 0.33 mg/ml.

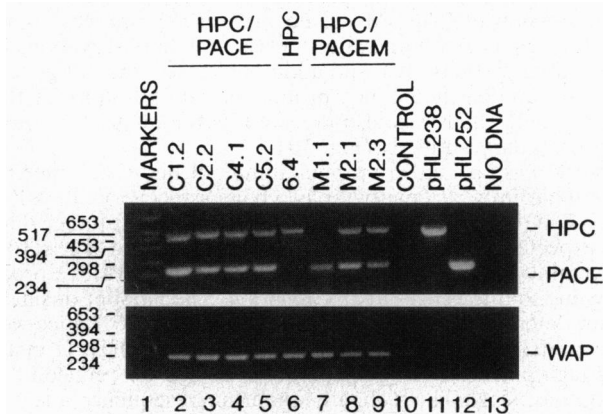


FIG. 2. Detection of HPC and PACE transgenes in founder mice by PCR analysis. Positions of the PCR products are on the right. Sizes of DNA markers are on the left (in nucleotides). Lanes: 1, DNA markers; 2–5, DNA from HPC-PACE; 6, HPC; 7–9, HPC-PACEM; 10, control mice; 11, plasmid pHL238; 12, plasmid pHL252; 13, negative control without DNA.

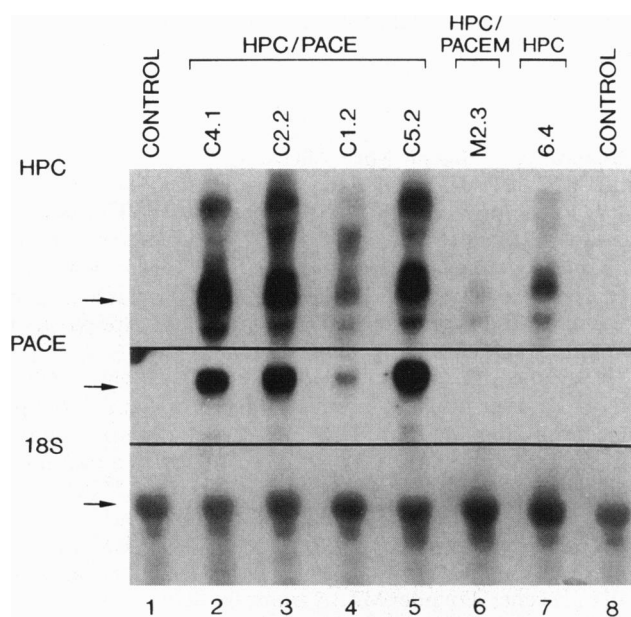


FIG. 3. Northern blot analysis of RNA. Total RNA from day 10 of lactation was electrophoresed at 15 μ g per lane. Lanes: 1 and 8, control mice; 2, C4.1.6.15; 3, C2.2.8.11.7; 4, C1.2.20.8.12; 5, C5.2.4.24.9; 6, M2.3.4.6; 7, 6.4.9.10. (Top) Detection of rHPC RNA. The arrow indicates mature HPC mRNA, 1725–1775 nt. Higher molecular weight bands of 2250, 2400 and 4800 nt represent precursor RNAs. (Middle) Detection of PACE RNA. The arrow indicates PACE mRNA, 2700 nt. (Bottom) Detection of 18S rRNA, as control for sample to sample variation.

Mice from bigenic lines C2.2, C5.2, and M2.3 were able to raise litters of 10–15 pups, indicating that the coexpression of PACE or PACEM and HPC did not impair nursing in these lines. Animals from lines C1.2 and C4.1 delivered litters of 10–15 pups but were able to sustain at most 6 of them. Transferring these litters to foster mothers within 12–24 h of birth resulted in survival, normal weight gain, and growth.

PACE Improves Processing of rHPC Precursor. Milk proteins from HPC–PACE and HPC–PACEM mice were separated by SDS/PAGE and compared to those from HPC mice. Western blot analysis using the 8861 antibody allowed estimation of the proportion of rHPC single and heavy chain forms (Figs. 4 and 5). rHPC from HPC mice consisted of 40–60% rHPC single chain form, as determined by scanning densitometry. In sharp contrast, expression of PACE reduced the amount of single chain form to <5% on an average, thus efficiently converting the precursor to the mature protein. Coexpression of PACEM did not improve the processing of rHPC single chain in one line, consistent with observations in cell culture systems (16), and additional lines were not generated. An unidentified mouse protein migrating faster than the rHPC single chain was also detected by the goat anti-mouse IgG secondary antibody (Fig. 4B).

rHPC was purified from pooled milk of line C.5.2 mice by immunoaffinity chromatography, giving a recovery of >80%. SDS/PAGE analysis of rHPC did not reveal bands other than the expected single, heavy, and light chains (Fig. 5). The rHPC polypeptides migrated faster than plasma-derived HPC, probably due to differences in glycosylation. The amount of single chain detected in rHPC purified from HPC–PACE mice was substantially lower than in rHPC purified from HPC mice, although purification by the earlier method (12) reduced the amount of single chain slightly. N-terminal sequence analysis of rHPC secreted by HPC–PACE mice confirmed appropriate cleavage of the rHPC propeptide and single chain (Table 2).

DISCUSSION

The recombinant expression of fully processed vitamin K-dependent plasma proteins is a challenge. The amount of rHPC

Table 1. Detection of rHPC in milk

Generation/mouse number	Lactation number and day	rHPC, μ g/ml
HPC–PACE		
F ₁ /C1.2.23	2L5	755 \pm 89
F ₁ /C1.2.20	3L5	896 \pm 44
F ₃ /C1.2.20.8.16	2L7	451 \pm 74
F ₄ /C1.2.20.7.21.6	1L13	660 \pm 59
F ₀ /C2.2	1L7	19 \pm 5
F ₁ /C2.2.8	3L10	308 \pm 27
F ₂ /C2.2.8.7	2L13	727 \pm 93
F ₃ /C2.2.8.7.18	3L13	1626 \pm 267
F ₀ /C4.1	3L15	75 \pm 8
F ₀ /C4.1	3L20	4 \pm 0.9
F ₂ /C4.1.6.48	1L7	908 \pm 140
F ₂ /C4.1.6.53	4L7	1352 \pm 123
F ₀ /C5.2	1L11	4 \pm 2
F ₀ /C5.2	3L15	1 \pm 0.4
F ₁ /C5.2.4	1L5	1154 \pm 113
F ₂ /C5.2.4.25	3L13	765 \pm 78
HPC–PACEM		
F ₂ /M2.3.10.10	1L7	260 \pm 51
F ₂ /M2.3.10.10	1L14	169 \pm 10
F ₂ /M2.3.4.14	1L7	331 \pm 59
HPC		
F ₀ /6.4	1L5	512 \pm 68
F ₀ /6.4	2L8	706 \pm 96
F ₁ /6.4.12	1L11	190 \pm 17
F ₂ /6.4.4.9	1L7	532 \pm 43

Lactation number and day are expressed, for example, as 1L7 for first lactation, day 7. Concentration of rHPC in defatted milk collected mainly between days 5 and 15 of lactation was assayed by ELISA. Values presented are an average of four to six dilutions of each sample, performed in duplicate assay. The lower limit of detection was 2 ng/ml.

precursor processed to the mature two-chain form in the human liver is 85–95%, \approx 50% in liver-derived HepG2, 30% in transfected baby hamster kidney, and 80% in human kidney 293 cell lines (1, 8, 9). Chinese hamster ovary (CHO) and C127 mouse fibroblast cell lines were not only unable to process the single chain but also secreted rHPC with the propeptide attached (8, 9), suggesting that the endogenous processing protease(s) may be saturated. Alternatively, as endoproteolytic cleavage of HPC occurs after the sequences Arg-Ile⁻⁴-Arg-Lys-Arg⁻¹- \downarrow and Arg-Ser-His¹⁵⁴-Leu-Lys-Arg¹⁵⁷- \downarrow , rHPC may be a poor substrate for the mammalian endoprotease(s), which prefers basic amino acids at the P4 position (19). Poor substrate specificity does not explain the secretion of both pro- and mature rHFIX by CHO cells (24, 25) or the retention of propeptide in C127 cells (26), as the Arg-Pro-Lys-Arg- \downarrow amino acid sequence in the HFIX propeptide corresponds to the Arg-Xaa-Lys/Arg-Arg- \downarrow PACE/furin cleavage motif. Low levels of endogenous PACE in CHO cells may partly explain the inefficient processing of rHFIX precursor (27). Moreover, expression of HFIX in heterologous tissues such as lymphoid cells led to the secretion of a protein with an aberrant electrophoretic pattern and partial activity (28), but when expressed in mouse liver the circulating rHFIX was correctly processed (29), emphasizing the limitations of heterologous cells in performing posttranslational modifications of complex foreign proteins.

This laboratory has described inefficient proteolytic processing of rHPC in the mammary gland of 12 lines of mice and two lines of pigs transgenic for the HPC cDNA or gene (10–12). We now report that the coexpression of PACE led to improved processing of the propeptide and single chain of rHPC, in contrast to the 20–30% of propeptide- or 40–60% single-chain-containing rHPC secreted by HPC mice (12). Processing occurred at the authentic sites, implying that PACE does not cleave after the other six basic amino acid pairs

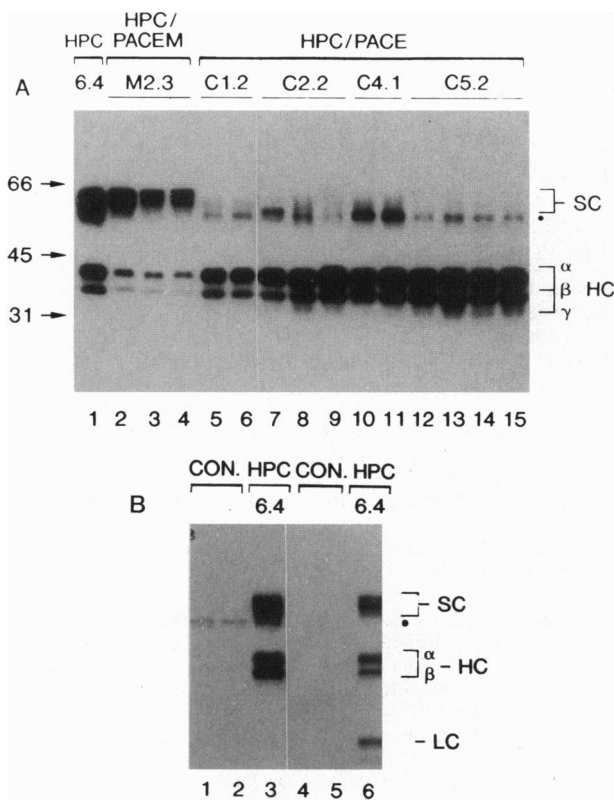


FIG. 4. Detection of rHPC in milk. Milk proteins (20 μ g) from days 7 and 15 of lactation were reduced, separated by SDS/PAGE in 10% gels, and analyzed on a Western blot with the 8861 antibody. (A) Lanes: 1, milk from mouse 6.4.4.9, first lactation, day 7 (1L7); 2 and 3, M2.3.10.18, 1L14 and 1L7; 4, M2.3.10.3, 1L7; 5, C1.2.20.7.21, 2L12; 6, C1.2.20.7.7, 1L13; 7, C2.2.8.7, 2L13; 8 and 9, C2.2.8.11.2, 1L7 and 1L13; 10 and 11, C4.1.6.37, 3L7 and 3L15; 12 and 13, C5.2.4.25.5, 3L7 and 3L13; 14 and 15, C5.2.4.25.5, 4L7 and 4L13, respectively. (B) Milk proteins from control and HPC mice were resolved on 8–16% SDS/PAGE gels and analyzed on a Western blot with the 8861 monoclonal (lanes 1–3) or sheep polyclonal (lanes 4–6) antibody. Lanes: 1, 2, 4, and 5, milk from two control mice; 3 and 6, 6.4.4.10, 3L7. The dot indicates a protein detected by the goat anti-mouse secondary antibody. SC, single chain; HC, heavy chain; LC, light chain; α , β , γ , HC glycoforms.

present in rHPC. However, mutation of the Lys¹⁵⁶-Arg¹⁵⁷ dipeptide and C-terminal sequence analysis will be necessary to determine whether a Kex2p-sensitive site after Lys¹⁵⁰-Lys¹⁵¹-Arg¹⁵² near the C-terminal end of the light chain is also cleaved (19). Additionally, coexpression of HPC with enzymatically inactive PACEM did not change the processing of the rHPC precursor. Thus these results suggest that the processing observed in HPC-PACE mice was due to active PACE. The premise that a PACE-like enzyme is responsible for the endogenous processing activity in HPC mice is supported by the extensive sequence homology of mouse and human PACE, 94% overall and 99% identity in the catalytic domain (27, 30). A calcium-dependent Golgi protease has been implicated in the processing of the precursors of prothrombin (31), factor X (32), and protein C (19). Our observations that PACE can cleave the rHPC precursor *in vivo* and a recent report that PACE processes the rHFIX precursor in CHO cells (17) imply that this enzyme is responsible for processing the propeptide and single chain of vitamin K-dependent plasma proteins.

PACE expression at levels an order of magnitude higher than endogenous mouse gene did not result in deleterious effects in two lines of HPC-PACE mice. The expression of PACE or the combination of rHPC and PACE seemed to affect only nursing in two other lines, while expression of rHPC

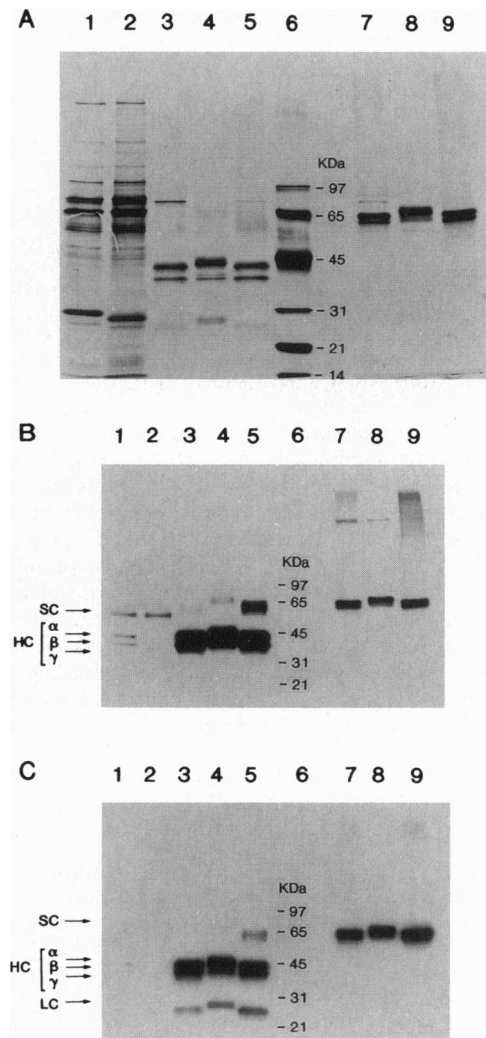


FIG. 5. Purification of rHPC. rHPC was isolated from milk by immunoaffinity chromatography. (A) Silver staining of proteins separated by SDS/PAGE in 8–16% gels and Western blot detection of rHPC using the 8861 monoclonal (B) or sheep polyclonal (C) antibody. Lanes: 1, starting material; 2, flow-through; 3 and 7, rHPC from HPC-PACE line C5.2; 4 and 8, plasma HPC; 5 and 9, rHPC from HPC mice (11); 6, protein standards. Samples 1–6 were reduced, and samples 7–9 were not. SC, α , β , γ -HC, and LC are as in Fig. 4. Note that the mouse protein was not captured on the affinity column (B, lane 2) or detected by the sheep polyclonal antibody (C).

alone did not (2). We do not have a good explanation for the underlying mechanism(s) yet. It is known that high-level expression of PACE/furin is toxic to cells (33). Expression of PACE may have had a similar impact, by increasing the processing of endogenous proteins. For example, precursors of the transforming growth factor β superfamily involved in the regulation of mammary gland development possess the consensus sequences for PACE-like enzymes (34). Certain other transgenes also affect the development and function of the mammary gland (35). This has been attributed to different transgene integration sites that alter the developmental regulation and expression level of transgenes, compared to milk protein genes. We have found that the 4.1-kb WAP promoter may not contain all the regulatory sequences for developmental regulation similar to the endogenous WAP gene and for position-independent expression (2). Changes in developmental regulation of either PACE or HPC, or of both, may affect lactation in some lines, especially as the effects do not correlate with the level of PACE expression.

Table 2. N-terminal sequence of rHPC

HPC source	Polypeptide	Sequence	% composition
Human plasma	Light chain	+ ¹ ANSFL $\gamma\gamma$ LRHSSL γ R γ C	100
	Heavy chain	+ ¹⁵⁸ DTEDQEDQVDPRLIDGK	100
rHPC mice	Propeptide	- ²⁴ TPAPLDSVFSSS	20–30
	Light chain	+ ¹ ANSFLEELRHSSLEREC	70–80
	Heavy chain	+ ¹⁵⁸ DTEDQEDQVDPRLIDGK	100
HPC-PACE mice	Light chain	+ ¹ ANSFLEELRHSSLEREC	100
	Heavy chain	+ ¹⁵⁸ DTEDQEDQVDPRLIDGK	100

Purified rHPC from C5.2 mice was subjected to automated Edman degradation and the sequence was compared to that of rHPC from HPC mice. Numbering indicates the first amino acid of the propeptide (-24), light chain (+1), and heavy chain (+158) of HPC. γ represents nondetected residues corresponding to γ -carboxyglutamic acid residues present in HPC. The identification of Glu residues in good yield at positions 6, 7, 14, 16, 19, 20, 25, 26, and 29 of the light chain suggests inefficient γ -carboxylation, consistent with earlier observations (12).

Transgenic animals are being explored as systems for the production of recombinant human proteins. However, expression of proteins in heterologous tissues at levels higher than in the tissue of origin can saturate processing enzyme activities (12, 28). Targeting PACE expression to the mammary gland is an example of the engineering of the posttranslational capacity of mammary epithelial cells for the secretion of well processed heterologous proteins. Coexpression of proteins with desired processing enzymes should have practical implications for the design of a "second generation" of transgenic animals and allow remodeling of organs like the mammary gland of livestock into more efficient bioreactors.

Note Added in Proof. The transforming growth factor β precursor has recently been shown to be processed by PACE, when coexpressed in LoVo cells (36).

We thank Dr. D.-W. Zhang for the generation of transgenic mice, M. H. Wroble for technical assistance, and M. Migliorini for sequence analysis. We are grateful to Dr. D. Scandella for the λ clones of HPC and Drs. C. Orthner and A.-M. Ralston for purified HPC and 8861 antibody.

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