Expression of functional recombinant human procathepsin B in mammalian cells

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Cathepsin B has been implicated in numerous pathobiological processes. In order to study its interactions with other proteins implicated in these processes, quantities of functional recombinant cathepsin B are needed. Therefore, we expressed recombinant human procathepsin B in mammalian cells (BSC-1 monkey kidney cells and HeLa human cervical carcinoma cells) using a vaccinia virus expression system. The recombinant human procathepsin B appeared to be authentic and expressed in its native conformation as indicated by: (1) N-terminal sequencing; (2) molecular size; (3) processing intracellularly to mature double-chain cathepsin B; (4) *in vitro* cleavage by pepsin to mature

cathepsin B coincident with appearance of activity against a selective synthetic substrate; and (5) substrate/inhibitor profiles. This is the first report of the expression of functional recombinant human procathepsin B in mammalian cells. We also report a single-step immunoaffinity purification procedure for the isolation of electrophoretically pure proenzyme. By the methodologies described, human procathepsin B can now be obtained in high yield. This should facilitate studies of its interactions with protease inhibitors, other proteases, extracellular matrices, cell-surface proteins and biological substrates that may be of relevance to the pathobiological functions of this enzyme.

INTRODUCTION

The lysosomal cysteine protease cathepsin B has been implicated in many pathobiological processes. Among these are tumour progression (for review, see [1]), hormone-induced tissue regression and apoptosis [2], myelocyte differentiation/macrophage activation [3,4], Alzheimer's disease [5], arthritis [6], antigen processing [7], cartilage degradation [8] and osteoclastic bone resorption [9]. In cancers, there are increases in the expression of cathepsin B at the mRNA, protein and activity levels as well as alterations in trafficking, localization and secretion of this enzyme (for a review, see [1]). The 5'-untranslated and translated regions of the cathepsin B gene are alternatively spliced, potentially altering the molecular forms expressed and the levels of expression [10,11]. Although transcriptional mechanisms for altering the expression of cathepsin B have not been identified, increased expression of cathepsin B in human tumours has been associated with malignant progression and/or poor prognosis (for a review, see [1]). Nevertheless, we know little about how either procathepsin B or mature cathepsin B might function in malignant progression or in other pathobiological processes.

We were interested in expressing functional human procathepsin B in amounts sufficient to facilitate study of interactions of this enzyme with protease inhibitors, other proteases, extracellular matrices, cell-surface proteins and biological substrates. Functional procathepsin B has not been produced with *Escherichia coli* expression systems [12,13] although Kuhelj et al. [13] have recently reported successful renaturation. Activatable forms of both rat and human procathepsin B have been produced with a yeast expression system [14,15]. The yeast expression system, however, has limitations as the procathepsin B is expressed as an α -factor fusion protein and is heterogeneously glycosylated. This

heterogenous glycosylation has been reported to reduce enzymic activity [14].

Vaccinia virus expression systems have proved useful for high levels of expression of genes in mammalian cells [16]. Vaccinia virus expression systems correctly synthesize and process proteins and can be used to study trafficking and localization of proteins, e.g. *ras* and *ras*-related proteins [17]. We report here the first production of functional human procathepsin B in mammalian cells using a vaccinia virus expression system. In addition, we report the development of a single-step immunoaffinity method for purification of human procathepsin B.

EXPERIMENTAL

Materials

The following cell lines were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.): BSC-1 monkey kidney cells, HeLaS3 human cervical carcinoma cells, CV-1 African green monkey kidney cells and TK⁻143B, a human sarcoma cell line lacking the thymidine kinase (TK) gene. The substrates benzyloxycarbonylarginylarginyl-7-amido-4-methylcoumarin (Z-Arg-Arg-NHMec), benzyloxycarbonylphenylalanyl-arginyl-7-amido-4-methylcoumarin (Z-Phe-Arg-NHMec) and L-arginyl-7-amido-4-methylcoumarin (L-Arg-NHMec) were purchased from Enzyme Systems Products (Livermore, CA, U.S.A.); *Nco*I and *Bam*HI restriction enzymes, Lipofectin, Opti-MEM and minimal essential medium (MEM) from GIBCO-BRL (Grand Island, NY, U.S.A.); methionine-free MEM from Quality Biological (Gaithersburg, MD, U.S.A.); [³⁵S]methionine (800–1000 Ci/mmol) from DuPont-New England Nuclear (Boston, MA, U.S.A.); peptide-*N*²-(*N*-acetyl- β -glucosaminyl)-asparagine amidase (PNGase F) and endoglycosidase H (endo H) from Boehringer-Mannheim (Indianapolis, IN, U.S.A.);

Abbreviations used: BUdR, 5-bromo-2'-deoxyuridine; E-64, 1-L-*trans*-epoxysuccinyl-leucylamido-3-(4-guanidino)butane; endo F or H, endoglycosidase F or H; L-Arg-NHMec, L-arginyl-7-amido-4-methylcoumarin; MEM, minimal essential medium; NP-40, Nonidet P-40; p.f.u., plaque-forming units; PNGase F, peptide-*N*²-(*N*-acetyl- β -glucosaminyl)-asparagine amidase; TK, thymidine kinase; Z-Arg-Arg-NHMec, benzyloxycarbonylarginylarginyl-7-amido-4-methylcoumarin; Z-Phe-Arg-NHMec, benzyloxycarbonylphenylalanylarginyl-7-amido-4-methylcoumarin.

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tunicamycin from Fluka (Ronkonkoma, NY, U.S.A.); aprotinin, Brij, 5-bromo-2'-deoxyuridine (BUdR), 3-(cyclohexylamino)-1-propanesulphonic acid, 1-L-*trans*-epoxysuccinyl-leucylamido-3-(4-guanidino)butane (E-64), Mops, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid, 2-(*N*-morpholino)ethanesulphonic acid, Nonidet P-40 (NP-40), pepstatin A, porcine pepsin, PMSF, Ponceau S, Triton X-100 and Tween from Sigma (St. Louis, MO, U.S.A.); Protein A-Sepharose, pI markers and precast isoelectric focusing gels from Pharmacia LKB (Piscataway, NJ, U.S.A.); Affigel-10, nitrocellulose membranes and horseradish peroxidase-conjugated goat anti-(rabbit IgG) from Bio-Rad (Melville, NY, U.S.A.); precast tricine gels, tricine/SDS running buffer and poly(vinylidene difluoride) membranes from Novex (San Diego, CA, U.S.A.); the enhanced chemiluminescence Western blot detection system from Amersham (Arlington Heights, IL, U.S.A.); primers for PCR from Operon (Alameda, CA, U.S.A.); and the Wizard DNA Clean-Up system from Promega (Madison, WI, U.S.A.). The source of molecular mass markers depended on the technique being used: for fluorography, ¹⁴C-methylated ovalbumin and carbonic anhydrase were obtained from DuPont-New England Nuclear; for SDS/PAGE, Dalton VII-L were from Sigma; and for immunoblotting, low-range biotinylated SDS/PAGE standards were from Bio-Rad and rainbow markers from Amersham. The monoclonal antibody (DC1) to a propeptide sequence from human procathepsin B was developed by Oncogene Science (Uniondale, NY, U.S.A.). The purification of human liver cathepsin B [18] and the characterization of the rabbit anti-(human liver cathepsin B) serum and IgG [18] and the rabbit anti-(human cathepsin B propeptide) IgG [19] have been described previously. All other chemicals were of reagent grade and were obtained from commercial sources.

Cloning of cDNA and construction of expression vector for human preprocathepsin B

The full-length coding sequence of human preprocathepsin B was amplified by PCR using a human preprocathepsin B cDNA isolated from a gastric adenocarcinoma cDNA library [20] as the template. A 5' oligonucleotide [5'-GGC GCCATGGCT TGG CAG CTC TGG with a recognition sequence for *Nco*I (underlined)], a 3' oligonucleotide [5'-CGGGATCCT TAG ATC TTT TCC CAG TA with a recognition sequence for *Bam*HI (underlined)] and a termination codon were designed based on our published human cDNA sequence [20]. The upstream primer inserts an extra alanine codon (GCT) immediately after the ATG initiation codon. The DNA was amplified in a Perkin-Elmer Geneamp 9600 DNA Thermal Cycler by a PCR cycle (95 °C for 30 s, 58 °C for 30 s, 75 °C for 1 min) repeated 30 times. The amplified full-length cDNA fragment of cathepsin B was digested with *Nco*I and *Bam*HI, purified with the Wizard DNA Clean-Up system and ligated into purified pTF7-EMCV-1 [21] that had been digested with *Nco*I/*Bam*HI. The resulting plasmid containing the cDNA for human preprocathepsin B under the control of the T7 promoter was designated pTF7CB3. The PCR-generated cDNA fragment was sequenced by the dideoxy method [22] to ensure that it was in frame and without any amino acid substitutions.

Vaccinia virus expression of human preprocathepsin B

Virus infection/plasmid transfection

BSC-1 cells (2×10^6 /35-mm-diam. dish) were infected with vTF7-3, a recombinant vaccinia virus containing the bacteriophage T7 RNA polymerase gene [23], at a multiplicity of 30 plaque-forming units (p.f.u.) per cell. The virus was allowed to adsorb

for 30 min and the inoculum replaced with 1 ml of Opti-MEM. The infected BSC-1 cells were then transfected with 10 µg of plasmid pTF7CB3 per dish using Lipofectin according to the directions of the manufacturer.

Isolation of recombinant vaccinia virus containing the human cathepsin B gene

CV-1 cells (2×10^6 /35-mm-diam. dish) were infected with 0.05 p.f.u./cell of wild-type vaccinia virus (strain WR) and transfected with 20 µg of plasmid pTF7CB3 as described above. Forty-eight hours after infection/transfection, the cells were harvested and a crude stock of vaccinia virus was prepared as described [24]. Recombinant viruses containing human preprocathepsin B, designated vTF7CB3, were selected by plaque assay on TK⁻143B cell monolayers in the presence of BUdR (25 µg/ml). TK⁻ recombinant virus plaques were distinguished from spontaneous TK⁻ mutant viruses by DNA dotblot hybridization. After three consecutive plaque purifications, the recombinant viruses were amplified by infecting TK⁻143B cell monolayers in the presence of BUdR, and then were propagated on HeLa cells without selection.

Virus co-infection

Suspension cultures of HeLa cells (7×10^6) were co-infected with a mixture of vTF7-3 and vTF7CB3 at a multiplicity of 5 p.f.u. per cell for each virus. After infection, the cells were cultured for 2 days in Opti-MEM. The media were collected and clarified by centrifugation (28000 g for 120 min at 4 °C). The supernatant was concentrated 10-fold in an Amicon model 8200 concentrator fitted with a YM 10 membrane (Danvers, MA, U.S.A.) and subjected to immunoaffinity chromatography as described below or subjected to SDS/PAGE, transferred to nitrocellulose membranes and analysed by immunoblotting with a rabbit anti-(human liver cathepsin B) IgG.

Isolation of recombinant human procathepsin B by immunoaffinity chromatography

Using a monoclonal antibody generated to a propeptide sequence of human procathepsin B (DC1), an immunoaffinity column was prepared by coupling the monoclonal antibody to Affi-Gel 10 (particle size 70–300 µm) overnight at 4 °C with gentle rotation in the presence of 100 mM Mops, pH 7.5. All subsequent steps were carried out at 4 °C. The buffer was removed by centrifugation at 1000 g for 2 min and the active ester residue blocked by incubation with 1 M ethanolamine, pH 8.0 (0.1 ml/ml of gel), for 1 h with rotation. The gel was then packed in a small column (1.6 cm × 20 cm) and equilibrated with loading buffer [10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulphonic acid (pH 7.2), 150 mM NaCl, 10 % (w/v) glycerol and 0.5 mM PMSF] at a flow rate of 25 ml/h. Concentrated medium from co-infected HeLa cells was applied over the affinity resin and recycled a second time. The unbound fraction was collected and the column washed with loading buffer at a flow rate of 0.5 ml/min until the A_{280} returned to zero. Bound procathepsin B was eluted with 100 mM glycine, pH 3.0, containing 0.5 mM PMSF. Twenty fractions of 1 ml were collected and neutralized to pH 7.4 with 1 M phosphate buffer, pH 8.5; the fractions were pooled and concentrated to a volume of 1–2 ml as described above and stored at –80 °C. The purity of the recombinant procathepsin B was confirmed by SDS/PAGE under reducing conditions followed by silver staining according to the method of Merrill et al. [25].

Characterization of recombinant human procathepsin B

Metabolic labelling and immunoprecipitation

Four to five hours after transfecting BSC-1 cells (2×10^6 /6-cm-diam. dish) with pTF7CB3 or co-infecting HeLa cells (2×10^6 /35-mm-diam. dish) with a mixture of vTF7-3 and vTF7CB3 (at a multiplicity of 5 p.f.u. per cell for each virus), methionine-free MEM containing dialysed 1% (v/v) fetal bovine serum was substituted for Opti-MEM for 15 min to starve the cells of methionine. The cells were then metabolically labelled with 50 μ Ci/ml [35 S]methionine for 18 h. Media were collected and clarified by centrifugation at 1000 *g* for 10 min. Cells were harvested with a lysis buffer [0.5% SDS, 50 mM Tris (pH 9.0), 100 mM NaCl and 2 mM EDTA], boiled for 3 min and the supernatant collected by centrifugation at 7800 *g* for 10 min at 4 °C. Media and cell lysates (50 μ l) were diluted to a final volume of 250 μ l with immunoprecipitation buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.05% Brij, 1 mM PMSF and 10 μ g/ml aprotinin] and incubated with 20 μ l of rabbit anti-(human liver cathepsin B) serum at 4 °C overnight. Antigen-antibody complexes were bound to Protein A-Sepharose, eluted with sample loading buffer (0.1 M Tris buffer, pH 6.8, containing 3% SDS, 20% glycerol and 0.002% Bromophenol Blue) and after boiling for 3 min were analysed by SDS/PAGE under reducing conditions and visualized by fluorography.

Immunoblotting

Media and cell lysates of HeLa cells co-infected with both vTF7-3 and vTF7CB3 were prepared as described above. Samples were electrophoresed on SDS/polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes. The membranes were developed with an enhanced chemiluminescence Western blot detection system using reconstituted dried milk (10%) and Tween as blocking agents, rabbit IgG raised against human liver cathepsin B or against a synthetic peptide derived from the N-terminus of the propeptide of human cathepsin B as the primary antibody, and horseradish peroxidase-conjugated goat anti-(rabbit IgG) as the secondary antibody [18,19].

Isoelectric focusing

Immunoaffinity-purified recombinant human procathepsin B (200 ng) and purified human liver cathepsin B (200 ng) were loaded on precast isoelectric focusing gels (pI range 3.0–9.0) and subjected to isoelectric focusing and silver staining according to the protocol for the Phast System (Pharmacia-LKB).

N-terminus sequence analysis

Immunoaffinity-purified recombinant human procathepsin B was run on 16% tricine/SDS/polyacrylamide gels, electrophoretically transferred to a poly(vinylidene difluoride) membrane (0.2 μ m pore size) using 10 mM 3-(cyclohexylamino)-1-propanesulphonic acid, pH 11.0, and stained with Ponceau S followed by a methanol/water destaining. Direct protein microsequencing from the poly(vinylidene difluoride) membrane was performed by Dr. Jan Pohl, Director of the Microchemical Facility of the Winship Cancer Center of the Emory University School of Medicine (Atlanta, GA, U.S.A.).

Pepsin processing

Media of HeLa cells containing recombinant human procathepsin B were incubated with pepsin (4 μ g/ml) in 50 mM sodium formate buffer, pH 3.0, at 37 °C for time periods from 0 to

90 min and analysed for activity against Z-Arg-Arg-NHMec as described below. Immunoaffinity-purified recombinant human procathepsin B (1 μ g) was incubated with pepsin (0.002 or 0.01 μ g) as described for media. Processing was analysed by separation of molecular forms on SDS/PAGE under reducing conditions, transfer to nitrocellulose membranes and immunoblotting as described above.

Analysis of glycosylation

To inhibit N-linked glycosylation during biosynthesis of cathepsin B, tunicamycin (2 μ g/ml) was added to infected BSC-1 cells immediately after their transfection with pTF7CB3. The cells were metabolically labelled and the molecular forms of cathepsin B immunoprecipitated and visualized by fluorography. To evaluate the types of oligosaccharides, recombinant human procathepsin B was incubated with PNGase F or endo H [18]. Immunoaffinity-purified recombinant procathepsin B (1 μ g) from HeLa cells was incubated for 16 h at 37 °C alone (pH 7.0) or with 600 m-units of PNGase F in 50 mM sodium phosphate buffer (pH 7.0), containing 5 mM EDTA and 0.5% NP40, or with 10 m-units of endo H in 50 mM sodium citrate buffer, pH 5.5. Cathepsin B was then subjected to SDS/12%-PAGE under reducing conditions followed by Coomassie Blue staining.

Determination of activity of recombinant human cathepsin B

The activity of cathepsin B in homogenates and conditioned media from BSC-1 cells was determined in triplicate against the selective substrate Z-Arg-Arg-NHMec (10 μ M final concentration) using our published protocol [26]. Enzyme activity is expressed as nmol of NHMec released/min per 10^5 cells. The presence of latent activity due to expression of procathepsin B was assessed by activation with pepsin (0.05 mg/ml) in 0.1 M formate buffer, pH 3.0, at 37 °C for 15 min [19]; latent activity is expressed as the difference between the total activity of both pro and mature forms of cathepsin B measured in the presence of pepsin and the activity of mature cathepsin B measured in the absence of pepsin. At 48 h after infection/transfection, conditioned media were collected and clarified by centrifugation at 1000 *g* for 10 min at 4 °C. Adherent cells were washed with PBS and harvested with a rubber policeman into prechilled (4 °C) homogenization buffer {25 mM 2-(*N*-morpholino)ethanesulphonic acid (pH 6.5), 0.25 M sucrose and 1 mM EDTA}. The cell suspension was homogenized on ice using 40 strokes of a Dounce homogenizer fitted with a type A pestle. Triton X-100 was added to the homogenate to a final concentration of 0.1%.

Active recombinant human cathepsin B was generated by incubation of immunoaffinity-purified recombinant human procathepsin B (2 μ g) with pepsin (0.01 μ g) at 37 °C for 20 min in the presence of 0.1 M formate buffer, pH 3.0, containing 1 mM EDTA. The activation reaction was stopped by adding pepstatin A at a final concentration of 10 μ M. Activities of recombinant human cathepsin B and of purified human liver cathepsin B against three substrates (Z-Arg-Arg-NHMec, Z-Phe-Arg-NHMec and L-Arg-NHMec) were then compared at a final substrate concentration of 10 μ M. Human liver cathepsin B was purified as described previously [18].

RESULTS AND DISCUSSION

Expression of recombinant human procathepsin B in mammalian cells

Human procathepsin B was expressed in BSC-1 cells by infection with a recombinant vaccinia virus containing the bacteriophage

Figure 1 Expression of recombinant human procathepsin B in mammalian cells

Each lane represents 1×10^5 cells; molecular masses (kDa) are shown on the left. (a) Recombinant human procathepsin B was expressed in BSC-1 cells by infection with vTF7-3, a recombinant vaccinia virus containing the bacteriophage T7 RNA polymerase gene, and transfection with pTF7CB3, a plasmid containing a human preprocathepsin B cDNA under the control of the T7 promoter. The biosynthesis, intracellular processing and secretion of recombinant human procathepsin B was followed by metabolic labelling, immunoprecipitation, SDS/12%-PAGE and fluorography of 50 μ l of conditioned media (lanes 1–3) and cell lysates (lanes 4–6). Lanes represent: 1 and 4, control uninfected cells; 2 and 5, cells infected with vTF7-3; and 3 and 6, cells infected with vTF7-3 and transfected with pTF7CB3. (b) Recombinant human procathepsin B was expressed in HeLa cells by co-infection with vTF7-3 and vTF7CB3, a recombinant vaccinia virus containing the human preprocathepsin B gene under the control of the T7 promoter. The biosynthesis and secretion of recombinant human procathepsin B was followed by metabolic labelling, immunoprecipitation, SDS/12%-PAGE and fluorography of 50 μ l of conditioned media (lanes 1–3) and cell lysates (lanes 4–6). Lanes represent: 1 and 4, cells infected with vTF7-3 alone; 2 and 5, cells infected with vTF7CB3 alone; and 3 and 6, cells co-infected with vTF7-3 and vTF7CB3.

T7 polymerase gene (vTF7-3) and transfection with pTF7CB3, a plasmid containing a human preprocathepsin B cDNA under the control of the T7 promoter. A protein of ~ 45 kDa was immunoprecipitated from the conditioned media of the transfected cells by an anti-(human cathepsin B) serum (Figure 1a, lane 3). In the cell lysate, we identified a 26/25 kDa doublet and a 45/43 kDa doublet (Figure 1a, lane 6). These proteins were not observed, under these conditions, in immunoprecipitates of media from untransfected BSC-1 cells (Figure 1a, lanes 1 and 2) or in immunoprecipitates of lysates from untransfected BSC-1 cells (Figure 1a, lanes 4 and 5). Endogenous monkey kidney cathepsin B was not immunoprecipitated by the anti-(human cathepsin B) serum (Figure 1a, lane 4). The 45/43 kDa bands are similar in size to the two bands of procathepsin B observed in pulse-chase studies of metabolically labelled human skin fibroblasts [27]. The

26/25 kDa doublet is comparable to the glycosylated and unglycosylated heavy-chain doublet of purified human liver and sarcoma cathepsin B [18]. The presence of a 26/25 kDa doublet in the lysates of BSC-1 cells suggests that the recombinant human procathepsin B was able to undergo complete processing to the mature double-chain form and, thus, had been correctly folded.

We further characterized the recombinant procathepsin B by immunoblotting of BSC-1 cell lysates with purified IgG fractions of two rabbit anti-(human cathepsin B) polyclonal antibodies. One of the antibodies was raised against purified human liver cathepsin B and recognizes all forms of the enzyme [18,19]; the second was raised against a synthetic peptide sequence at the N-terminus of the human cathepsin B propeptide and recognizes only procathepsin B [19]. The ~ 45 kDa proteins cross-reacted with both antibodies, indicating that they were indeed procathepsin B (results not shown). A 36 kDa protein of unknown origin was immunoprecipitated from the cell lysates of infected and infected/transfected BSC-1 cells (Figure 1a, lanes 5 and 6). This protein was not immunoprecipitated from the uninfected cells (Figure 1a, lane 4), nor was it recognized by either of the anti-(cathepsin B) antibodies (results not shown).

We constructed a recombinant vaccinia virus containing the human preprocathepsin B gene (vTF7CB3) under the control of the T7 promoter and co-infected HeLa cells with this virus and vTF7-3 in order to obtain milligram quantities of procathepsin B for biochemical studies. Co-infection of HeLa cells resulted in expression of procathepsin B in the media and cell lysates as determined by the presence of a 46/43 kDa doublet after immunoprecipitation (Figure 1b, lanes 3 and 6). The 26/25 kDa doublet of mature cathepsin B was not observed in the lysates of HeLa cells. The level of expression of recombinant human procathepsin B in the HeLa cells was ~ 0.2 mg/ 10^8 cells as determined by comparison with known amounts of mature single-chain and double-chain human liver cathepsin B (results not shown). This is comparable with the level of expression of other proteins with vaccinia virus expression systems, e.g. 72 kDa progelatinase A [28]. Our results indicate that most of the recombinant procathepsin B expressed in co-infected HeLa cells was not processed to mature cathepsin B. This may reflect expression of a lysosomal protease in a cancer cell line, as cathepsin D in human breast carcinomas [29] and cathepsin L in malignant transformed fibroblasts [30,31] are primarily secreted as pro forms with little or no enzyme processed intracellularly.

Enzymic activity of recombinant human cathepsin B

Cathepsin B activity

Z-Arg-Arg-NHMec-hydrolysing activity was measured in the conditioned media and cell homogenates of control, infected and infected/transfected BSC-1 cells (Figure 2), before and after activation by pepsin with the difference representing the latent activity (i.e. the activity due to procathepsin B). Prior to treatment with pepsin, there was little activity against the cathepsin B substrate in the media of any of the BSC-1 cells (Figure 2: Media, mature). After treatment with pepsin, there was a 25-fold increase in cathepsin B activity in the media of infected/transfected cells (Figure 2: Media, latent), i.e. in those cells shown by immunoprecipitation to secrete recombinant procathepsin B (cf. lane 3 in Figures 1a and 1b). There was little activity in the media of control and infected cells after treatment with pepsin (Figure 2: Media, latent), indicating that these cells secreted little or no endogenous procathepsin B. There was endogenous cathepsin B activity in the homogenates of control cells (Figure 2: Cells, mature), but this could not be increased by pepsin, suggesting that the endogenous enzyme was in the mature form. The activity

Figure 2 Analyses of cathepsin B activity in BSC-1 cells and conditioned media

Mature activity represents the activity measured against Z-Arg-Arg-NHMec prior to incubation with pepsin, whereas latent activity represents the activity due to procathepsin B (assessed as the difference in activities measured before and after incubation with pepsin). The stippled bars represent activity in control cells; the striped bars in cells infected with vTF7-3; and the open bars activity in cells infected with vTF7-3 and transfected with pTF7CB3. Activity is expressed as nmol of NHMec/min per 10⁵ cells; mean $\pm$ S.D.

of mature recombinant cathepsin B in homogenates of infected/transfected cells was 70 % greater than in homogenates of control BSC-1 cells (Figure 2: Cells, mature) and that of latent or procathepsin B in infected/transfected cells was 200 % greater (Figure 2: Cells, latent). These results support the conclusion drawn from our immunoprecipitation studies that both mature and pro forms of recombinant cathepsin B were present in infected/transfected BSC-1 cells.

Figure 3 Activation of recombinant procathepsin B by pepsin

Concentrated (10 $\times$) medium (2 ml) from co-infected HeLa cells was incubated with pepsin (8 μ g) at pH 3.0 for the indicated time periods at 37 $^{\circ}$ C and then assayed for activity against Z-Arg-Arg-NHMec. Activity is expressed as nmol of NHMec/min per 10⁵ cells.

Figure 4 Molecular forms of recombinant cathepsin B generated by incubation with pepsin

Immunoaffinity-purified recombinant human procathepsin B (1 μ g) was incubated with pepsin (10 ng) at pH 3.0 and 37 $^{\circ}$ C for the time periods indicated. Samples were separated on SDS/12 % polyacrylamide gels, transferred to nitrocellulose membranes and analysed by immunoblotting with a rabbit anti-(human liver cathepsin B) IgG. Molecular masses (kDa) are shown at the left.

Pepsin activation of recombinant human procathepsin B

There is some controversy in the literature as to the enzyme(s) responsible for activation of procathepsin B *in vivo* [15,32]. Nevertheless, procathepsin B secreted into the media of tumour cells can be activated *in vitro* by incubation of the media with pepsin [19,33]. Therefore, we determined whether the recombinant human procathepsin B expressed in mammalian cells with the vaccinia virus system was synthesized in a conformation that could be activated by pepsin. We demonstrate that pepsin was able to cleave recombinant procathepsin B to yield active cathepsin B and protein products of the correct size whether the substrate for pepsin was recombinant procathepsin B secreted into conditioned medium and thus in a complex mixture of other proteins (Figure 3 and results not shown), or was immunoaffinity-purified recombinant procathepsin B (Figure 4 and results not shown). In Figure 3, a time-dependent increase in cathepsin B activity is depicted; this was maximal (1.4 nmol/min per 10⁵ cells) after 30 min of incubation. When immunoaffinity-purified recombinant procathepsin B (see below) was incubated with pepsin, single-chain cathepsin B could be observed within 1 min and both single- and double-chain cathepsin B within 5 min of incubation (Figure 4). The ability of pepsin to generate cathepsin B activity and the appropriate mature forms of cathepsin B indicates that the recombinant enzyme produced with the vaccinia virus expression system was in a native conformation.

Characterization of recombinant human cathepsin B: substrate specificity and inhibition

We compared the activities of immunoaffinity-purified recombinant human cathepsin B and human liver cathepsin B against three synthetic substrates. Cathepsin B had previously been shown to have a greater k_{cat}/K_m against a Z-Phe-Arg substrate than against a Z-Arg-Arg substrate [18]. The activities of recombinant and liver cathepsin B were 40–50 % greater against Z-Phe-Arg-NHMec than against Z-Arg-Arg-NHMec. Activities of both enzymes (tested against the di-Arg substrate) could be inhibited > 99 % by 10 μ M E-64, an irreversible cysteine protease inhibitor often used as an active-site titrant for cathepsin B and other cysteine proteases [34]. Neither enzyme was able to cleave

Figure 5 Reducing SDS/PAGE of recombinant human procathepsin B secreted from co-infected HeLa cells and purified by immunoaffinity chromatography

Molecular masses (kDa) are shown on the left. (a) Samples (20 μ l) from various purification steps were separated on SDS/12% polyacrylamide gels, transferred to nitrocellulose membranes and analysed by immunoblotting with a rabbit anti-(human liver cathepsin B) IgG. Lanes represent: 1, concentrated conditioned medium; 2, unbound fraction; 3, wash fraction; 4, pool of recombinant procathepsin B eluted with glycine; and 5, 25 ng of human liver cathepsin B. (b) Affinity-purified procathepsin B (200 ng) visualized by silver-staining.

the unblocked aminopeptidase substrate L-Arg-NHMec. Thus, the substrate/inhibitor profiles for recombinant human cathepsin B and human liver cathepsin B were similar, again suggesting that the recombinant cathepsin B had been correctly folded.

Immunoaffinity purification of recombinant human procathepsin B

Recombinant procathepsin B bound to an immobilized monoclonal antibody generated against a human cathepsin B propeptide sequence and could be eluted with 100 mM glycine at pH 3.0 (Figure 5a, lane 4). Recovery of recombinant human procathepsin B from concentrated conditioned media of HeLa cells was $\geq 95\%$ (as estimated by quantification of immunoblots) with a yield of ~ 1.0 mg of purified procathepsin B per 5×10^8 cells. Affinity-purified recombinant human procathepsin B ran as a diffuse band of ~ 45 kDa in silver-stained SDS/polyacrylamide gels under reducing conditions (Figure 5b). Our yield of purified recombinant human procathepsin B from HeLa cell medium was an order of magnitude greater (12–13-fold) than has been reported for the yield of purified recombinant human procathepsin B from yeast culture medium by Mach et al. [15]. Our yield was, however, 4–5-fold less than has been reported for that of a proenzyme mutant of rat cathepsin B produced in yeast [35]. The latter is an active-site mutant in which the active-site Cys has been mutated to Ala and thus the expressed enzyme is not functional. Furthermore, the rat and human procathepsin B expressed in yeast are α -factor fusion proteins, carrying N-terminal extensions of four amino acids [35]. We confirmed that the human procathepsin B expressed in mammalian cells was authentic procathepsin B by N-terminal sequence analysis of the first 28 amino acids (positions 18–45) of immunoaffinity-purified recombinant human procathepsin B. Twenty-four amino acids corresponded to those predicted by the cDNA [20]. The N-terminal sequence analysis was unable to distinguish between His and Ala at positions 24, 43 and 45 and predicted that position 41 was either Trp or Cys; the amino acids predicted by the cDNA for these positions were His, Ala, His and Trp, respectively [20]. The vaccinia virus expression of human procathepsin B would appear to be advantageous in that authentic human procathepsin B is expressed, the yields are high and the proenzyme can be purified to homogeneity in a single step by an immunoaffinity method. Furthermore, if one required mature forms of recombinant cathepsin B, the recombinant human procathepsin B can be activated *in vitro* with pepsin or other proteases and active cathepsin B purified by affinity ligand chromatography (results

Figure 6 Isoelectric focusing of recombinant human procathepsin B and human liver cathepsin B

Samples (200 ng each) of immunoaffinity-purified recombinant human procathepsin B and human liver cathepsin B were loaded on precast isoelectric focusing gels (pI range 3.0–9.0) and silver stained. Lanes represent: 1, pI markers from 2.5 to 6.5; 2, recombinant human procathepsin B; 3, mature human liver cathepsin B; and 4, pI markers from 3 to 9.

not shown), a method used for purification of cathepsin B from a wide variety of tissues [18,36]. Thus, with the combination of vaccinia virus expression and immunoaffinity chromatography, one can obtain quantities of native human procathepsin B or cathepsin B.

Isoelectric point of recombinant human procathepsin B

Purified mature cathepsin B has previously been shown to consist of multiple isoforms. For example, crystalline rat liver cathepsin B consists of four isoforms with pIs from 4.9 to 5.3 [37]. The diffuse band observed upon silver staining of immunoaffinity-purified recombinant human procathepsin B suggested that recombinant human procathepsin B may consist of more than one isoform. The number of isoforms and their pI(s) have not been reported for recombinant procathepsin B. By isoelectric focusing, recombinant human procathepsin B was found to consist of multiple isoforms ranging from a pI of 4.0 to a pI of 6.2 (Figure 6, lane 2), whereas purified human liver cathepsin B (i.e. mature cathepsin B) consisted of one main isoform of 4.8 with other minor isoforms ranging from 4.6 to 5.2 (Figure 6, lane 3). Thus, the recombinant human procathepsin B appeared to have additional isoforms in both the more acidic and more basic range. Complicating the interpretation of these data is the finding that the isoelectric points being compared are for isoforms of mature liver cathepsin B and for isoforms of procathepsin B expressed in HeLa cells, a human cervical carcinoma line. Furthermore, the mature human liver cathepsin B was purified from tissue and presumably had high-mannose carbohydrates for targeting to the lysosomes. The recombinant human procathepsin B was secreted into conditioned media and may have acquired complex carbohydrates in the secretory pathway.

Glycosylation of recombinant human procathepsin B

Since there were multiple isoforms of recombinant human procathepsin B and there are three potential N-linked oligosaccharide sites in the human procathepsin B gene [20,38], we determined the contribution of carbohydrate to the molecular size of recombinant human procathepsin B. Procathesin B immunoprecipitated from lysates of infected/transfected BSC-1 cells ran as a single 36 kDa band in cells labelled in the presence of tunicamycin as compared with a 45/43 kDa doublet in cells

Figure 7 Analyses of glycosylation of recombinant human procathepsin B

Molecular masses (kDa) are shown on the left. Immunoaffinity-purified recombinant human procathepsin B (1 μ g) from HeLa cells was digested with endoglycosidases and subjected to SDS/12%-PAGE followed by staining with Coomassie Blue. Lanes represent: 1, control; 2, digestion with endo H; and 3, digestion with PNGase F. The arrowheads in lane 2 indicate the three bands generated by digestion with endo H.

labelled in the absence of tunicamycin (results not shown). Treatment with PNGase F, an endoglycosidase that cleaves N-linked oligosaccharides from the peptide backbone [39], also reduced the molecular size of procathepsin B from 45 to 36 kDa (results not shown and lane 3 in Figure 7). A molecular size of 36 kDa for unglycosylated or deglycosylated procathepsin B would be predicted by the cDNA sequence [20]. In cells treated with tunicamycin, no further processing of unglycosylated recombinant procathepsin B was observed over a 48 h time period. Similar observations have been made for unglycosylated procathepsin B produced in the presence of tunicamycin in human skin fibroblasts [27]. Our results thus verify that N-linked glycosylation of procathepsin B is necessary for intracellular processing, presumably because high-mannose oligosaccharides are required for generation of the phosphomannosyl recognition marker that binds to mannose-6-phosphate receptors for transport to the lysosomes [40].

Recombinant human procathepsin B might be glycosylated on one, two or all three of the putative N-linked glycosylation sites [20,38]. In addition, the nature of the oligosaccharides on any one site may differ from that on another site. Although cathepsin B as a lysosomal enzyme would be expected to have high-mannose oligosaccharides [40], Pagano et al. [38] have reported that tumour procathepsin B secreted into ascites fluid has complex oligosaccharides. To explore this, we analysed the nature of the oligosaccharides on recombinant human procathepsin B affinity-purified from the media of co-infected HeLa cells (Figure 7). Incubation of the recombinant enzyme with endo H, an endoglycosidase that cleaves high-mannose-type oligosaccharides between the two proximal GlcNAc residues of asparagine-linked carbohydrate chains [41] resulted in three molecular mass forms: an undigested form of 45 kDa, an intermediate form of 39 kDa and a deglycosylated form of 36 kDa (Figure 7, lane 2). This suggests that the procathepsin B secreted from HeLa cells was heterogeneously glycosylated: one form with high-mannose-type oligosaccharides, one form with high-mannose-type oligosaccharides on some of the N-linked glycosylation sites, and one form without high-mannose-type oligosaccharides. Pulse-chase studies of the biosynthesis and processing of cathepsin B in normal and *ras*-transformed human breast epithelial cell lines also suggest that procathepsin B

acquires both high-mannose and complex oligosaccharides (M. Sameni and B. F. Sloane, unpublished work). Similar findings have been reported for cathepsin D (e.g. [42,43]), possibly reflecting pathways alternative to the mannose phosphate receptor pathway for targeting of lysosomal proteases to the lysosome [43,44].

Recombinant procathepsin B and pathobiological function of this enzyme

The literature implicating cathepsin B in the pathology of human diseases is growing and thus there is a need to explore the interactions between pro and mature forms of cathepsin B and other molecules that play functional roles in these disease states. Immunofluorescence studies of breast cancer cell lines and *ras*-transformed breast epithelial cells have revealed cathepsin B staining on the surface of these cells [19,45], suggesting that there may be a cell-surface binding protein for cathepsin B. Access to recombinant procathepsin B will facilitate analysis of cell-surface binding. Recombinant procathepsin B will also be useful as an antigen for production and characterization of antibodies suitable for analysis of cathepsin B in tissue samples and biological fluids. Since alternative splicing of the 5' region of the cathepsin B gene can produce a truncated procathepsin B that may be specific to tumours [10], antibodies specific to the truncated propeptide might serve as tumour markers. The studies herein report for the first time the expression of functional authentic human procathepsin B in mammalian cells and in addition report a new method for immunoaffinity purification of procathepsin B. Both should contribute to biochemical studies of procathepsin B and to the production of antibodies with specificities for various molecular forms of cathepsin B.

This work was supported by U.S. Public Health Service grant CA 36481 to B. F. S. W.-P. R. is a National Cancer Institute Cancer Center Oncology Research Faculty Development Fellow. We thank Drs. Clive Dennison and Edith Elliott (University of Natal, Pietermaritzburg, South Africa) and Dr. Kamiar Moin (Wayne State University, Detroit, MI, U.S.A.) for valuable suggestions regarding the manuscript.

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