Probing cathepsin K activity with a selective substrate spanning its active site

Fabien LECAILLE*, Enrico WEIDAUER†, Maria A. JULIANO‡, Dieter BRÖMME† and Gilles LALMANACH*[1]

*INSERM EMI-U 00-10 'Protéases et Vectorisation', Laboratoire d'Enzymologie et Chimie des Protéines, Faculté de Médecine, Université François Rabelais, 2 bis, Boulevard Tonnellé, F-37032 Tours Cedex, France, †Department of Human Genetics, Mount Sinai School of Medicine, New York, NY 10029, U.S.A., and ‡Escola Paulista de Medicina, Universidade Federal de São Paulo, 04044-020 São Paulo, Brazil

The limited availability of highly selective cathepsin substrates seriously impairs studies designed to monitor individual cathepsin activities in biological samples. Among mammalian cysteine proteases, cathepsin K has a unique preference for a proline residue at P2, the primary determinant of its substrate specificity. Interestingly, congopain from *Trypanosoma congolense* also accommodates a proline residue in its S2 subsite. Analysis of a congopain model showed that amino acids forming its S2 subsite are identical with those of cathepsin K, except Leu^{67} which is replaced by a tyrosine residue in cathepsin K. Furthermore, amino acid residues of the congopain S2′ binding pocket, which accepts a proline residue, are strictly identical with those of cathepsin K. Abz-HPGGPQ-EDN$_2$ph [where Abz represents *o*-aminobenzoic acid and EDN$_2$ph (= EDDnp) represents *N*-(2,4-dinitrophenyl)-ethylenediamine], a substrate initially developed for trypanosomal enzymes, was efficiently cleaved at the Gly–Gly bond by cathepsin K ($k_{cat}/K_m = 426\,000$ M^{-1} · s^{-1}). On the other hand, Abz-HPGGPQ-EDN$_2$ph was resistant to hydrolysis by cathepsins B, F, H, L, S and V (20 nM enzyme concentration) and the Y67L (Tyr^{67} → Leu)/L205A cathepsin K mutant (20 nM), but still acted as a competitive inhibitor. Taken together, the selectivity of Abz-HPGGPQ-EDN$_2$ph to cathepsin K primarily depends on the S2 and S2′ subsite specificities of cathepsin K and the ionization state of histidine at P3. Whereas Abz-HPGGPQ-EDN$_2$ph was hydrolysed by wild-type mouse fibroblast lysates, its hydrolysis was completely abolished in the cathepsin K-deficient samples, indicating that Abz-HPGGPQ-EDN$_2$ph can be used to monitor selectively cathepsin K activity in physiological fluids and cell lysates.

Key words: cathepsin, cysteine protease, fluorogenic substrate, trypanosome.

INTRODUCTION

Mammalian lysosomal papain-like CPs (cysteine proteases) have been described traditionally as housekeeping enzymes mostly restricted to non-specific intracellular protein turnover [1]. However, an increasing number of studies using specific inhibitors and/or probes as well as protease-deficient mouse models have demonstrated that these enzymes, including cathepsins B, K, L or S, also fulfil more specific physiological functions in MHC-II antigen presentation, in extracellular-matrix turnover, and in prohormone processing (see [2] for a review). In addition, thiol-dependent cathepsins are involved in a variety of diseases such as inflammation, angiogenesis, tumour metastasis, osteoporosis and rheumatoid arthritis, and represent promising drug targets of clinical interest [3]. However, a major drawback is the lack of highly specific and selective substrates capable of monitoring their individual enzymic activities.

Among mammalian CPs, cathepsin K exhibits the highest capability to degrade components of the extracellular matrix. Although cathepsin K shows a restricted pattern of tissue expression and is predominantly expressed in osteoclasts and multinucleated giant cells [4], it is also found in lung epithelial cells, in cancer prostate cells, in differentiated macrophages or in the serum of Gaucher patients [5–8]. The emerging role of cathepsin K in bone remodelling led us to believe that the development of peptidomimetics as cathepsin K inhibitors would provide new therapeutics to treat osteoporosis [4,9,10]. Despite the recent development of potent drug candidates [2,11], a better knowledge of the physiological role of cathepsin K and its substrate specificity remains of major interest for supporting the design of more efficient cathepsin K inhibitors. The individual monitoring of cathepsin K activity in biological fluids or in tissue extracts is seriously hampered by the lack of a selective substrate. Z-GPR-AMC (where Z stands for benzyloxycarbonyl and AMC for 7-amino-4-methyl-coumarin) shows some selectivity for cathepsin K, but it is also well hydrolysed by cathepsin B, granzyme A, trypsin, thrombin and tryptase [4,12,13]. Analyses of cleavage sites of cathepsin K in type I and II collagens [14,15], as well as profiling of its substrate specificity using positional scanning (P1–P4) tetrapeptide libraries [16], confirmed that cathepsin K exhibits a unique preference for a proline residue at P2 among mammalian cathepsins, which has been attributed to the presence of Tyr^{67} and Leu^{205} in the S2 binding pocket, the major determinant for substrate specificity. Interestingly, CPs from *Trypanosoma cruzi* (cruzipain) and *T. congolense* (congopain), which play a key role in the pathogenicity of the parasites, also accommodate a proline residue in S2 and a proline residue in S2′ [17,18]. We have recently designed an IQF (intramolecularly quenched fluorescence) substrate, namely Abz-HPGGPQ-EDN$_2$ph [where Abz represents *o*-aminobenzoic acid

Abbreviations used: Abz, *o*-aminobenzoic acid; AMC, 7-amino-4-methyl-coumarin; cat K$^{-/-}$, cathepsin K knockout; CP, cysteine protease; C-4S, chondroitin-4 sulphate; DTT, DL-dithiothreitol; E-64, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane; EDN$_2$ph (= EDDnp), *N*-(2,4-dinitrophenyl)ethylenediamine; IQF, intramolecularly quenched fluorescence; KO, knockout; RP, reversed phase; TFA, trifluoroacetic acid; WT, wild-type; Z, benzyloxycarbonyl.

[1] To whom correspondence should be addressed (e-mail lalmanach@univ-tours.fr).

and EDN_2ph (= EDDnp) represents *N*-(2,4-dinitrophenyl)ethylenediamine], which is specifically hydrolysed by the two trypanosomal CPs at the Gly–Gly bond [19].

On the basis of these results and supported by structural analysis of the active sites of congopain and cathepsin K, the aim of the present study was to determine if a fluorogenic substrate, originally designed for trypanosomal CPs, might be a selective substrate for human cathepsin K.

EXPERIMENTAL

Materials

DTT (DL-dithiothreitol), E-64 [L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane] and C-4S (chondroitin-4 sulphate) were obtained from Sigma–Aldrich (St. Quentin le Fallavier, France). Z-FR-AMC and Arg-AMC were purchased from Bachem (Weil am Rhein, Germany). All other reagents were of analytical grade.

Enzymes

Human cathepsins B, L and S were supplied by Calbiochem (VWR International, Pessac, France). WT (wild-type) human cathepsin K and the Y67L ($Tyr^{67} \rightarrow Leu$)/L205A cathepsin K mutant were expressed in *Pichia pastoris* as described elsewhere [16]. Recombinant human cathepsins F and V were produced as reported previously [20,21]. Cathepsin H was prepared from rat liver [22]. Congopain (CP2 type) was purified from *T. congolense* lysates [23]. The activation buffers for enzyme assays were: 0.1 M phosphate buffer (pH 6.0), containing 2 mM DTT and 2 mM EDTA for cathepsins B, H, L, K and S and the Y67L/L205A mutant; 0.1 M acetate buffer (pH 5.5), containing 2.5 mM DTT and 2.5 mM EDTA for cathepsins V and F; and 0.1 M phosphate buffer (pH 6.0), containing 6 mM DTT and 2 mM EDTA for congopain. The molarity of the proteases was determined using E-64 as the active-site titrant [24].

Structural analysis of cathepsin K and congopain

A homology-based model of the catalytic domain of congopain (GenBank® accession no. L25130) was developed previously, using papain, actinidin, papaya protease ω and cruzain as molecular templates [25]. The three-dimensional structure of human cathepsin K corresponded to a pyrrolidinone inhibitor–cathepsin K complex (accession number 1AU3). Both structures were superimposed using the Homology program (INSIGHT II software, Molecular Simulations, San Diego, CA, U.S.A.), as described previously [16]. All calculations were performed on a Silicon Graphics O2 workstation.

Peptide synthesis

Abz-HPGGPQ-EDN_2ph, a substrate with resonance energy transfer properties due to a donor group (Abz) and a fluorescence quencher group (EDN_2ph), was synthesized by fluoren-9-ylmethoxycarbonyl solid-phase peptide synthesis procedure as described by Serveau et al. [19]. A stock solution of 2 mM Abz-HPGGPQ-EDN_2ph, dissolved in dimethylformamide, was stored at − 80 °C until use.

Kinetic measurements

The enzymic activity of 1 nM cathepsin K was followed by monitoring the hydrolysis of Abz-HPGGPQ-EDN_2ph at an excitation wavelength of 320 nm and an emission wavelength of 420 nm (Kontron SFM 25 spectrofluorimeter). Fluorescence calibration was performed as reported previously [17]. Experiments were performed in triplicate at 30 °C in the presence or absence of 0.15 % (w/v) C-4S. Kinetic results were determined by running the Enzfitter software (Biosoft, Cambridge, U.K.) and were reported as means ± S.D.

Second-order rate constants (k_{cat}/K_m) were first measured under pseudo-first-order conditions, i.e. using a substrate concentration far below the K_m. Michaelis constant and catalytic constant values were obtained graphically from a Hanes linear plot using various concentrations of substrate (0.1–10 μM), and the accuracy of K_m and k_{cat} values was confirmed by non-linear regression analysis (substrate concentration: 0.2–6 μM) [26]. The same procedure was applied for 1 nM congopain. Resistance of Abz-HPGGPQ-EDN_2ph to hydrolysis by cathepsins B, F, H, L, S and V and by Y67L/L205A cathepsin K mutant was measured using a 20 nM enzyme concentration.

Determination of inhibitory constant K_i

Cathepsin L (5 nM) was incubated with Abz-HPGGPQ-EDN_2ph (1–100 μM) in the activating buffer at 25 °C for 10 min, in a final volume of 300 μl. The residual enzymic activity was recorded in the presence of 3–10 μM Z-FR-AMC at an excitation wavelength of 350 nm and an emission wavelength of 460 nm. The K_i value was calculated by plotting $1/v$ against inhibitor concentration [I] (Dixon plot). The competitive inhibition of cathepsins B and S and the Y67L/L205A mutant (5 nM each) by Abz-HPGGPQ-EDN_2ph was measured using the same method. In addition, Michaelis constants for 2 nM cathepsin L, 2 nM cathepsin B and 3 nM cathepsin S were also deduced from non-linear regression analysis, using Z-FR-AMC as a substrate (0.5–50 μM) [26].

Reversed phase (RP)-HPLC analysis

Cathepsin K (10 nM) was incubated with 100 μM Abz-HPGGPQ-EDN_2ph in its activation buffer at 30 °C (final volume: 30 μl) for 1 h, before adding 100 μl of ethanol. After removal of the precipitate, the supernatant containing the native peptide and/or its proteolytic fragments was evaporated, and re-dissolved in 0.1 % TFA (trifluoroacetic acid). An aliquot of each sample was fractionated by RP-HPLC on a C_{18} OD-300 column (Brownlee ABI, Weiterstadt, Germany), using a 30 min linear gradient (0–60 %) of acetonitrile in 0.1 % TFA at a flow rate of 0.3 ml/min. Proteolysis products were identified by comparison with intact Abz-HPGGPQ-EDN_2ph, and the elution profiles were analysed by running the ChromQuest chromatography workstation (ThermoFinnigan, Les Ulis, France). Cleavage sites were located by N-terminal sequencing (Procise sequencer, Applied Biosystems). The same procedure was repeated for cathepsins B, L, S and Y67L/L205A (10 nM).

pH–activity profiles

The pH–activity profiles of human cathepsin K and congopain were determined at 0.3 μM substrate concentration ($S < K_m$, where the initial rate v_o is directly proportional to the k_{cat}/K_m value). Measurements were performed using 100 mM NaH_2PO_4/50 mM citric acid buffers (pH range 3.0–8.0), containing 2 mM DTT and 2 mM EDTA for cathepsin K and 10 mM DTT and 2 mM EDTA for congopain.

Activity assays of cathepsin K on cultured fibroblasts

Cathepsin K knockout (cat $K^{-/-}$) mice (C57BL/6J background) were generated by targeted disruption of the cathepsin K gene

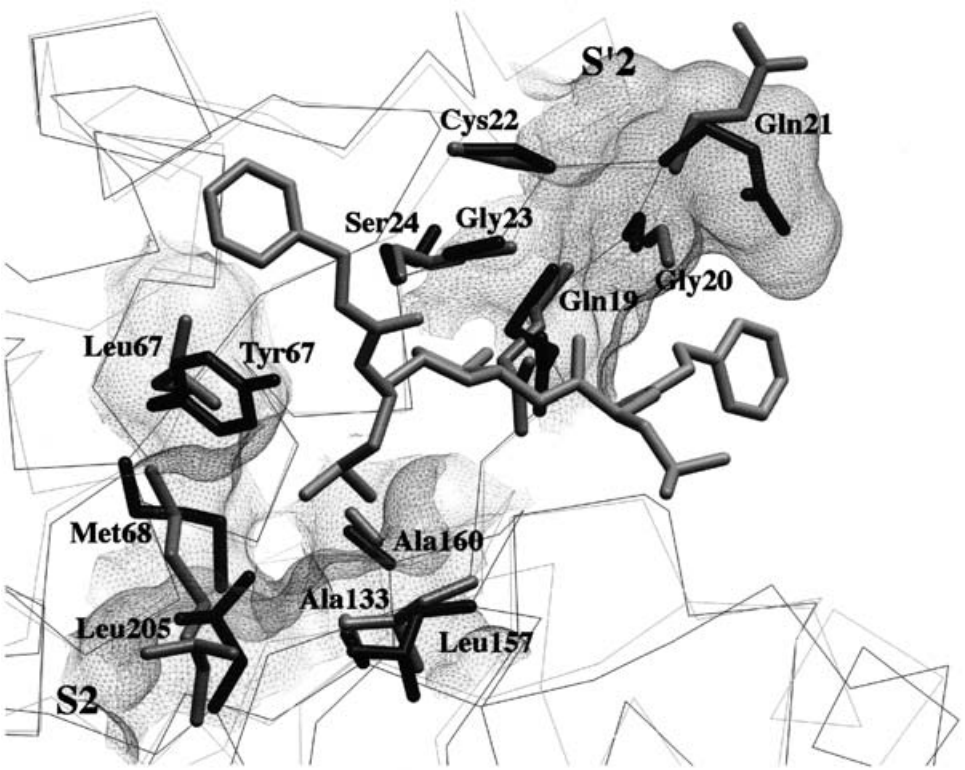

Figure 1 Substrate binding clefts of cathepsin K and congopain

A three-dimensional model of the catalytic domain of congopain [25] was superimposed to the X-ray structure of pyrrolidinone-bound cathepsin K (accession number 1AU3). Only the side chains of residues lining the S2 and S2′ pockets (Connolly surface) are shown (cathepsin K, dark; congopain, light grey). The covalently bound inhibitor (stick representation; dark grey) is shown interacting with the binding sites S3-S3′.

[27] and were kindly provided by Dr Paul Saftig (University of Kiel, Kiel, Germany). Skin specimens (5 mm × 5 mm) were collected from WT and cat $K^{-/-}$ mice. After treatment for 3 h at room temperature (22 °C) with Dispase II (Roche Diagnostics, Mannheim, Germany) and in the presence of 1 % penicillin G, amphotericin B and streptomycin B (Cellgro, Mediatech, Herdon, VA, U.S.A.), samples were placed at 37 °C on 25 cm^2 culture flasks in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD, U.S.A.), containing 1 % penicillin G, amphotericin B and streptomycin B and supplemented with 20 % (v/v) foetal calf serum (Gibco BRL). Cells were grown at 37 °C in an atmosphere containing 5 % CO_2. When cells reached confluence, they were detached with 0.25 % trypsin and divided in the ratio 1:3. The amount of foetal calf serum was then decreased to 10 %. Cell monolayers were washed twice with PBS, trypsinized, resuspended in PBS and washed again twice before centrifugation at 800 ***g*** for 10 min at 4 °C. Finally, cell lysates were prepared in 100 mM sodium acetate buffer (pH 5.5), containing 2.5 mM DTT, 2.5 mM EDTA, 10 μM PMSF and 10 μM pepstatin. After centrifugation at 13 000 ***g*** for 10 min at 4 °C, protein concentrations in the supernatants of WT and knockout (KO) cells were determined using the Bradford kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Extracted proteins (1 μg) were resolved by SDS/PAGE (10 % gel) and transferred on to PVDF membranes (Millipore, Bedford, MA, U.S.A.). Non-specific binding was blocked by 2 h incubation in PBS/0.1 % Tween 20, containing 5 % (v/v) non-fat dried milk. The membranes were incubated overnight with a rabbit polyclonal antibody directed against mouse cathepsin K (1:1000 in PBS/0.1 % Tween 20) as described previously [4]. The membranes were then incubated with a goat anti-rabbit IgG(H + L)–peroxidase conjugate (Southern Biotechnology Associates, Birmingham, AL, U.S.A.) for 1 h, before detection using the chemiluminescent ECL® kit (Amersham Biosciences, Little Chalfont, Bucks., U.K.).

Residual enzymic activity of lysate supernatants (10 μg of protein/assay) was monitored using 5 μM Z-FR-AMC as the substrate at 25 °C in 100 mM sodium acetate buffer (pH 5.5), containing 2.5 mM DTT and 2.5 mM EDTA. Enzymic activities towards 5.6 μM Abz-HPGGPQ-EDN_2ph were determined under the same conditions, except that 50 μg of protein/assay was used.

RESULTS AND DISCUSSION

Comparative structural analysis of cathepsin K and congopain

The substrate specificity of papain-like CPs primarily depends on P2–S2 interactions. Most of them exhibit a marked preference for bulky hydrophobic or aromatic residues at P2, whereas cathepsin K preferentially accommodates a proline residue at its S2 binding site [16]. Similarly, we have reported previously that both cruzain, the major CP of *T. cruzi* (the etiological agent of Chagas disease in South America), and congopain, its homologue in *T. congolense*, accept a proline residue in the S2 pocket [19]. The catalytic domain of congopain shares more than 65 % similarity in the sequence with cathepsin K and has structural features similar to that of cathepsin K. Amino acids constituting the congopain S2 binding pocket are identical with those of cathepsin K, with the noticeable exception of Leu^{67}, which is replaced by a tyrosine residue in cathepsin K (Figure 1). In addition, constitutive residues

Table 1 Kinetic parameters for the hydrolysis of Abz-HPGGPQ-EDN$_2$ph and Z-GPR-AMC

Experiments were performed as described in the Experimental section. K_m and k_{cat} values for Abz-HPGGPQ-EDN$_2$ph hydrolysis were determined by non-linear regression analysis. n.d., not determined.

	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	
Enzyme	Abz-HPGGPQ-EDN$_2$ph	Z-GPR-AMC*
Cathepsin K	426 000 $K_m = 1.2 \pm 0.2$ μM $k_{cat} = 0.5 \pm 0.03$ s^{-1} 285 000 $\pm$ 43 000†	117 600 $K_m = 68 \pm 21$ μM $k_{cat} = 8 \pm 1.8$ s^{-1}
Cathepsin K + C-4S‡	294 000 $\pm$ 2000†	n.d.
Y67L/L205A	nsh§	122‖
Cathepsin B	nsh§	9490
Cathepsin L	nsh§	370
Cathepsin S	nsh§	34
Cathepsins F, H and V	nsh§	n.d.
Congopain	110 000 $\pm$ 6000†	n.d.

* From [4].
† Determined under pseudo-first-order conditions.
‡ 0.15 % (w/v) C-4S [28].
§ nsh, no significant hydrolysis (enzyme concentration 20 nM).
‖ From [16].

of the putative S2′ subsite of congopain (namely Gln[19], Gly[20], Gln[21], Cys[22], Gly[23] and Ser[24]) are strictly identical with those of cathepsin K (Figure 1). The active sites of both enzymes display a very similar structural framework, with the exception of Gln[21] in congopain whose side chain shifts after exposure to solvent. However, this different orientation may not be significant and may have been induced by additional interactions between cathepsin K and its covalently bound peptidyl pyrrolidinone inhibitor (accession number 1AU3).

Specific hydrolysis of Abz-HPGGPQ-EDN$_2$ph by cathepsin K

On the basis of the similar structural properties of cathepsin K and congopain (see above) and previous substrate specificity studies [17–19], the ability of cathepsin K to cleave Abz-HPGGPQ-EDN$_2$ph has been determined. K_m and k_{cat} values were determined from Hanes plots and non-linear regression analysis, whereas the specificity constant (k_{cat}/K_m) was deduced from individual K_m and k_{cat} values and from analysis under pseudo-first-order conditions. Analysis of the second-order rate constant showed that cathepsin K accommodated well Abz-HPGGPQ-EDN$_2$ph in its active site (Table 1) and hydrolysed it approx. 4-fold better than Z-GPR-AMC ($k_{cat}/K_m = 118\,750$ $M^{-1} \cdot s^{-1}$). This was mostly due to a dramatic improvement of affinity (1.2 versus 68 μM). Cleavage occurred at the Gly–Gly bond, similar to that observed with congopain and cruzipain [19]. Michaelis constants are very similar for cathepsin K and congopain. Whereas C-4S, a major component of extracellular matrixes, enhances the collagenolytic activity of cathepsin K and increases its stability at neutral pH [28,29], we found that the presence of C-4S did not modify the specificity constant for Abz-HPGGPQ-EDN$_2$ph (Table 1), as reported already for other small synthetic substrates [28]. In the presence of 20-fold higher amounts of cathepsins B, F, H, L, S and V (20 nM), no detectable release of fluorescence was observed, indicating that Abz-HPGGPQ-EDN$_2$ph was not susceptible to hydrolysis by these enzymes. Furthermore, no cleavage products were detected by RP-HPLC, confirming the resistance of Abz-HPGGPQ-EDN$_2$ph to proteolysis. The serine proteases cathepsin G, chymotrypsin and leucocyte elastase were also unable to cleave Abz-HPGGPQ-EDN$_2$ph, despite their known capability to accommodate a proline residue at P2 [30]. Interestingly, the Y67L/L205A cathepsin K mutant was also unable to hydrolyse Abz-HPGGPQ-EDN$_2$ph. The fact that this mutant excludes the binding of a proline residue at P2 [16] supports the idea that interactions between cathepsin K and Abz-HPGGPQ-EDN$_2$ph critically depend on P2 proline–S2 interactions. On the other hand, cathepsin L, as well as WT and Y67L/L205A cathepsin K [16], accommodates a histidine residue in their S3 binding site, suggesting that this residue at P3 does not play a key role in terms of selectivity for Abz-HPGGPQ-EDN$_2$ph hydrolysis. The presence of a glycine residue at P1 of Abz-HPGGPQ-EDN$_2$ph confirms that the nature of the amino acids in adjacent positions could have an influence on the specificity [19,31]. Interestingly, the IQF substrate displayed striking differences with peptidyl coumarylamide substrates. No cleavage at the Gln-EDN$_2$ph bond was observed, although glycine at P1, proline at P2 and glycine at P3 were reported as favoured residues for cathepsin K [16]. The resistance to cleavage might rely on the influence of the chemical nature of the leaving group [32] or, alternatively, EDN$_2$ph might not fit into the P1′ position. Nevertheless, the present finding emphasizes the important contribution of primed subsites, particularly the S2′ subsite, to define cathepsin K specificity in addition to the primary role of the S2 subsite. The presence of amino acids in the prime position may have synergistic effects of binding at different subsites [31] and affects the specificity on the non-prime side of the scissile bond [16]. Subsequently, results confirm that IQF substrates are more relevant to investigate the substrate specificities of endoproteases and that human cathepsin K exhibits close similarity in substrate specificity for trypanosomal congopain, including a unique preference for proline residues at both P2 and P2′ among mammalian CPs.

Competitive inhibition by Abz-HPGGPQ-EDN$_2$ph

Owing to the presence of a histidine residue with a theoretical pK value close to that of the experimental pH, the influence of the ionization state of the P3 residue on enzymic activity has been studied. The bell-shaped pH–activity profiles of cathepsin K for the hydrolysis of Z-FR-AMC (used as reference) and Abz-HPGGPQ-EDN$_2$ph showed two different trends (Figure 2). Whereas cathepsin K cleaved the non-ionizable Z-FR-AMC substrate over a broad pH range 4.0–8.0, Abz-HPGGPQ-EDN$_2$ph hydrolysis occurred over a narrow pH range (approx. pH 5.5–7.0), with a maximal efficiency at pH 6.0. Compared with congopain (maximal activity at pH 5.0), Abz-HPGGPQ-EDN$_2$ph hydrolysis by cathepsin K shifted to a less acidic pH (ΔpH = 1), suggesting that the histidine residue at P3 might be deprotonated (imidazole form) to allow cleavage. Hydrolysis of Z-FR-AMC by human cathepsins L, B and S and the Y67L/L205A mutant was decreased in the presence of increasing amounts of Abz-HPGGPQ-EDN$_2$ph. Abz-HPGGPQ-EDN$_2$ph inhibited cathepsins B, L and S and the Y67L/L205A mutant with K_i values of 11.3 $\pm$ 0.9, 2.1 $\pm$ 0.2, 0.8 $\pm$ 0.2 and 3.6 $\pm$ 2.0 μM respectively. These K_i values were very similar to the K_m value reported for cathepsin K (1.2 μM), except for cathepsin B, which is by one order of magnitude less efficiently inhibited. Lack of cleavage by cathepsins B, L or S (as shown by RP-HPLC analysis; results not shown) indicates that Abz-HPGGPQ-EDN$_2$ph bound to their active site, leading to a stable Michaelis complex, but the k_{cat} value was impaired. Similarly, it has been reported that the presence of histidine at P3 dramatically decreased the k_{cat} value for papain [33]. In summary, the results suggest that the ionization state of the side chain of histidine at P3 appears to be essential for the catalysis of Abz-HPGGPQ-EDN$_2$ph. Although the substrate has a similar affinity

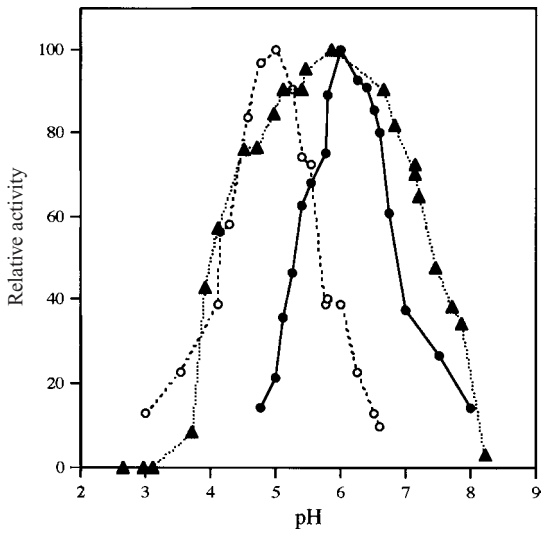

Figure 2 pH–activity profiles of congopain and cathepsin K on Abz-HPGGPQ-EDN$_2$ph and Z-FR-AMC

Relative k_{cat}/K_m values for the hydrolysis of Abz-HPGGPQ-EDN$_2$ph (●) and Z-FR-AMC (▲) by cathepsin K and relative k_{cat}/K_m values for the hydrolysis of Abz-HPGGPQ-EDN$_2$ph (○) by congopain were expressed as normalized rates and plotted against the pH values of the assay.

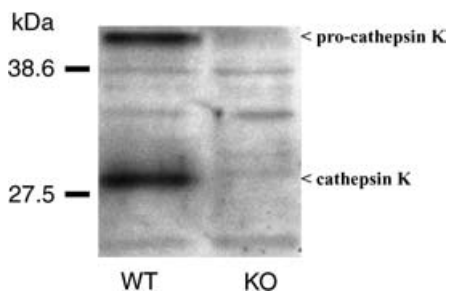

Figure 3 Immunoblotting of fibroblast lysates using an anti-(cathepsin K) polyclonal antibody

Extracted proteins (1 μg) of fibroblast lysates from WT and cat K$^{-/-}$ (KO) mice were subjected to SDS/PAGE (10 % gel) under reducing conditions and transferred on to PVDF membranes. Bands were revealed by chemiluminescence after incubation with a rabbit polyclonal antibody directed against mouse cathepsin K (1:1000) [4], as described in the Experimental section.

to mammalian CPs, only cathepsin K hydrolyses Abz-HPGGPQ-EDN$_2$ph efficiently.

Monitoring of cathepsin K activity in biological samples

The specificity of Abz-HPGGPQ-EDN$_2$ph towards cathepsin K activity was further investigated by monitoring fluorescence release. Cell lysates of skin fibroblasts from WT and cat K$^{-/-}$ (KO) mice were prepared as described in the Experimental section. Although it is predominantly expressed in osteoclasts, cathepsin K is also found in other cells, such as lung epithelial cells, activated macrophages or fibroblasts (see [2] for a review). Western-blot analysis showed the presence of two bands in normal skin fibroblasts (Figure 3), which corresponded to procathepsin K and to its mature processed form. By contrast, no immunoreactivity was detected in fibroblasts from cat K$^{-/-}$ mice. Supernatants of WT and cat K$^{-/-}$ cell extracts were further analysed for their ability to cleave Abz-HPGGPQ-EDN$_2$ph and Z-FR-AMC. Both WT and cat K$^{-/-}$ supernatants (10 μg of protein/assay) hydrolysed Z-FR-AMC. Proteolytic activity was slightly higher

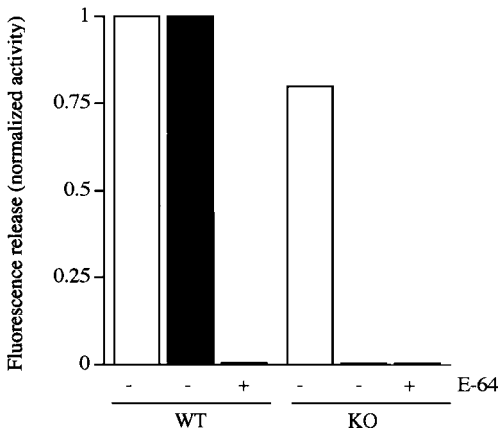

Figure 4 Selective hydrolysis of Abz-HPGGPQ-EDN$_2$ph by cathepsin K from skin fibroblasts

Fibroblast lysates from WT and cat K$^{-/-}$ (KO) mice were prepared as described in the Experimental section. The protein concentration of cell extract supernatants was determined using the Bradford kit, and their CP activity (10 μg of total protein/assay) was recorded with 5 μM Z-FR-AMC (5 μM; open bar) at 25 °C in 100 mM sodium acetate buffer (pH 5.5), containing 2.5 mM DTT and 2.5 mM EDTA. Monitoring of Abz-HPGGPQ-EDN$_2$ph hydrolysis (5.6 μM; black bar) was performed using 50 μg of total protein/assay. Substrate hydrolysis (fluorescence release) by fibroblast lysates from WT and cat K$^{-/-}$ mice were normalized.

in WT than in cat K$^{-/-}$ samples and was fully abolished by the addition of E-64, indicating that hydrolysis of Z-FR-AMC related specifically to CPs. By contrast, fibroblast lysates from WT mice, but not from cat K$^{-/-}$ (KO) mice, cleaved Abz-HPGGPQ-EDN$_2$ph (Figure 4). Assuming that Abz-HPGGPQ-EDN$_2$ph is cleaved similarly by human and mouse cathepsin K, these results confirm unambiguously that Abz-HPGGPQ-EDN$_2$ph can be used to monitor selectively cathepsin K activity in biological samples. Preliminary studies support the idea that Abz-HPGGPQ-EDN$_2$ph hydrolysis by broncho-alveolar fluids from patients suffering from inflammatory disorders depends on their cathepsin K concentration (F. Lecaille and G. Lalmanach, unpublished work). In conclusion, Abz-HPGGPQ-EDN$_2$ph may be adequate to probe the presence of cathepsin K activity and to measure directly and selectively its concentration in physiological fluids and/or soluble fractions of tissue/cell lysates, such as osteoclasts, bronchial epithelial cells or cancer prostate cell lines.

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