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ORIGINAL RESEARCH

Protein processing by the placental protease, cathepsin P

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ABSTRACT: Cathepsin P is a member of a family of placentally expressed cathepsins (PECs). The closest human homolog of cathepsin P is cathepsin L, a broad specificity enzyme that has functions in many tissues in addition to placenta. The gene duplications that gave rise to the PECs provide a rare opportunity to define proteolytic functions in placenta, a transient organ unique to mammals. Peptidyl substrate and inhibitor libraries have shown that cathepsin P has evolved an unusually restricted preference for substrates containing hydrophobic amino acids. Proteomic techniques were used to probe for substrates of this enzyme. Recombinant cathepsin P was incubated with rat choriocarcinoma (Rcho-1) cell proteins to identify substrates using two-dimensional difference gel electrophoresis. Substrate proteins were excised from gels and characterized by trypsin digestion and MALDI MS/MS. Two endoplasmic reticulum (ER) proteins, gp96 and calreticulin, emerged as potential substrates, and western blotting showed that these proteins are processed by cathepsin P from their C-terminus, removing the KDEL ER retention signal. Immunohistochemistry showed that a portion of cathepsin P co-localizes with calreticulin in Rcho-1 cells. Extracellular calreticulin induces differentiation of Rcho-1 cells, indicating a potential role of cathepsin P in processing and secretion of calreticulin during differentiation of trophoblast giant cells.

Key words: placenta / cathepsin / proteolysis / processing

Introduction

Proteolysis by cathepsins plays critical roles in processes related to early development, including fertilization, embryonic and fetal development, tissue remodeling, antigen presentation, cell-cycle regulation, angiogenesis, apoptosis and inflammatory responses (Turk *et al*., 2001; Puente *et al*., 2003). Pharmacological targeting of cysteine proteases with broad-based inhibitors such as E-64 and leupeptin indicates a critical role for cathepsins in blastocyst hatching, implantation and normal embryonic development in mouse, rat and hamster (Babiarz *et al*., 1992; Afonso *et al*., 1997; Sireesha *et al*., 2008). Originally, cathepsins L and B were proposed to be the primary targets of these inhibitors, but genetic deletion of both of these enzymes in mice has no obvious effect on normal fetal development (Deussing *et al*., 1998; Roth *et al*., 2000; Felbor *et al*., 2002). Subsequently, a family of placentally expressed cathepsins (PECs) were discovered in rodent genomes (Sol-Church *et al*., 1999; Hemberger *et al*., 2000; Deussing *et al*., 2002; Sol-Church *et al*., 2002; Mason, 2008). This family of proteases are well conserved in rodent genomes; orthologs of cathepsins 6, M, P, Q and R are found in both old world and new world mice (Sol-Church *et al*., 2002; Glenn *et al*., 2008). The closest human homolog of these genes is cathepsin L, an enzyme that is expressed in all tissues.

Cathepsin P was the first PEC to be discovered and mRNA for this gene can be detected as early as Day 7.5 in the ectoplacental cone of the mouse conceptus and even earlier in the hamster blastocyst (Sol-Church *et al*., 1999; Hemberger *et al*., 2001; Deussing *et al*., 2002; Sireesha *et al*., 2008). Cathepsin P mRNA is up-regulated during gestation in a developmentally dependent manner in mouse (Sol-Church *et al*., 1999; Deussing *et al*., 2002). Later in gestation, it is localized to the labyrinth layer of the definitive placenta (Nakajima *et al*., 2000; Hemberger *et al*., 2001).

Recombinant pro-cathepsin P autoactivates at neutral pH, which is a unique property for a lysosomal cysteine protease (Mason *et al*., 2004; Puzer *et al*., 2005). The recombinant enzyme is optimally active at neutral pH (6.5–7.5) and preferentially hydrolyzes small synthetic substrates containing hydrophobic amino acids (Mason *et al*., 2004; Puzer *et al*., 2005). A screen with a combinatorial library of small peptidyl inhibitors confirmed the preferred specificity of cathepsin P for hydrophobic aromatic amino acids in six contiguous-binding sites spanning the enzyme's active site cleft (Hassanein *et al*., 2007). Like other cathepsins, cathepsin P is found in the lysosome, partially co-localizing with cathepsin B in the rat placental cell line, Rcho-1 (Hassanein *et al*., 2007). Cathepsin P immunoreactivity was also detected in cellular compartments devoid of cathepsin B, indicating that the enzyme

might also function outside the lysosome. mRNA and enzyme activity of cathepsin P increase during differentiation of Rcho-1 trophoblast stem cells to a trophoblast giant cells phenotype, indicating a potential role in these invasive cells (Hassanein *et al*., 2007).

Most studies of the role of lysosomal proteases in placental function have focused on general proteolysis of extracellular matrix proteins to facilitate invasive implantation (Afonso *et al*., 1997; Cheon *et al*., 2004). Such general proteolysis could be performed by several broad specificity proteases, such as the matrix metalloproteases and serine proteases (Lamarca *et al*., 2005). The tissue-specific expression, narrow specificity and unusual pH activity profile of cathepsin P indicate that this enzyme may fulfill a much more specific placental proteolytic role. The preferred peptide sequence for binding to cathepsin P is Trp-Trp-Phe-Val-Trp-Phe (Puzer *et al*., 2005; Hassanein *et al*., 2007). A bioinformatics search of the NCBI protein database reveals no exact matches with this peptide. Biological substrates are defined not only by peptide sequence but also by conformational access of enzyme to hydrolysable sequences within proteins. We therefore utilized the resolving power, quantitative capability and sensitivity of two-dimensional difference gel electrophoresis (2D-DIGE) to identify protein substrates of cathepsin P.

Materials and Methods

Materials

NCTC-135 medium was purchased from Sigma Aldrich, (St Louis, MO, USA). Fetal bovine serum (FBS), horse serum, trypsin and HBSS were purchased from Mediatech Inc. (Herndon, VA, USA). Rcho-1 cells were a kind gift from Michael J. Soares, Kansas Medical Center, Kansas City, KS, USA. Sheep anti-calreticulin antibody to aa 270–390 for immunohistochemistry was from BD Biosciences (San Jose, CA, USA). Polyclonal rabbit anti-calreticulin raised to aa 405–417, polyclonal goat anti-calreticulin raised to the N-terminus of the protein and polyclonal rabbit anti-Gp96 raised to the N-terminus of the protein were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibody to cathepsin P was prepared as described previously (Mason *et al*., 2004). Texas Red-labeled mouse anti-rabbit IgG antibody, FITC-labeled donkey anti-sheep IgG antibody and horseradish peroxidase secondary antibodies were from Jackson Immunoresearch Inc. (West Grove, PA, USA). Recombinant mouse cathepsin P was expressed in *Pichia pastoris*, purified and activated as described previously (Mason *et al*., 2004). Cy dyes, ECL kits and IPG strips were purchased from GE Healthcare (Piscataway, NJ, USA). Gel Code Blue stain was from Pierce Biotechnology Inc. (Rockford, IL, USA). Sequencing grade modified trypsin was from Promega (Madison, WI, USA). C-18 Zip Tips were from Millipore (Billerica, MA, USA). Other reagents used in this study were analytical grade and were obtained from Sigma Aldrich.

Proteolysis of Rcho-1 cell proteins by cathepsin P

Rcho-1 trophoblast stem cells were maintained in a proliferative state by culturing in hormone and growth-factor-rich medium (NCTC-135 containing 20% FBS, 50 μM β-mercaptoethanol and 1% pyruvic acid). Cells were split every 2–3 days as described (Faria and Soares, 1991; Sahgal *et al*., 2006). Confluent cells were scraped and washed three times with ice-cold PBS to remove serum and other media components. Cells were then lysed in cathepsin P activity buffer 1 (20 mM Tris, 1 mM EDTA, 1 mM dithiothreitol and 4% CHAPS, pH 7.4) by freeze-thawing (Bredemeyer *et al*., 2004). After removal of insoluble material by centrifugation at 12 000*g* for 5 min, supernatant protein (50 μg) was incubated with or without cathepsin P (0.2 μg) for 6 h at 37°C. The enzymatic reaction was stopped by placing the sample immediately on ice and by adding a 3-fold volume of denaturing isoelectric focusing buffer (7 M urea, 2 M thiourea, 4% CHAPS and 30 mM Tris buffer, pH 8.5).

For western blotting, a similar digestion protocol was used: Rcho-1 protein extracts were incubated with cathepsin P for 1–20 h, digestion was stopped by placing samples on ice and they were then boiled in SDS–PAGE sample buffer.

Two-dimensional electrophoresis and DIGE labeling

Equal amounts of control and treated samples were trace-labeled with Cy-3-red or Cy-5-green according to standard protocols (GE Healthcare). A pool of equal amounts of all control and treated samples were labeled with a third dye, Cy-2-blue, to be used as a standard for each gel. Four separate samples of both treated and controls were prepared to determine significance of quantitative differences in levels of individual proteins due to cathepsin P treatment. Pairs of control and treated samples were combined with a portion of the standard sample and proteins were separated on 24-cm immobilized gradient gels IPG (3–10) strips. Strips were focused for 50 000 Vh and then proteins were reduced and alkylated with iodoacetamide and equilibrated for SDS–PAGE separation (12% polyacrylamide gels, 200 × 230 × 1.0 mm).

Image analysis and differential display of cathepsin P digested proteins

2D SDS–PAGE gels were scanned using a Typhoon Trio imager (GE Healthcare) to identify proteins labeled by the three dyes. Images were analyzed using Decyder software to map protein spots and compare levels of proteins in digested samples with undigested samples (Alban *et al*., 2003). Statistical analysis using Student's *t*-test was applied to identify the biologically significance of differentially processed proteins. Protein spots that were reduced or increased on cathepsin P treatment and received *P*-value of <0.05 were considered significant and included in a potential substrate list (products of hydrolysis may appear as new spots). A separate picking gel was run simultaneously using aliquots from the standard samples and was stained with Gel Code Blue stain.

In-gel trypsin digestion and mass spectrometric identification

Selected spots were excised from gels using a 1.5 mm Spot Picker Plus (The Gel Company, San Francisco, CA, USA). Picked spots were destained by washing in 50% acetonitrile. Gel plugs were sequentially treated with 10 mM dithiothreitol (45 min, 56°C), 50 mM iodoacetamide (30 min, 22°C), 100 mM NH_4HCO_3 (10 min) and 100% acetonitrile (10 min). Plugs were dried using speed vacuum and protein was digested with sequencing grade modified trypsin (200 ng in NH_4HCO_3, 16 h, 37°C). Peptides were purified with C-18 Zip Tips prior to loading onto 192 spot MALDI plates in 1 μl matrix solution (cyano-4-hydroxycinamic acid in 70% acetonitrile, 0.1% trifluoroacetic acid, 5 mg/ml). MS measurements were carried out on a 4700 proteomic analyzer, a MALDI-TOF-TOF-MS spectrometer (Applied Biosystems, Foster City, CA, USA). Mass spectra were obtained on a mass range of 800–3200 Da, using a laser beam (337 nm, 200 Hz) as ionization source. The instrument was used in reflector-positive mode with an acceleration voltage of 20 kV. The TOF-TOF mass spectra were acquired by the Data Dependent Acquisition method with the five strongest precursor ions selected from one MS scan. MS accuracy was externally calibrated with trypsin-digested peptides of horse myoglobin. The database searches were performed at a peptide mass tolerance of ± 0.3 Da and a fragment

mass tolerance of ±0.4 Da. All data analysis and database searching were performed by the GPS Explorer software using the NCBInr rodent subset of protein sequences (Applied Biosystems). Molecular weights and pIs of identified proteins were calculated from primary sequence using the protein sequence analysis tools on the ExPASy proteomics server (http://ca.expasy.org/cgi-bin/pi_tool).

Western blot analysis

Equal amounts (20 μg) of cathepsin P treated or control protein extracts from Rcho-1 cells were separated by 10% SDS–PAGE and then transferred onto PVDF membranes. Primary antibodies were: polyclonal rabbit anti-calreticulin raised to aa 405–417 (1:2000 in blocking solution); polyclonal goat anti-calreticulin raised to the N-terminus of the protein (1:2000 in blocking solution) and polyclonal rabbit anti-Gp96 raised to the N-terminus of the protein (1:1000 in blocking solution). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit or anti-goat IgG. ECL kits were used to detect horseradish peroxidase.

Immunohistochemistry

Rcho-1 cells were grown to confluence in four-chambered glass slides. Cells were washed with PBS, fixed in methanol/acetone (1:1) at −20°C for 5 min, permeabilized with 0.25% Triton 100-X for 15 min and then washed with PBS to remove detergent (Zheng *et al.*, 2001). Rabbit anti-mouse cathepsin P antibody (1:300 in blocking solution) (Mason *et al.*, 2004) and sheep anti-calreticulin antibody to aa 270–390 (1:200 in blocking solution) were then added and incubated at 4°C for 24 h. Texas Red-labeled anti-rabbit IgG and FITC-labeled anti-sheep IgG (each 1:500 dilution) were used to visualize the primary antibodies. 4,6-Diamidino-2-phenylindole (Dapi) was used as a nuclear marker. Cells incubated without primary antibodies or with pre-immune serum were used to control for non-specific signals.

Calreticulin treatment of Rcho-1 cells

Rcho-1 cells were cultured in proliferative medium in the presence or absence of purified calreticulin (1 or 2 μg/ml) for 7 days. For comparison, a group of cells were cultured in differentiation medium for 7 days. Cells were evaluated morphologically using a Motic AE31 phase contrast light microscope with a ×20 objective.

Identification of mRNA in Rcho-1 cells

Expression of actin and markers of differentiation were determined in control cells and cells treated with calreticulin (2 μg/ml in proliferative media for 6 days, with fresh media added at Day 3). RT–PCR was performed as described previously (Hassanein *et al.*, 2007). Primers used were: GCTCTCTTCCAGCCTTCCTT and CTTCTGCATCCTGTCAG CAA for β- and γ-actin, 168 bp product; TGAAGAGTTGAGTCTGTG GAGGACC and GCCAGTTTTTGAGAACATCTGACC for cathepsin P, 526 bp product; CTCTGAAACACTTGGTCGGC and CGGCACAGGT TACAAATGGC for placental lactogen, 247 bp product; CTTCAA GAAGTCCGCAGGTC and ACCAAAGAGGAAGGGTTCGT for Hand I, 252 bp product; and GGAGTGGAAGAGGAACAATGCG and TGGGACAACAAAAAAGCGGG for cathepsin I, 592 bp product.

Cell counting

Cells were harvested, mixed with an equal volume of counting solution (0.4% Trypan Blue) and counted using a hemocytometer (Hausser Scientific, Horsham, PA, USA).

Results

Identification of cathepsin P substrates by 2D-DIGE proteomics

In this study, the rat choriocarcinoma cell line Rcho-1 was chosen as a reproducible source of proteins for digestion by recombinant rat cathepsin P. 2D-DIGE analysis revealed a number of potential substrates and products, although most proteins are not affected by cathepsin P treatment (Fig. 1A). Not all of the spots seen to change in individual gels proved to be altered significantly on batch analysis of multiple gels using the biological variation analysis program of the Decyder software package. After background subtraction, image cropping to remove poorly resolved areas of gel images and batch analysis, only 25 spots changed in abundance due to cathepsin treatment with a *P*-value of <0.05. A separate picking gel loaded with 1 mg Rcho-1 cell protein was stained with Gel Code Blue. Sixteen of the identified spots were matched with spots on the picking gel. After picking, enzymatic digestion and MALDI MS/MS, nine of these spots were characterized based on a Mascot score >100 and a close match of predicted molecular weight and pI with experimental values. Spots that did not reach these criteria were either limited in abundance or contained more than one protein so they could not be accurately identified or quantified. Identified spots are shown in Fig. 1B and Table I. ATP synthase was identified as a digestion product and the picked spot appeared to be smaller than the calculated size of the intact protein. The identified proteins are typically found in the cytoplasm, mitochondria or endoplasmic reticulum (ER) of cells (Table I). Three-dimensional representations of spots from digested and undigested samples show reduced volumes of digested spots (Fig. 1C). These results show that cathepsin P can degrade a limited number of cellular proteins *in vitro*. Cathepsin P is packaged into vesicular compartments of cells so it is not likely to encounter the cytoplasmic and mitochondrial proteins under normal cellular conditions. This leaves two ER proteins Gp-96 and calreticulin, as the only potentially physiologically relevant substrates for cathepsin P discovered in this study.

Validation of Gp-96 as a substrate for cathepsin P

Western blotting was used to show processing of Gp-96 by cathepsin P in Rcho-1 cell extracts (Fig. 2). Under cathepsin P assay conditions at pH 6.5 (or 7.5), Gp-96 was not processed by endogenous proteases. After 1 h treatment with cathepsin P, an initial cleavage product of 84 kDa was observed. This band increased in intensity after 6 h and decreased after 20 h incubation. After 6 h of digestion, a new band of 72 kDa appeared and the intensity of this band increased at 20 h. No other bands appeared and the combined intensity due to all three bands did not change appreciably during this time course. These results indicate that Gp-96 is sequentially processed by cathepsin P at two sites toward the C-terminus of the protein. Analysis of the primary amino acid sequence of rat Gp-96 reveals two hydrophobic regions, Asn-Tyr-Tyr-Ala-Ser and Leu-Ala-Val-Val-Leu-Phe, cleavage of which would yield products of ~72 and 84 kDa, respectively (Fig. 2B).

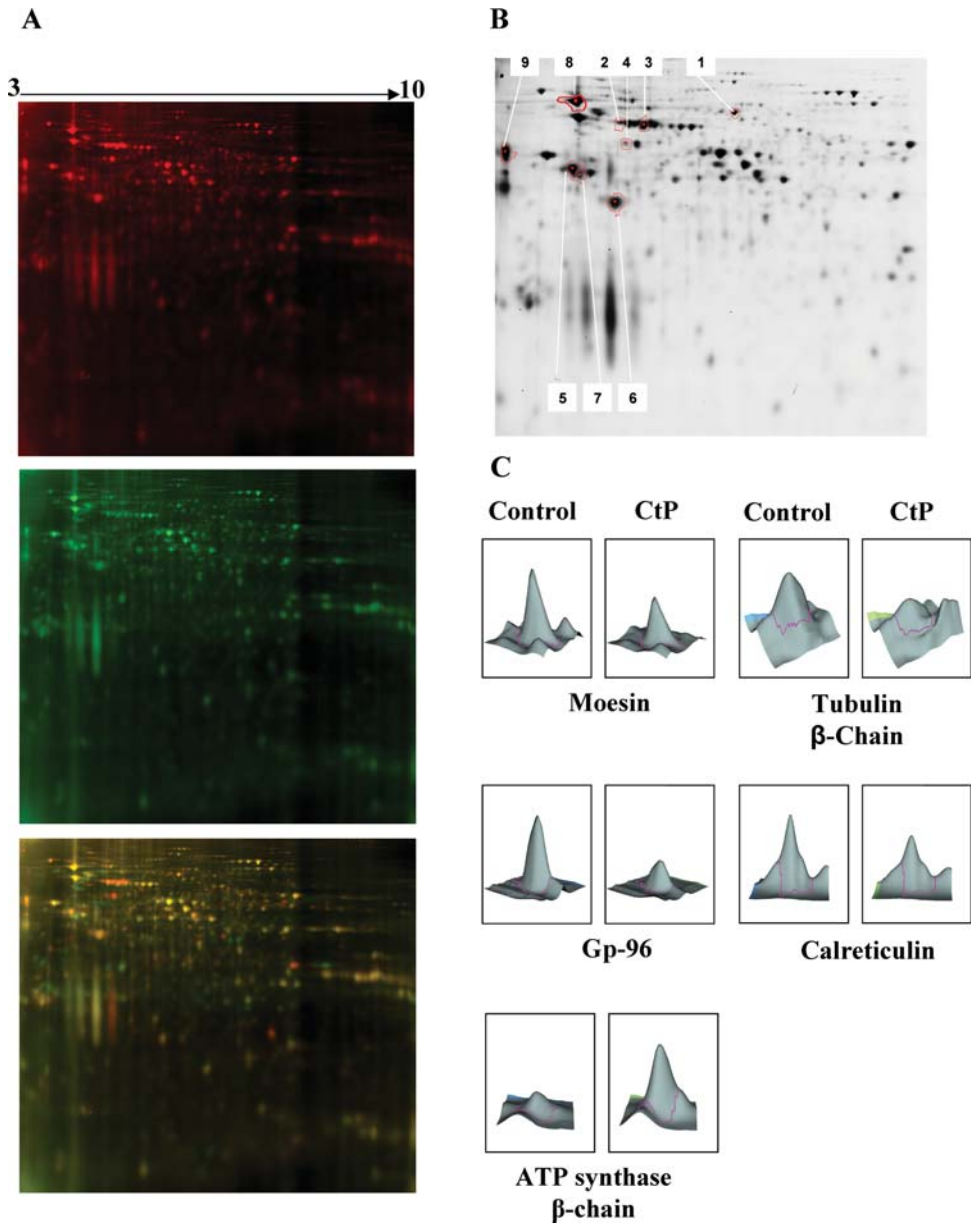

Figure 1 2D-DIGE analysis of cathepsin P-mediated proteolysis of rat trophoblast extracts.

One example of the 2D gels is shown in (**A**). Control Rcho-1 proteins incubated for 6 h at 37°C in the absence (upper panel) or presence of cathepsin P (middle panel) labeled with Cy 3 (red) and Cy 5 (green), respectively. The merged images (lower panel) show protein spots that are reduced (red) or increased (green) in relative abundance due to cathepsin P proteolysis. Yellow spots indicate proteins that are not significantly altered. Red spots are potential substrates, whereas green spots are potential products. A picking gel stained with Gel Code Blue (**B**) shows the nine spots that were identified as *in vitro* substrates for cathepsin P (Table I). Three-dimensional representations of spots of control and digested proteins are shown in (**C**).

Validation of calreticulin as a substrate for cathepsin P

Western blot analysis of cathepsin P digestion of protein extracts of rat Rcho-1 cells using an antibody directed to the C-terminus of calreticulin showed a time-dependent reduction of the intact 58 kDa protein (Fig. 3A). Increasing the concentration of cathepsin P also increased loss of the intact protein (Fig. 3B). Control incubations without added cathepsin P show no loss of immunoreactive protein, demonstrating that endogenous enzymes do not significantly degrade calreticulin in the time frame and assay conditions of these experiments. The time and enzyme concentration-dependent effect on the hydrolysis of the rat protein confirms calreticulin as a substrate for cathepsin P. A lack of appearance of any intermediates indicates that either cathepsin P completely digests calreticulin down to small peptides and amino acids or that cathepsin P processes calreticulin from its C-terminus.

Table 1 Trophoblast proteins processed by cathepsin P *in vitro*

ID	Protein name[2]	Protein ID[2]	MW[3]	PI[3]	*t*-test[4]	Fold change[5]	Subcellular distribution[6]	Mascot score[7]	Coverage (%)[8]
1	Moesin	MOES_RAT	67 565.7	6.2	1.6E−05	↓1.85	Cytoplasm	146	14
2	Heat shock-related 70 kDa	HSP7C_RAT	69 485.6	5.4	6.3E−07	↓2.74	Cytoplasm	400	37
3	Stress-70 protein	GRP75_RAT	73 813.8	6.0	7.6E−06	↓3.05	Mitochondria	291	27
4	60 kDa heat shock protein	CH60_RAT	60 917.4	5.9	1.1E−05	↓3.70	Mitochondria	439	31
5	Tubulin β-5 chain	TBB2A_RAT	49 639.0	4.8	1.2E−04	↓1.87	Cytoplasm	125	22
6	Actin, α skeletal muscle	ACTG_RAT	42 023.9	5.2	1.2E−03	↓1.63	Cytoplasm	295	31
7	ATP synthase β chain	ATPB_RAT	56 318.5	5.2	1.8E−05	↑2.08	Mitochondria	329	33
8	Gp96	EDM17052	92 418.2	4.7	7.5E−06	↓2.18	ER	453	30
9	Calreticulin	CALR_RAT	48 137.0	4.4	1.1E−01	↓1.27	ER	138	32

[2]Substrate proteins are identified by name and Uniprot ID.
[3]MW and pI were calculated from the primary amino acid sequence found in the NCBI database.
[4]*t*-tests were used to determine significantly altered spots on 2D-DIGE by analysis of eight separate samples. Only spots that changed significantly and closely matched predicted MW and pI (with the exception of ATP synthase which is a potential product that migrated faster than predicted for the full-length protein in the SDS dimension) are shown.
[5]Average fold decrease (or increase) in proteins are indicated.
[6]Cellular locations of each protein reported in the Swiss Atlas protein database are listed.
[7]Mascot scores for MS data are listed.
[8]Percent total protein sequence identified are also listed.

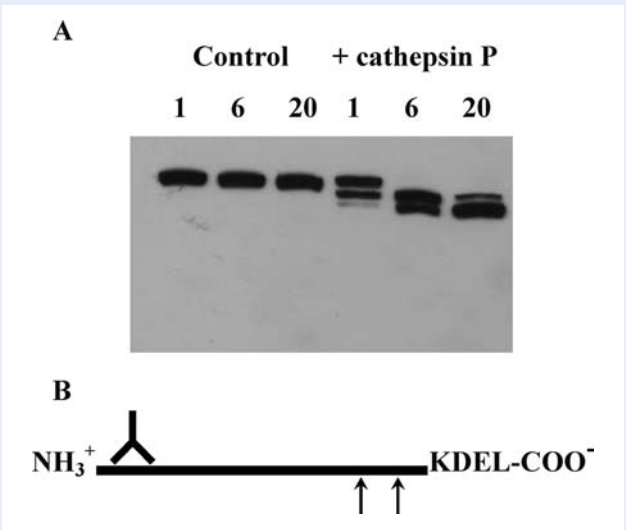

Figure 2 Gp-96 proteolysis by cathepsin P.
Western blotting of time-dependent hydrolysis of Rcho-1 proteins by cathepsin P using an antibody to Gp-96 shows sequential processing to a 72 kDa product via a 84 kDa intermediate after digestion for 1, 6 and 20 h (**A**). No processing is observed in the control samples. Analysis of the primary sequence of rat Gp-96 reveals two hydrophobic domains (arrows) that are potential cleavage sites for cathepsin P that would yield products of the observed molecular weights (**B**). The antibody can recognize C-terminally processed products.

The latter process was revealed by western blotting using an antibody directed to the N-terminus of calreticulin (Fig. 3C). The time-dependent experiments revealed four distinct proteolytic products of sizes ranging from 52 to 34 kDa. Total immune-reactive calreticulin was not significantly diminished over 24 h. Native calreticulin consists of three distinctive domains: a globular N-domain, a proline-rich central P-domain and a Glu/Asp-rich C-domain (Ellgaard *et al.*, 2001; Hojrup *et al.*, 2001). The N- and P-domains constitute the chaperone functions of calreticulin, whereas the C-domain is a high-capacity Ca^{2+}-binding domain (Michalak *et al.*, 1999). At the C-terminus, there is a KDEL ER retention signal. The sizes of the cleavage products indicate that processing occurs in the P-domain and at the start of the C-domain. Cleavage sites are difficult to predict from primary amino acid sequence but there is one hydrophobic region at the beginning of the C-domain, FAVLGL, that may be cleaved by cathepsin P. However, there are no long stretches of hydrophobic amino acids at other sites in the C-terminus of calreticulin, indicating that the structure of this protein must allow alternative less specific cleavage by cathepsin P. Nevertheless, as seen for Gp-96, calreticulin undergoes limited processing by cathepsin P from its C-terminus (Fig. 3D).

Topological co-localization of calreticulin and cathepsin P

For calreticulin or Gp-96 to be physiologically significant substrates for cathepsin P, the enzymes and substrates must be physically in the same cellular compartment. We have previously shown that cathepsin P co-localizes with cathepsin B in lysosomes (Hassanein *et al.*, 2007). This co-localization was not 100%, indicating that cathepsin P might also be in other cellular compartments. Immunohistochemistry shows that cathepsin P (red) and calreticulin (green) are largely co-localized in Rcho-1 cells (yellow, Fig. 4).

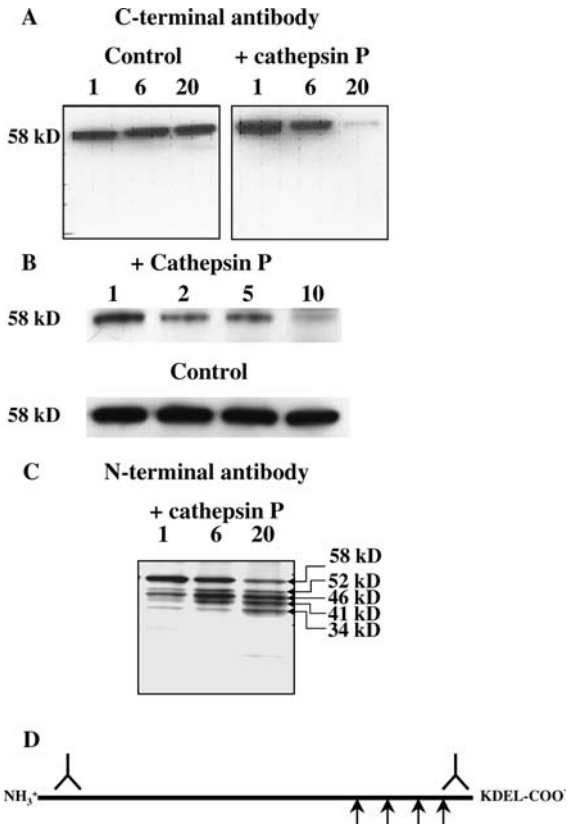

Figure 3 Calreticulin proteolysis by cathepsin P.
Western blotting of time-dependent (1, 6 and 20 h) hydrolysis of Rcho-1 proteins by cathepsin P using an antibody to the C-terminus of calreticulin showed a time-dependent reduction of full-length calreticulin (**A**). No detectable proteolysis of calreticulin was observed in the absence of cathepsin P. The amount of full-length calreticulin was also reduced by increasing amounts of cathepsin P (**B**). Relative amounts of cathepsin P added to a constant amount of cellular protein are indicated above each lane, 1 representing a protein/enzyme ratio of 250:1. Probing of similar blots using an antibody to the N-terminus of calreticulin shows time-dependent (1, 6 and 20 h) appearance of discreet proteolytic fragments with of 34, 41, 46 and 52 kDa (**C**). A schematic view of processing of calreticulin is shown in (**D**). Representative gels of at least three separate experiments are shown.

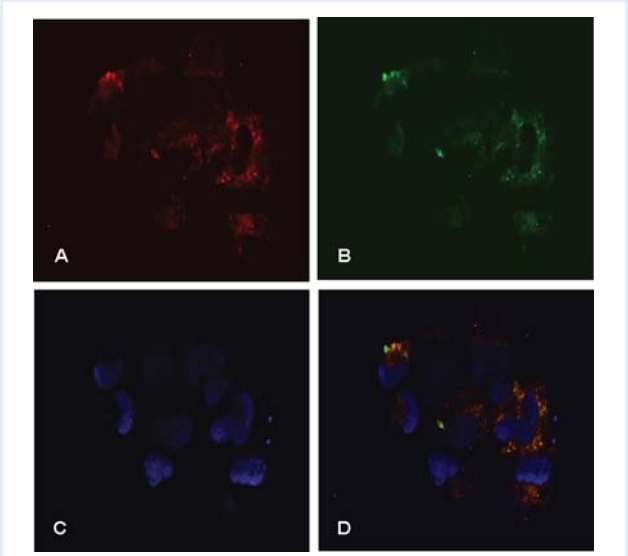

Figure 4 Cellular co-localization of cathepsin P and calreticulin in Rcho-1 cells.
Rcho-1 cells were fixed, permeabilized and incubated with rabbit anti-cathepsin P and sheep anti-calreticulin-specific antibodies. Species-specific fluorescent antibodies (Texas Red-anti-rabbit IgG and FITC-anti-sheep IgG) were used to identify the primary IgGs. Calreticulin is shown as green (**B**) and cathepsin P as red (**A**). Dapi stain was used as a nuclear marker (**C**). Merged images are shown in (**D**).

Calreticulin promotes trophoblast differentiation to a giant cell phenotype

Calreticulin is well established as an ER chaperone but it is also proposed to have extracellular functions. Calreticulin is secreted by an Epstein–Barr virus immortalized human B cell line and the protein was shown to inhibit proliferation and adhesion of endothelial cells of the vasculature, both *in vitro* and *in vivo* (Pike *et al.*, 1998; Yao *et al.*, 2002). The mechanism by which the protein is secreted or how the protein inhibits proliferation and adhesion of the endothelial cells is not yet established, but the protein (also termed vasostatin) is a promising regulator of angiogenesis (Ma *et al.*, 2007). The vasostatin properties of calreticulin reside in the N-terminal portion of the protein and both intact and truncated forms of the protein are active *in vitro* (Pike *et al.*, 1999). We discovered that bovine calreticulin induces differentiation of Rcho-1 cells into a giant cell phenotype in the presence of media that normally maintains cells in a proliferative state (Fig. 5). Although the undifferentiated cells have an angular shape and proliferate *in vitro*, the calreticulin-treated cells are much larger and have an enlarged nuclei, multiple nucleoli and appear very granular. These cells are indistinguishable from cells differentiated by removal of growth-factor-rich media (Fig. 5B). Cell numbers continue to increase in undifferentiated cells, whereas cell growth is impaired in cells differentiated by calreticulin treatment or growth factor withdrawal (Fig. 5E). RT–PCR shows that expression of giant cell differentiation markers, cathepsin P, placental lactogen, hand1 and cathepsin 1 are all increased in the treated cells (Fig. 5F).

Discussion

Cathepsin P is a cysteine protease that is expressed exclusively in placenta (Sol-Church *et al.*, 1999). mRNA for this gene is detected in trophoblasts of the ectoplacental and expression levels increase in a developmentally dependent manner toward the end of fetal development (Sol-Church *et al.*, 1999; Deussing *et al.*, 2002). Rcho-1 cells are a well-established model for differentiation of trophoblast stem cells into a giant cell phenotype (Faria and Soares, 1991). Differentiation of these cells is associated with up-regulation of cellular levels of mRNA and enzymatic activity of cathepsin P, making these cells a valuable model system to dissect the biological function of cathepsin P (Hassanein *et al.*, 2007).

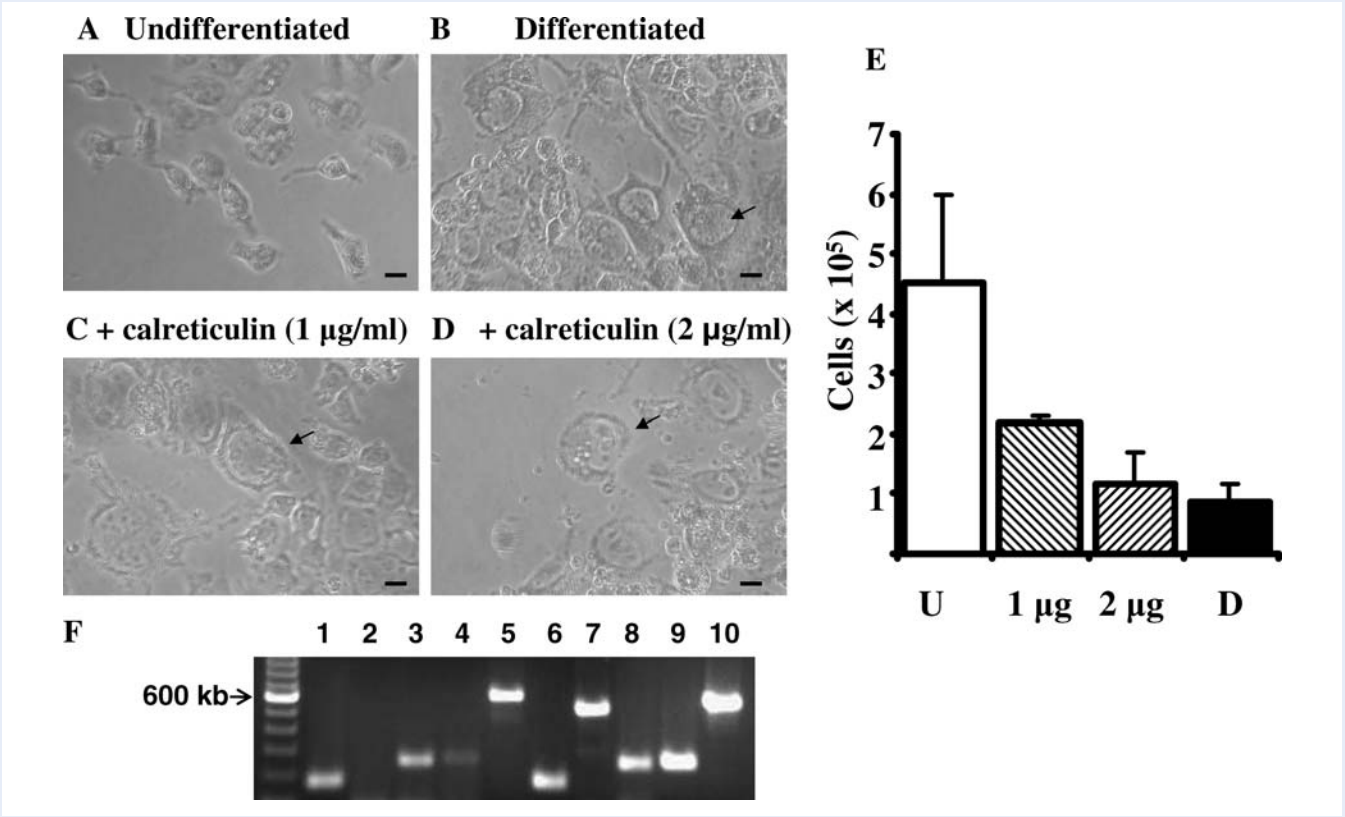

Figure 5 Calreticulin-induced Rcho-1 differentiation.
Phase contrast images of undifferentiated Rcho-1 cells (**A**), differentiated cells (**B**) and cells treated with calreticulin (**C** and **D**) are shown. Size bars are 10 µm. Cells were seeded at 10^5 per well of 12-well plates. Cells were counted and shown as mean with error bars showing standard deviations (**E**) for undifferentiated cell (U), differentiated cells (D) and calreticulin-treated cells (1 and 2 µg). Cell numbers were significantly reduced ($P < 0.05$, Student's *t*-test) when treated with calreticulin or differentiation media compared with controls. This experiment is representative of three separate experiments that gave similar results. Expression of actin and markers of cell differentiation were determined for control cells and cells treated with 2 µg of calreticulin for 6 days (**F**). Lanes 1–5 show RT–PCR products from control cells and lanes 6–10 show products from treated cells. Lanes 1 and 6, actin; lanes 2 and 7, cathepsin P; lanes 3 and 8, placental lactogen; lanes 4 and 9, hand1; lanes 5 and 10, cathepsin 1. A 100 bp ladder is shown on the left.

Our first approach was to perform a proteomic analysis of the major proteins extracted from Rcho-1 cells that are sensitive to hydrolysis by cathepsin P. Although a total cell extract was hydrolyzed with cathepsin P under optimal conditions for enzyme activity, relatively few proteins were degraded during a 6 h treatment. Our earlier work with peptide libraries of substrates and inhibitors indicated that cathepsin P would have a restricted specificity (Puzer *et al*., 2005; Hassanein *et al*., 2007), but this proteomic study provides the first evidence that cathepsin P has a restricted specificity for cellular proteins. Despite the marked preference of cathepsin P for peptidyl substrates and inhibitors that contain hydrophobic amino acids, several of the cleavage sites in calreticulin are in the very hydrophilic C-terminal end of the protein. Cleavage specificity for linear peptides is not a good predictor for processing of native proteins and most hydrophobic regions of proteins are not accessible to proteases. In contrast to its closest human homolog, cathepsin L, it appears that the degradome of cathepsin P will be quite limited (Kirschke *et al*., 1982; Lopez-Otin and Overall, 2002). Although cathepsin L has a restricted activity against some native proteins (Johnson *et al*., 1986), it has a very broad specificity and can degrade proteins down to small peptides and amino acids (Barrett and Kirschke, 1981). Low abundance protein substrates are likely to be missed by proteomic screens and several substrates in total cell homogenates will not be physiologically relevant if they do not encounter the enzyme in living tissues. Nevertheless, this proteomic screen has shown that two potentially significant protein substrates undergo limited processing by cathepsin P.

Placental tissue contains many proteases and endogenous proteases, which cause rapid degradation of calreticulin (see Supplementary Fig. 1). Neither a cocktail of broad-based inhibitors nor a specific inhibitor of cathepsin P could arrest this proteolysis, indicating that many proteases can degrade this protein. The identity and physiological significance of this proteolysis is not known, but this precluded placental tissue as a source of protein to determine specificity of cathepsin P.

The co-localization of cathepsin P with calreticulin indicates that cathepsin P may function in the ER as a processing enzyme. Such a location for a lysosomal enzyme is unusual but not without precedent; cathepsin W is predominantly localized in the ER (Wex *et al*., 2001). Unlike other lysosomal cathepsins, the pro-peptide of cathepsin P is

processed at neutral pH and the enzyme is optimally active at neutral pH, so it is ideally suited for an ER function (Mason *et al*., 2004).

Gp-96 (also known as GRP94) processing revealed only two cleavage products, indicating that cathepsin P processes this protein in a limited, sequential fashion. Endogenous cathepsins B and L rapidly degrade this protein down to small peptides and amino acids at pH 5.5 (M.H. and R.W.M., unpublished data). Processing of Gp-96 by cathepsin P is from the C-terminus, cleaving within the dimerization domain of this protein (Facciponte *et al*., 2006). This C-terminal processing would remove the KDEL ER retention signal, allowing release of Gp-96 from the cell. Extracellular Gp-96 acts as a potent adjuvant, eliciting anti-tumor immunity but a role in placental function is not known (Srivastava *et al*., 1986; Facciponte *et al*., 2006).

Calreticulin is expressed at its highest level in the definitive placenta and appears to play an important role in trophoblast maturation and embryonic development (Hershberger and Tuan, 1999; Su *et al*., 2004). The C-terminal domain of calreticulin is sensitive to proteolysis and can be degraded by a range of digestive serine proteases (Hojrup *et al*., 2001). Genetic deletion of the C-terminal domain and KDEL signal of calreticulin has been shown to stimulate secretion of this protein from transfected cells (Sonnichsen *et al*., 1994). Although intracellular processing of calreticulin would be expected to impair the chaperone and calcium-binding functions of the protein (Hershberger and Tuan, 1998; Wainwright *et al*., 1998), the function of the secreted processed calreticulin may be more significant.

Our data indicate that proteolytic processing by cathepsins may be an important mediator of calreticulin secretion by the placenta. Calreticulin released by removal of its KDEL ER retention signal could induce differentiation of trophoblasts in an autocrine fashion. This differentiation would further induce expression of cathepsin P (Hassanein *et al*., 2007) to create a feedback loop to enhance efficiency of differentiation of adjacent cells in a paracrine fashion to ensure coordinated differentiation of trophoblast cells invading the maternal uterus during placentation. Differentiation of primary trophoblast to highly invasive and phagocytic giant cells is critical during implantation into the maternal decidua during early gestation (Zybina *et al*., 2000).

The differentiated giant cells secrete a wide array of angiogenic factors and vasodilators (Cross *et al*., 2002) to promote continuous blood flow from maternal arteries throughout fetal development. During the latter half of pregnancy, endovascular trophoblast giant cells target the endothelial cells lining the maternal arteries, replacing them and enhancing vasodilatation. Calreticulin could contribute to this arterial remodeling as it disrupts endothelial cell attachment to laminin of basement membranes (Yao *et al*., 2002). The released endothelial cells can then be replaced by trophoblast giant cells to promote vasodilation and enhanced placental blood supply. This contrasts with the anti-angiogenic action of calreticulin that impairs endothelial cell proliferation (Pike *et al*., 1998). Calreticulin levels are increased 5-fold in plasma of pregnant women and are further elevated in plasma from women with pre-eclampsia, leading to a proposal that the anti-angiogenic function of this protein may be a cause of increased blood pressure in this disorder (Gu *et al*., 2008). A significant proportion of calreticulin isolated from human placenta is devoid of six C-terminal amino acids, indicating proteolytic removal of the KDEL ER retention signal (Hojrup *et al*., 2001). In rodents, cathepsin P may be the protease that regulates placental release of calreticulin to promote placental blood flow without elevating maternal blood pressure. Other members of the cathepsin family are also expressed in trophoblast giant cells, such as cathepsin I in this study, but these enzymes have not yet been characterized, so cathepsin P may not be the only enzyme that can process calreticulin in placenta. These or other uncharacterized proteases may contribute to calreticulin processing in the mature placenta (see Supplementary Fig. 1). There is no direct placenta-specific ortholog of cathepsin P in the human genome, but a functional ortholog may be a less specialized enzyme such as cathepsin L.

In summary, we have used a proteomic approach to identify Gp-96 and calreticulin as protein substrates for cathepsin P. The study indicates that cathepsin P has limited proteolytic activity and acts as a processing enzyme, unlike its closest human homolog, cathepsin L. Processing of Gp-96 and calreticulin by cathepsin P removes the C-terminal KDEL ER retention signal that will permit secretion of the proteins during differentiation of Rcho-1 cells into a giant cell phenotype. The secreted calreticulin may enhance this differentiation and promote placental vasodilation. Secreted calreticulin may also play a role in the maternal vasoconstriction that causes pre-eclampsia, indicating that proteolytic-mediated release of this protein must be tightly regulated. Detailed comparative studies are required to determine whether proteolytic-mediated release of calreticulin in this rodent system provides a model to study the role of calreticulin in human physiology and pathology.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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