Expression and characterization of cathepsin P

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The mouse genome contains a family of clan C1A proteases that appear to be restricted to rodents within Eutherian (placental) mammals. mRNA analysis has shown that these genes are expressed exclusively in placenta. Sequence analysis predicts that the expressed proteins will be functional and consequently it was proposed that this family of proteases may have evolved to perform subspecialized functions of the closely related protease, cathepsin L, that is expressed in placental tissues of all mammalian species. In the present study, it was shown that cathepsin P can be expressed in *Pichia pastoris* as an inactive zymogen that can be activated with proteinase K, chymotrypsin or pancreatic elastase at neutral pH. Unlike other mammalian cathepsins, cathepsin P could also be autoactivated at neutral pH, but not at acidic pH. The activated enzyme was capable of hydrolysing peptidyl substrates and the protein substrates azocasein and transferrin, with optimal activity at pH 6.5–7.5. Little activity could be detected at pH 5.0 and below. Salts such as Na_2SO_4 and hyaluronate stimulated the activity of the protease against peptidyl substrates. The properties of cathepsin P appear to be quite distinct from those of cathepsin L, indicating that the duplication that gave rise to cathepsin P has probably not yielded an enzyme that provides a subfunction of cathepsin L in rodents. It seems probable that cathepsin P has evolved to perform a function that is performed by an evolutionarily unrelated protease in other mammalian species.

Key words: activation, cathepsin, expression, inhibition, placenta, protease.

INTRODUCTION

The placenta is an organ unique to the Eutherian (placental) mammals and has developed to enable the growing foetus to obtain nutrients from the maternal blood supply during gestation. In evolutionary terms, the appearance of the placenta is very recent, occurring nearly 135 million years ago. Most of the specific placental functions are probably achieved by co-opting functions of pre-existing proteins that have evolved over many more years. Indeed, the rapid speciation of the early mammals has precluded the evolution of placenta-specific genes in all mammals. Unique functions of the placenta include implantation, vascularization and immune modulation. Each of these processes appears to involve proteases, indicating that the placenta has co-opted functions of proteases that originally evolved to perform proteolytic functions in other organs [1–9]. Gene duplications have occurred in several branches of Eutheria, and two of these duplications involve protease genes. In Artiodactyls (cloven-hooved mammals), a large family of aspartic proteases is specifically expressed in placenta [10,11], whereas rodents express a large family of placentally expressed cysteine proteases [12–14]. This family of cysteine proteases is closely related to cathepsin L, an enzyme expressed in all mammalian species. All of the mouse enzymes are found on chromosome 13 and have been designated cathepsin M, P, Q, R, 1, 2, 3 and 6 [13,14]. Virtual translation of the mRNA sequences of the expressed cysteine proteases indicates that all of the critical structural components of the proteins are conserved enabling the new genes to code for functional proteases. Cathepsin P was originally identified in rat placenta [15] and is also expressed in other rodent species [12]. It seems probable that maintenance of the structure of cathepsin P is driven by some evolutionary advantage imparted by the function of this protease. It was proposed that cathepsin P may have evolved a placenta-specific subfunction of the closely related protease, cathepsin L, an enzyme found in all mammalian species [13]. The purpose of this study was to express and characterize the catalytic properties of cathepsin P; first to determine whether it is a functional protease and second to determine whether it could have evolved to perform a restricted function of cathepsin L in placenta.

EXPERIMENTAL

Materials

Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2, Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH_2 [where Dnp- stands for 2,4-dintrophenyl-, Dpa- for *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl- and Mca- for (7-methoxycoumarin-4-yl) acetyl-], and Dnp-Pro-cyclohexyl-Ala-Gly-Cys(Me)-His-Ala-Lys(Nma)NH_2 (where Nma stands for *N*-Me-2-aminobenzoyl) were from Biomol (Plymouth Meeting, PA, U.S.A.). Mca-Pro-Leu-Gly-Leu-Dnp(Dpa)-Ala-Arg-NH_2, Fmoc-Tyr-Ala-OH, Z-Gly-Gly-OH, Z-Leu-Tyr-OH (where Fmoc stands for fluoren-9-ylmethoxycarbonyl) and -NHMec (aminomethyl coumarylamide) substrates were from Bachem (King of Prussia, PA, U.S.A.). Primers for PCR and sequencing were from Integrated DNA Technologies (Coralville, IA, U.S.A.). Restriction enzymes were purchased from Promega (Madison, WI, U.S.A.). Proteinase K, chymotrypsin, pancreatic elastase, chondroitin sulphates A, B, C and hyaluronate were from Sigma (St. Louis, MO, U.S.A.).

Expression of cathepsin P in *Pichia pastoris*

Cathepsin P was expressed using the constitutive promoter of glyceraldehyde-3-phosphate dehydrogenase in *P. pastoris* (Invitrogen, Carlsbad, CA, U.S.A.). Two primers, 5′-CCACGTGGCCCAAGCACATGATCCCAAATTGGATGC-3′ and

Abbreviations used: Dnp, 2,4-dintrophenyl; Dpa, *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; DTT, dithiothreitol; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; LC-LC, 6-hexanamido hexanoate; Mca, (7-methoxycoumarin-4-yl)acetyl; NHMec, aminomethyl coumarylamide; Nma, *N*-Me-2-aminobenzoyl; PNGase F, peptide N-glycosidase F.

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5′-CATCTAGATCAAAAGATGTTGGGATAGCTGGCAAGTGAAGC-3′, containing *Pml*I and *Xba*I cleavage sites (underlined), were used to amplify mouse pro-cathepsin P by reverse transcriptase PCR of 17-day mouse placental RNA. The resultant cDNA lacked sequence coding for the 17 N-terminal amino acids that presumably form the signal peptide of cathepsin P. The remainder of the PCR product was full-length up to and including the natural stop codon for cathepsin P. The PCR product was then digested with *Xba*I and *Pml*I. Vector pGAPZαC was digested with *Pml*I and *Xba*I and ligated to the pro-cathepsin P product with T4 ligase (Promega) to generate a construct that was in frame with the yeast α-factor pheromone signal sequence. After transformation into *Escherichia coli* and selection with Zeocin, colonies with inserts were sequenced. A plasmid with the correct in-frame sequence was selected for further study. The recombinant plasmid was linearized with *Avr*II and electroporated into *P. pastoris*, strain KM71 (Invitrogen). High-productivity clones were selected with increasing concentrations of Zeocin up to 1000 μg/ml, and production of secreted protein was determined by SDS/PAGE of culture supernatant from test cultures. One clone was selected and protein produced in 250 ml of cultures of YPD [1 % (w/v) yeast extract, 2 % (w/v) peptone and 2 % (w/v) glucose] media in shaker flasks.

Deglycosylation of cathepsin P

Culture supernatant from transfected cells (procathepsin P) or purified mature cathepsin P was denatured by boiling in SDS (0.1 %) and 50 mM 2-mercaptoethanol, cooled, SDS-complexed with Triton X-100 and then the glycosylated proteins were digested with PNGase F (peptide N-glycosidase F; Sigma) for 3 h at 37 °C. Processed proteins were identified by SDS/PAGE followed by either direct staining of proteins or Western blotting with anti-cathepsin P antibody.

Activation and purification of cathepsin P

Conditioned YPD media were harvested and adjusted to pH 7.5 with 1 M Tris base. Culture supernatant was then concentrated 5-fold by ultrafiltration, and dialysed against 20 vol. of 20 mM Tris/HCl buffer (pH 7.5). In the initial experiments, cathepsin P was activated by the addition of proteinase K, chymotrypsin or pancreatic elastase (see the Results section). For bulk preparation, the dialysed crude sample of procathepsin P was incubated at 4 °C for 6 days and autocatalytic conversion to mature form determined by SDS/PAGE. Mature cathepsin P was separated from contaminating proteins by ion-exchange chromatography on a column of Protein Pak Q (2 cm × 10 cm) using a Waters protein purification system. After application of sample to the column, bound protein was eluted with a linear gradient of NaCl in 20 mM Tris/HCl buffer (pH 7.5; 0–1 M, 125 ml). Protein concentrations were determined by Coomassie Blue staining using a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, U.S.A.).

Western blotting of cathepsin P

A polyclonal antibody was raised to the peptide YEGKDGPCRYRSENASANIT conjugated to keyhole-limpet haemocyanin in a New Zealand White rabbit (Covance, Denver, PA, U.S.A.). The peptide was designed to be unique to cathepsin P and to be exposed on the surface of mature cathepsin P. The close similarity of cathepsin P to the other placentally expressed cathepsins and cathepsin L greatly restricted the choice of peptides suitable for immunization. *E. coli*-produced recombinant cathepsins M, P, Q and L were used to determine the specificity of the antibody. The primary antibody was diluted 1:2000 or 1:4000 in non-fat milk and incubated with the blot for 16 h at 4 °C. Bound antibody was detected using a 1:8000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG and an ECL® detection system (Amersham Biosciences, Little Chalfont, Bucks, U.K.). For detection of biotinylated proteins, mouse anti-biotin was used as the primary antibody and horseradish peroxidase-conjugated goat anti-mouse IgG was used as the secondary antibody. Antibodies were from Jackson ImmunoResearch Labs (West Grove, PA, U.S.A.) unless otherwise stated.

Peptidolytic assay of cathepsin P

Cathepsin P was preincubated in 100 mM sodium phosphate buffer (pH 6.5), containing 1 mM EDTA and 1 mM DTT (dithiothreitol) at 37 °C for 30 min. Assays were initiated by the addition of the quench fluorescent substrate, Mca-Pro-Leu-Gly-Leu-Dnp(Dpa)-Ala-Arg-NH_2, and fluorescence-monitored continuously in a PerkinElmer LS50B spectrofluorimeter using an excitation wavelength of 328 nm and an emission wavelength of 393 nm [16]. In some experiments, salts were added during preincubation, and inhibitors were added after a steady initial rate of activity was determined. k_{cat} and K_m values were determined by non-linear regression analysis of the data fitted to the Michaelis–Menten equation, and when $[S] < K_m$, k_{cat}/K_m values were determined by analysis of substrate hydrolysis under first-order conditions and calculated as k_{obs}/E_t [17]. Cleavage products were analysed by HPLC.

Proteolytic activity of cathepsin P

Azocasein (Sigma) was used to assay the proteolytic activity of cathepsin P. The enzyme was incubated with 2.5 mg/ml azocasein in 20 mM sodium phosphate buffer (pH 6.5), containing 1 mM EDTA and 1 mM DTT at 37 °C. Activity was stopped by the addition of trichloroacetic acid to give a final concentration of 0.33 %, and incubation on ice for 30 min. Precipitated protein was removed by centrifugation, and the solubilized protein determined by measuring the absorbance of the supernatant at 440 nm. Holotransferrin (1 mg/ml; Sigma) was incubated with cathepsin P (4 μM) in 100 mM sodium phosphate buffer (pH 6.5), containing 1 mM EDTA and 1 mM DTT at 37 °C. At timed intervals, samples (10 μl) were taken and analysed by SDS/PAGE and Coomassie Blue staining.

Active-site titration of cathepsin P

Enzyme was titrated with E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane]. Auto-activated cathepsin P was incubated with an equal volume of E-64 (0–100 μM) in 100 mM sodium phosphate buffer (pH 6.5), containing 1 mM EDTA, 1.5 M Na_2SO_4 and 1 mM DTT for 3 h at 25 °C. An aliquot of each sample was then taken and residual activity against Mca-Pro-Leu-Gly-Leu-Dnp(Dpa)-Ala-Arg-NH_2 determined as described above. For inhibition constants, k_{+2} and K_i were calculated by non-linear regression analysis of the data fitted to the equation, $k_{obs} = [I]k_{+2}/([I] + K_i)$, using substrate concentrations significantly lower than K_m.

RESULTS

Expression of procathepsin P

Sequencing of the recombinant DNA showed that the *Pml*I had not cut the cathepsin PCR product and that blunt-end ligation

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had successfully created an in-frame insertion of cathepsin P into the vector. The sequence coding for the yeast α-factor pheromone signal sequence was followed by seven additional amino acids (Ser-Met-Asn-Ser from the vector and Pro-His-Val from the PCR product) and then full-length pro-cathepsin P. The natural stop codon for cathepsin P was intact and immediately preceded the vector sequence. A clone that was 100 % identical with the published sequences of the vector and procathepsin P was selected for electroporation into *P. pastoris* strain KM71. To produce cultures of *P. pastoris* that expressed and secreted high levels of procathepsin P, it was necessary to select colonies that were resistant to 1000 μg/ml Zeocin. Colonies resistant to lower concentrations of Zeocin had been successfully transfected, but they expressed low levels of mRNA and protein. Test cultures of selected colonies highly resistant to Zeocin were prepared in YPD media in the absence of Zeocin and shown to express high levels of protein (Figure 1A). Total protein concentrations in culture supernatant from transfected cells typically reached 0.1–0.2 mg/ml. Protein levels in culture supernatant from mock-transfected cells were low but a range of proteins could be detected by SDS/PAGE after concentrating the culture supernatant (Figure 1A, lane 3). These proteins could also be detected in culture supernatant from the transfected cells, but three additional bands were expressed at higher levels with approximate molecular masses of 31, 35 and 38 kDa (Figure 1A, lanes 1 and 2). The additional proteins were identified as cathepsin P by Western-blot analysis using an antibody raised to a peptide sequence found exclusively in cathepsin P (Figure 1B, lane 2). The antibody does not recognize the closely related proteases, cathepsins L, M or Q (Figure 1C).

The multiple protein bands in the culture supernatant were reduced to a single band of approximate molecular mass of 28 kDa by deglycosylation with PNGase F and were shown to be cathepsin P by Western-blot analysis (Figures 2A and 2B). The lower protein band in Figure 2(A) is the added PNGase F that does not react with the antibody (lane 4). With lower concentrations of PNGase F, time-dependent processing shows progressive decrease in the size of the multiple glycosylated forms to the final deglycosylated product (Figure 2C). The low amount of PNGase F is not detected by silver staining (Figure 2C, lane 1). PNGase F was unable to deglycosylate procathepsin P that was not denatured before treatment (results not shown).

Activation of procathepsin P

Cathepsins are synthesized as inactive zymogens and the propeptide has to be removed for enzymes to exhibit activity. When procathepsin P (10 μg) was incubated with proteinase K (2 μg), it was rapidly processed into three smaller molecular-mass forms of approx. 22, 24 and 27 kDa (Figure 3A). The processed proteins reacted with a peptidyl diazomethane inhibitor, biotin-LC-LC-Tyr-Ala-CHN_2 (where LC-LC stands for 6-hexanamido hexanoate), whereas only limited reaction of the inhibitor could be seen with the unprocessed procathepsin P (Figure 3A, lane 4). The processed proteins were stable to further processing. When procathepsin P was incubated with proteinase K in the continuous presence of biotin-LC-LC-Tyr-Ala-CHN_2, some degradation products could be seen with disappearance of the smaller of the three mature proteins (Figure 3A, lanes 5–7). Efficient processing could be achieved with as little as 50 ng of proteinase K within 1 h (Figure 3B). Proteinase K could also process procathepsin P at pH values as low as 5.5 (Figure 3C). However, at pH 5.5, the processed cathepsin P was less reactive with biotin-LC-LC-Tyr-Ala-CHN_2. At pH 5.0, procathepsin P was neither processed nor reactive with inhibitor (Figure 3C, lanes 7 and 9).

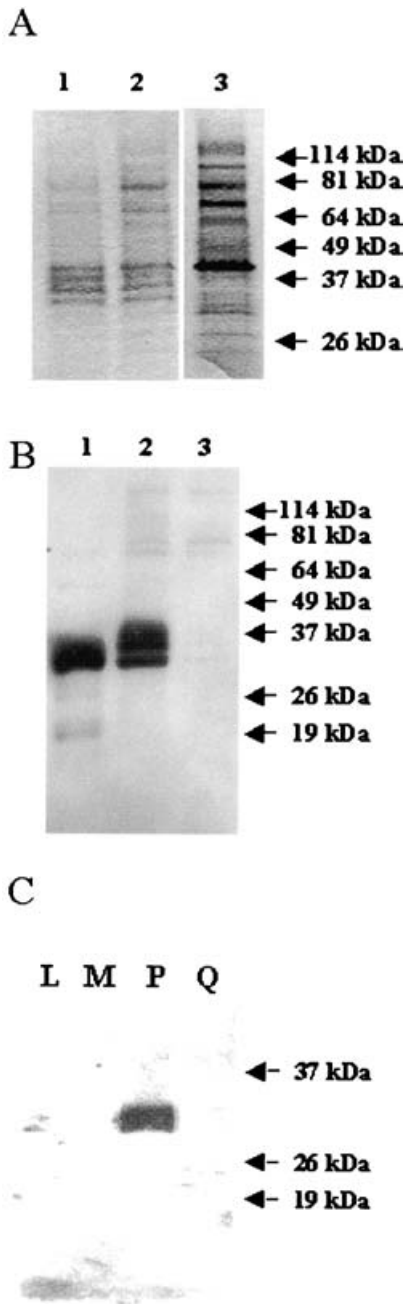

Figure 1 Expression of procathepsin P

(**A**) Proteins expressed and secreted by transfected cells were analysed by separation using SDS/PAGE (12 % gel) followed by staining with Coomassie Blue. Undiluted culture supernatant (20 μl) from one transfected culture was loaded on to lane 1, 20 μl of a 5× concentration of culture supernatant from a different sample of transfected cells was loaded on to lane 2 and 20 μl of 20× concentrated culture supernatant from mock-transfected cells was loaded on to lane 3. (**B**) Procathepsin P expressed in *E. coli* (lane 1) or *P. pastoris* (lane 2) and mock-transfected *P. pastoris* (lane 3) were separated by SDS/PAGE, transferred to PVDF and then blotted with anti-cathepsin P antibody. (**C**) Western blot using the same antibody with cathepsin L, M, P and Q proteins expressed in *E. coli*.

Pancreatic elastase and chymotrypsin (Figure 3D) could also activate procathepsin P. The processed cathepsin P was capable of reacting with biotin-LC-LC-Tyr-Ala-CHN_2 in a thiol-dependent manner. Under similar conditions, trypsin was unable to activate procathepsin P and pepsin was unable to activate procathepsin P at pH 4.0 (results not shown).

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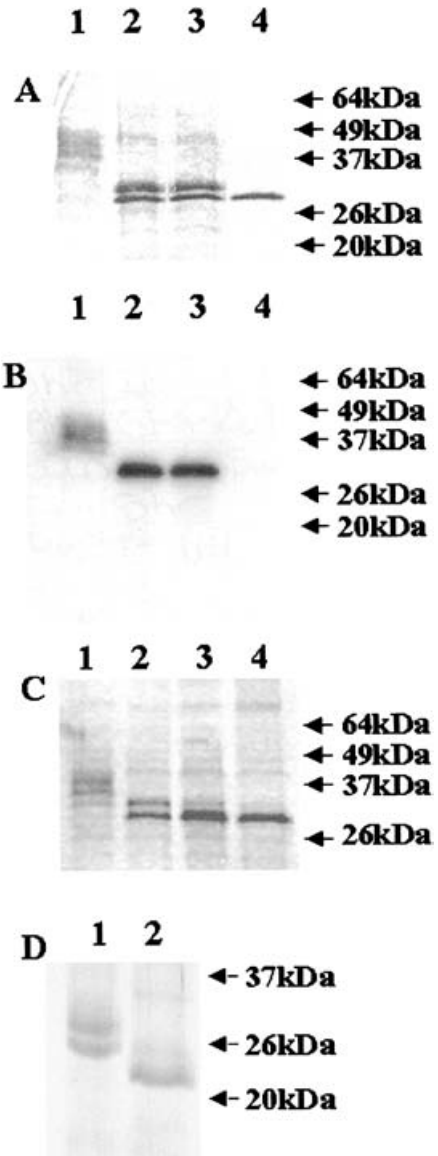

Figure 2 Deglycosylation of procathepsin P

(**A**, **B**) Culture supernatant from transfected cells was digested with PNGase F for 3 h (lanes 2 and 3) or untreated (lane 1) and then separated by SDS/PAGE. Lane 4 shows PNGase alone. (**A**) Total silver-stained proteins and (**B**) Western blot of a duplicate gel probed with anti-cathepsin P. (**C**) Culture supernatant was incubated with a 20-fold lower quantity of PNGase F for 0, 1, 6 and 24 h (lanes 1–4 respectively) and protein separated by SDS/PAGE and silver-stained. (**D**) Mature cathepsin P treated for 3 h in the presence (lane 2) or absence (lane 1) of PNGase F and proteins stained with Coomassie Blue.

A common feature of lysosomal proteases is their ability to be autoactivated at acidic pH. We were unable to demonstrate such acidic autoactivation for procathepsin P. However, during purification of procathepsin P, we discovered that autoactivation occurred at pH 7.5. Culture supernatant containing procathepsin P was adjusted to pH 7.5 with 1 M Tris base and then concentrated by ultrafiltration. The concentrated extract was then dialysed against a 20-fold excess of 20 mM Tris/HCl buffer (pH 7.5). The dialysed culture supernatant was then incubated at 4 °C for 6 days. The procathepsin P was processed to mature-sized forms (Figure 3E, lane 2). This processing was blocked when the proenzyme was incubated at 4 °C in the presence of 200 μM biotin-LC-LC-Tyr-Ala-CHN_2 (Figure 3E, lane 3). Similar processing was seen for procathepsin P purified by ion-exchange chromatography (results not shown).

When autoactivated mature cathepsin P was treated with PNGase F, the two bands were processed to a single protein of an apparent molecular mass of 22 kDa (Figure 2D). This shows that the two proteins are differentially glycosylated forms of the same protein.

Purification of mature cathepsin P

The high expression levels of procathepsin P by the transfected *P. pastoris* enabled the autocatalysed mature enzyme to be purified to 95 % homogeneity by a single step of ion-exchange chromatography. The purified product consisted of two bands of molecular masses 27 and 24 kDa (Figure 3E, lane 5). The N-terminal amino acid sequence of both of the processed forms of the enzyme was SIGLPD, showing that the processed cathepsin P contained an additional three amino acids N-terminal to the N-terminus that we originally predicted by alignment of the cathepsin P sequence with the known N-terminus of the lysosomal form of the related enzyme, cathepsin L [12]. For a typical purification procedure, 15 mg of purified mature cathepsin P is obtained from a 250 ml of culture of transfected *P. pastoris*.

Peptide substrate hydrolysis by cathepsin P

The first reactivity detected for processed cathepsin P was with the active-site-directed inhibitor, biotin-LC-LC-Tyr-Ala-CHN_2 (Figure 3A). During the initial purification, activity was screened against a range of peptidyl substrates typically hydrolysed by other cysteine proteases (Z-Phe-Arg-NHMec, Z-Arg-Arg-NHMec, Arg-NHMec, Bz-Phe-Val-Arg-NHMec, succinyl-Ala-Ala-Phe-NHMec, Z-Val-Ala-Asp-NHMec), but no hydrolysis could be detected. No hydrolysis of bradykinin, somatostatin, Fmoc-Tyr-Ala-OH, Z-Gly-Gly-OH or Z-Leu-Tyr-OH, potential carboxypeptidase substrates, could be detected by HPLC analysis. A number of quenched fluorescent substrates were then screened. No significant hydrolysis of Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2, Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH_2 or Dnp-Pro-cyclohexyl-Ala-Gly-Cys(Me)-His-Ala-Lys(Nma)NH_2 could be detected, but Mca-Pro-Leu-Gly-Leu-Dnp(Dpa)-Ala-Arg-NH_2 was hydrolysed by cathepsin P, with cleavage between the Gly-Leu bond (determined by HPLC of cleavage products compared with standards). In 100 mM sodium phosphate buffer (pH 6.5), containing 1 mM EDTA and 1 mM DTT, slow hydrolysis of this substrate was detected. This hydrolysis was significantly stimulated by addition of 1.5 M Na_2SO_4 to the incubation buffer. The K_m value for this substrate in the presence or absence of the salt was calculated to be in excess of 10 μM, and hence the activity was determined as k_{cat}/K_m using continuous monitoring of substrate hydrolysis in 0.5–2 μM substrate. In the low-salt buffer, k_{cat}/K_m was calculated to be $400 \pm 54\ M^{-1} \cdot s^{-1}$, whereas in high salt it was $20000 \pm 1500\ M^{-1} \cdot s^{-1}$. Cathepsin P was found to be optimally active at pH 7.0 against this substrate in the presence of salt (Figure 4). Significant activity could be detected at pH 8.5, but activity was greatly decreased at pH 5 and below. Activity was also stimulated 4-fold by incubation in 1 mg/ml hyaluronate, but not by chondroitin sulphates A, B or C.

Protein hydrolysis by cathepsin P

The general protein substrate azocasein was used to determine the proteolytic capacity of cathepsin P. Both time- and enzyme-concentration-dependent hydrolysis of azocasein by cathepsin

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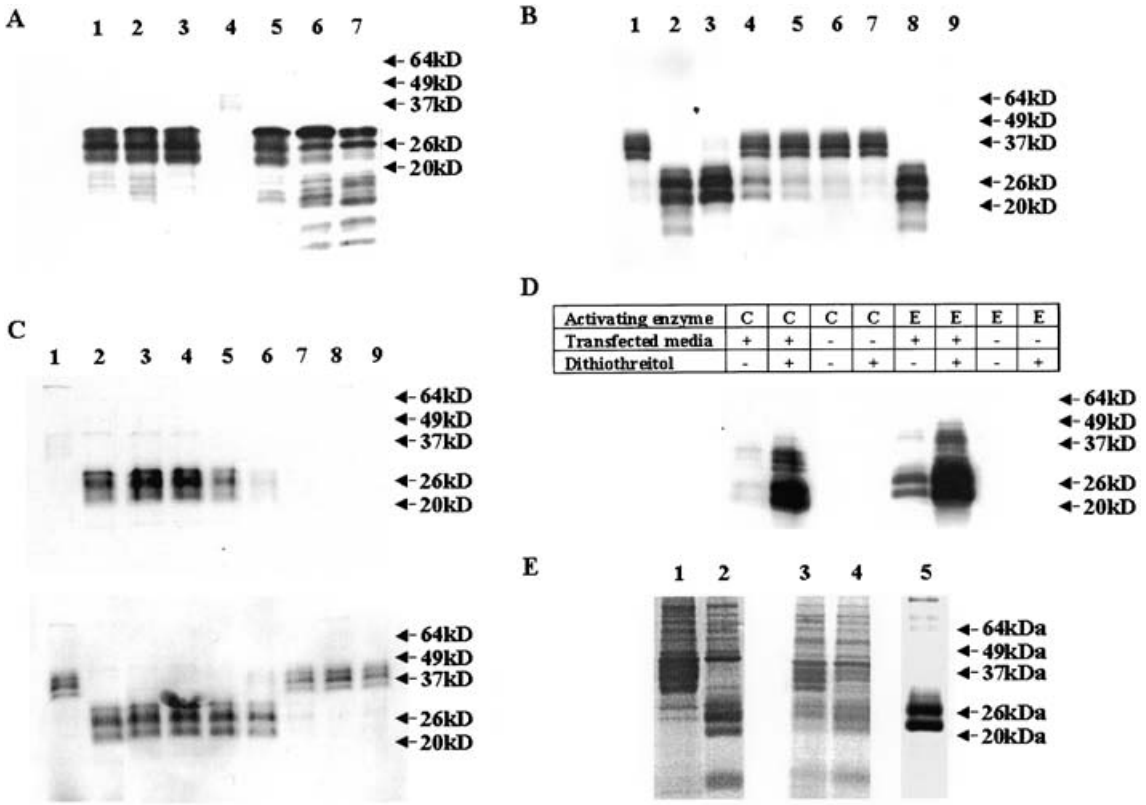

Figure 3 Activation of procathepsin P

(**A**) Culture supernatant from transfected cells (10 μg) was incubated with proteinase K (2 μg) in 100 mM sodium phosphate buffer (pH 7.5), containing 1 mM DTT at 30 °C for 2 h (lanes 1 and 5), 6 h (lanes 2 and 6) or 17 h (lanes 3 and 7). Biotin-LC-LC-Tyr-Ala-CHN_2 (2 μM) was added either at the start of the experiment (lanes 5–7) or for the last 1 h of incubation (lanes 1–3). Proteins were then separated by SDS/PAGE, blotted on to PVDF membranes and biotin was identified by ECL® using an antibody to biotin. Lane 4 shows culture supernatant from transfected cells incubated for 17 h in the presence of inhibitor alone. (**B**) Culture supernatant from transfected cells (10 μg) incubated with 100, 50, 25, 12.5, 6.25, 3.12 and 100 ng of proteinase K in 100 mM sodium phosphate buffer (pH 7.5), containing 1 mM DTT at 30 °C for 1 h (lanes 2–8 respectively). Lane 1, culture supernatant from transfected cells without proteinase K; lane 9, culture supernatant from non-transfected cells incubated with 100 ng of proteinase K. Proteins were separated by SDS/PAGE and identified by Western blotting with anti-cathepsin P antibody. (**C**) Culture supernatant from transfected cells (10 μg) was incubated at 30 °C for 1 h with 50 ng of proteinase K in 100 mM sodium phosphate buffer (pH 7.5, lane 2; 7.0, lane 3; 6.5, lane 4) or sodium acetate buffer (pH 6.0, lane 5; 5.5, lane 6; 5.0, lanes 7 and 9), each containing 1 mM DTT. Lanes 1 and 8 contain culture supernatant from transfected cells incubated without proteinase K in pH 7.5 and 5.0 buffers respectively. Each sample was then incubated with 2 μM biotin-LC-LC-Tyr-Ala-CHN_2 for an additional 1 h before separation by SDS/PAGE and blotting to PVDF membrane. The upper panel shows a blot for biotin and the lower panel is the same blot re-probed with antibody to cathepsin P. (**D**) Culture supernatant from transfected and non-transfected cells (1 ml) was adjusted to pH 7.5 with 1 M Tris base and incubated at 37 °C for 4 h with 10 μg of chymotrypsin or 10 μg of elastase. A 10 μl of aliquot of each sample was then incubated with 25 μM biotin-LC-LC-Tyr-Ala-CHN_2 for 1 h in the presence (lanes 1, 3, 5 and 7) or absence (lanes 2, 4, 6 and 8) of 1 mM DTT. Elastase (**E**) and chymotrypsin (**C**) treated samples of culture supernatant from transfected and non-transfected cells. Samples were then separated by SDS/PAGE and reactive proteins identified by Western blotting and ECL® detection of biotin. (**E**) Protein samples separated by SDS/PAGE and stained for protein with Coomassie Blue. Lane 1 shows 10 μl of culture supernatant stored frozen for 6 days and lane 2 shows 10 μl of the same culture supernatant incubated at 4 °C for the same period of time. The sample loaded on to lane 3 was 5 μl of culture supernatant incubated with an equal volume of 400 μM biotin-LC-LC-Tyr-Ala-CHN_2 for 6 days at 4 °C and the sample loaded on to lane 4 is the same amount of culture supernatant incubated in the absence of the inhibitor. Similar processing was seen for procathepsin P purified by ion-exchange chromatography (results not shown). Lane 5 shows 20 μg of purified mature cathepsin P.

P was demonstrated (Figure 5). This activity depended on the activation of the enzyme with DTT and was inhibited by pretreatment of the enzyme with E-64. This activity was unaffected by concentrations of Na_2SO_4 up to 1 M (higher concentrations caused precipitation of the substrate). The rate of hydrolysis of azocasein under the conditions used was calculated to be 34 ± 6 μg of protein $\cdot h^{-1} \cdot$ (nmol of cathepsin P)$^{-1}$. Under similar conditions, papain was approx. 1000 times more efficient in hydrolysing this denatured protein substrate. Cathepsin P could also degrade transferrin, yielding smaller fragments that could be detected by SDS/PAGE (Figure 5C).

Inhibition of cathepsin P

Although the original identification of activation of procathepsin P was determined by the reaction with biotin-LC-LC-Tyr-Ala-CHN_2, the rate of reaction of the inhibitor with the autoactivated mature enzyme was too slow to be determined accurately, being $< 20\ M^{-1} \cdot s^{-1}$. Reactivity with E-64 was similarly slow. Reactivity of both biotin-LC-LC-Tyr-Ala-CHN_2 and E-64 with cathepsin P was stimulated by the addition of salt to the assay. In the presence of 1.5 M Na_2SO_4, K_i and k_{+2} were determined to be 2 ± 0.4 μM and 0.0036 s^{-1} for E-64 to give a k_{+2}/K_i of $1780\ M^{-1} \cdot s^{-1}$. For biotin-LC-LC-Tyr-Ala-CHN_2, k_{obs} was linearly related to inhibitor concentration using 1–5 μM inhibitor, indicating that $K_i \gg [S]$. k_{+2}/K_i was calculated to be $k_{obs}/[I]$ to give $353 \pm 11\ M^{-1} \cdot s^{-1}$.

DISCUSSION

The constitutive expression system was shown to be an effective system for the expression of high levels of procathepsin P. A single

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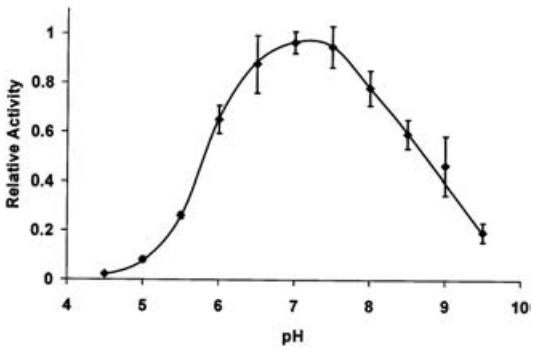

Figure 4 Effect of pH on the activity of cathepsin P against Mca-Pro-Leu-Gly-Leu-Dnp(Dpa)-Ala-Arg-NH_2

Cathepsin P (150 pmol) was incubated in 100 mM sodium acetate (pH 5–6) or sodium phosphate (pH 7–9.5) buffers containing 2 M Na_2SO_4 and 1 mM DTT, and hydrolysis of Mca-Pro-Leu-Gly-Leu-Dnp(Dpa)-Ala-Arg-NH_2 (1 μM) measured continuously at 37 °C. Initial rates at each pH were determined and rates of hydrolysis relative to the maximal rate at pH 7.0 are shown.

protein product was identified after deglycosylation with PNGase F, indicating that the expression system generates multiple N-glycosylated forms of procathepsin P. There are five potential N-glycosylation sites in procathepsin P and two of them are in the peptide used to raise an antibody to the enzyme. Differences seen in protein staining and antibody reaction may be partially caused by differences in N-glycosylation. Deglycosylated procathepsin P migrates with an apparent molecular mass of 28 kDa, which is somewhat smaller than the calculated mass of the deglycosylated protein (35.5 kDa). The secreted protein was processed by proteinase K, pancreatic elastase and chymotrypsin to yield mature forms of cathepsin P that were resistant to further proteolysis. The molecular masses of the processed forms of cathepsin P (22, 24 and 27 kDa) and the deglycosylated mature form (22 kDa) were similar to the predicted mass of the mature enzyme (25 kDa). Trypsin was unable to activate or process procathepsin P. Homology modelling shows that the linker region between the propeptide and mature form of cathepsin P is devoid of basic amino acids and therefore cannot be processed by trypsin [13]. High concentrations of proteinase K were capable of degrading slowly the mature enzyme bound to an active-site-directed inhibitor, although most of the enzyme remained resistant for at least 17 h. Resistance to proteolysis indicates that the expressed protein is most probably folded correctly. Procathepsin P was also capable of autoprocessing to give similar mature enzymes. The 22 kDa form of cathepsin P was not generated by autolytic processing, and is probably a partial degradation product. Autolytic processing occurred most efficiently at neutral pH and autoprocessing did not occur at acidic pH. Indeed, the optimal activity of the processed enzyme was found to be in the neutral pH range, with little activity at pH 5.0 and below. Most lysosomal proteases have evolved to be optimally stable and active at acidic pH, although cathepsins S and K, which are proposed to have non-lysosomal functions, do exhibit some activity at neutral pH [18,19]. These results indicate that unlike other typical lysosomal proteases [20–22], the activation of cathepsin P is not triggered by acidification during packaging within cells.

Initial studies indicated that cathepsin P is a poor protease, with very low specific activity against synthetic peptidyl substrates and slow reactivity with peptidyl inhibitors. This activity and reactivity were significantly stimulated by high-salt concentrations. These results indicate that the addition of salt increases

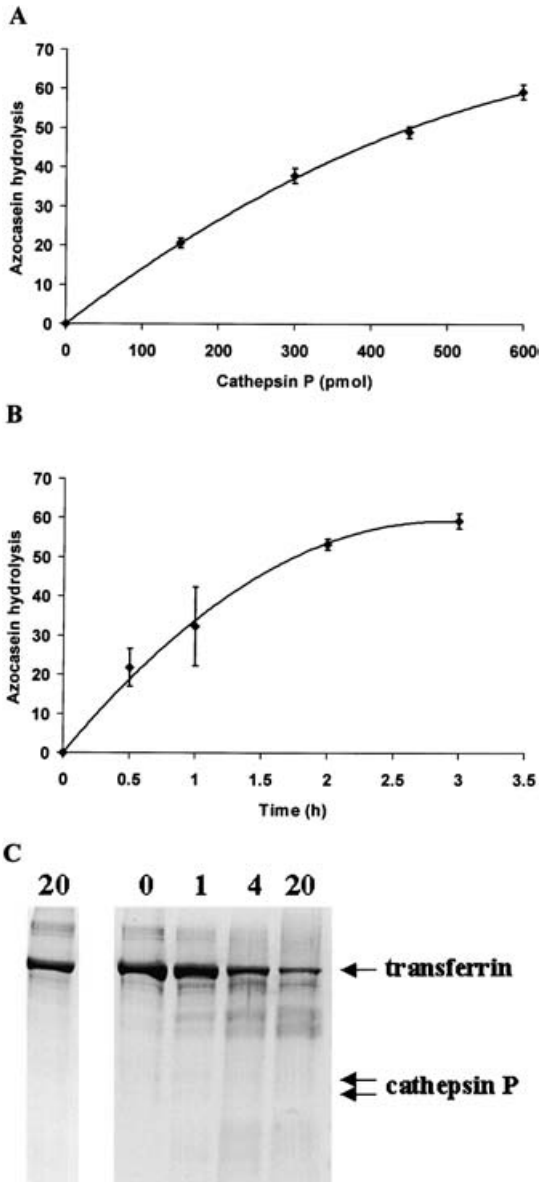

Figure 5 Activity of cathepsin P against protein

Cathepsin P was incubated with azocasein for up to 3 h (see the Experimental section). Hydrolysis was determined by reading absorbance of trichloroacetic acid-soluble peptides at 440 nm. The values on the *y*-axis in (**A** and **B**) were calculated to denote μg of azocasein hydrolysed during the assay. The influence of enzyme concentration (**A**) and incubation time (**B**) on azocasein hydrolysis is shown. (**C**) Degradation of transferrin by incubation with cathepsin P for 0–20 h. The left lane shows transferrin incubated alone for 20 h.

access of small molecules to the active site of cathepsin P. Structural models indicate that the active site of cathepsin P is more restricted than that of cathepsin L and a charged amino acid may restrict access to the active site [13]. Hyaluronic acid, but not chondroitin sulphates, was also capable of stimulating activity of cathepsin P against the synthetic substrate. These results indicate that environmental conditions will influence the peptidolytic activity of cathepsin P and that the accessibility of substrates to the active site is restricted. When azocasein was used as a protein substrate, salts did not significantly affect proteolysis, indicating that the salt dependence of activity is substrate-dependent. Processing of transferrin into fragments visible by SDS/PAGE indicates that cathepsin P is a true endopeptidase.

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Mca-Pro-Leu-Gly-Leu-Dnp(Dpa)-Ala-Arg-NH_2 was originally designed as a substrate for matrix metalloproteases, with k_{cat}/K_m values for MMP1, MMP2 and MMP3 of 14800, 629000 and 23000 $M^{-1} \cdot s^{-1}$ respectively [16]. Thus the efficiency of hydrolysis of this substrate by cathepsin P is comparable with that of MMP1 and MMP3. The catalytic activity of protein by cathepsin P is also comparable with that of several proteases, but it is approximately three orders of magnitude weaker than the related proteases, papain and cathepsin L (R.W. Mason and G. Lu, unpublished work). Cathepsin P also exhibits low reactivity with active-site-directed inhibitors, especially in the presence of low-salt concentrations. The low catalytic activity of the enzyme when compared with the more ubiquitously expressed cathepsins may reflect a more specific function for this protease.

This study has confirmed the original prediction that the evolutionarily conserved structural features of the placenta-specific protease cathepsin P yield an active protease. Conservation of the structure of cathepsin P is, quite probably, driven by evolution of a placenta-specific proteolytic function for the new gene product. Since cathepsin P and the related placental proteases are expressed only in rodents, it had been proposed that cathepsin P probably have evolved a sub-specialized function of the more ubiquitously expressed structural homologue, cathepsin L. However, this may not be the case because the expressed protein has novel catalytic properties not shared by cathepsin L. In species that do not express cathepsin P, the function of this enzyme could be performed by an unrelated protease. The evolution of cathepsin P has created an enzyme that is activated and functional at neutral pH, giving it characteristics that are more similar to secreted proteases such as the MMPs, a family of proteases that have been implicated in human placental implantation [23,24]. Cathepsin P may have evolved to perform a placenta-specific function that is performed by one of these enzymes in non-rodents.

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