Quantification of cathepsins B and L in cells

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A method for quantifying active cysteine proteinases in mammalian cells has been developed using an active-site-directed inhibitor. Fluoren-9-ylmethoxycarbonyl(di-iodotyrosylalanyl)-diazomethane (Fmoc-[I_2]Tyr-Ala-CHN_2) was prepared and shown to react irreversibly with cathepsins B and L, but not with cathepsin S. The non- and mono-iodo forms of the inhibitor reacted with all three enzymes. These results demonstrate that, unlike cathepsins B and L, cathepsin S has a restricted S_2-binding site that cannot accommodate the bulky di-iodotyrosine. Fmoc-[I_2]Tyr-Ala-CHN_2 was able to penetrate cells and react with active enzymes within the cells. A radiolabelled form of the inhibitor was synthesized and the concentration of functional inhibitor was established by titration with papain. This inhibitor was used to quantify active cysteine proteinases in cultured cells. Active cathepsin B was found to be expressed by all of the cells studied, consistently with a housekeeping role for this enzyme. Active forms of cathepsin L were also expressed by all of the cells, but in different quantities. Two additional proteins were labelled in some of the cells, and these may represent other non-characterized proteinases. Higher levels of active cathepsins B and L, and an unidentified protein of M_r 39000, were found in breast tumour cells that are invasive, compared with those that are not invasive. From the data obtained, it can be calculated that the concentrations of both active cathepsins B and L in lysosomes can be as high as 1 mM, each constituting up to 20% of total protein in the organelle. This new technique provides a more direct procedure for determining the proteolytic potential of cellular lysosomes.

INTRODUCTION

Cathepsins B and D were the first lysosomal proteinases to be purified. This was possible because mammalian tissues do not contain any significant inhibitors of cathepsin D, and the inhibitors of cathepsin B bind only weakly to the enzyme [1]. Quantitative analyses of steps in purification have been used to calculate the quantities of these enzymes in the original tissue [2]. These early experiments led to the conclusion that cathepsins B and D are major lysosomal proteinases, and each enzyme can contribute up to 10% of the total lysosomal protein. Subsequent studies have led to the discovery of several other lysosomal proteinases, including cathepsins S, L, O, K and W [3–8]. Cathepsins L and S have been purified in small quantities from tissues [3,4,6]. Analysis of purification data from human liver cathepsin L suggests that the quantities of this enzyme are approximately 20 times less than cathepsins B and D [6]. Such calculations are complicated by both the instability of the enzymes and by inhibition with naturally occurring inhibitors. We have developed a series of membrane-permeant, covalently binding inhibitors of cathepsins B and L, and used radiolabelled analogues of these to demonstrate the presence of the active enzymes in living cells [9,10]. These inhibitors bind to all active forms of the proteinases, including the light chain of a two-chain form of cathepsin B, and the heavy chain of a two-chain form of cathepsin L [9–11]. This work led to the concept that the quantities of active cysteine proteinases could be determined within cells using these reagents. We now report a technique that we have developed that permits the relative concentrations of active cathepsins B and L in live cells to be determined for the first time. We chose a peptidyl diazomethane as the probe because of prior work that has demonstrated the specificity of such reagents, and used a peptide blocked at the N-terminus with a fluoren-9-ylmethoxycarbonyl (Fmoc) group to permit further modifications of the probe without damaging the diazomethane group [12]. Results from these studies show that cathepsin L is actually as abundant as cathepsin B in some cell types, and thus will make a major contribution to the proteolytic capacity of lysosomes within these cells.

EXPERIMENTAL

Materials

Human cell lines were purchased from America Type Culture Collection (Rockville, MD, U.S.A.). Kirsten-virus-transformed 3T3 (KNIH 3T3) cells were from M. M. Gottesman (National Cancer Institute, Bethesda, MD, U.S.A.). Sheep anti-(human cathepsin B) IgG was a generous gift provided by D. J. Buttle, University of Sheffield Medical School, Sheffield, U.K. Rabbit anti-(human cathepsin L) was obtained as described previously [6]. Papain was purchased in a suspension form of 25 mg/ml from Sigma (St. Louis, MO, U.S.A.). Iodobeads and BCA Protein Assay Reagent were purchased from Pierce (Rockford, IL, U.S.A.). Fmoc-Tyr-Ala-OH was obtained from Bachem Feinchemikalien Inc. (Budendorf, Switzerland). Carbobenzoxy (Z)-Arg-Arg-NH-Mec was purchased from Bachem Bioscience Inc. (King of Prussia, PA, U.S.A.). $Na^{125}I$ was purchased from DuPont NEN Products (Boston, MA, U.S.A.). Diazald (*N*-nitroso-*N*-methyl-4-toluenesulphonamide) was from Aldrich (Milwaukee, WI, U.S.A.). All other chemicals were ACS grade. Human cathepsin B was purchased from Sigma. Cathepsin S was prepared from bovine spleen as previously described [13]. Cathepsin L was purified from sheep liver [14]. The purity of each

Abbreviations used: diazald, *N*-nitroso-*N*-methyl-4-toluenesulphonamide; E-64, L-3-carboxy-*trans*-2,3-epoxypropionyl-L-leucine 4-guanidinobutylamide; Fmoc, fluoren-9-ylmethoxycarbonyl; Z, carbobenzoxy.

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proteinase was determined by SDS/PAGE and staining with Coomassie Brilliant Blue.

Preparation of inhibitor

Ethereal diazomethane was made from diazald according to the manufacturer's instructions. Fmoc-Tyr-Ala-OH was converted to the diazomethane on the basis of the method of Green and Shaw [15]. Briefly, 1 mmol of Fmoc-Tyr-Ala-OH was incubated with 0.8 mmol of *N*-methylmorpholine and 0.8 mmol of isobutyl chloroformate at −20 °C for 15 min in dry, oxygen-free conditions. Ethereal diazomethane (1.5 mmol) was then added and incubated for a further 15 min at 0 °C. The peptidyl diazomethane was purified by preparative HPLC.

Preparative HPLC

Fmoc-Tyr-Ala-CHN_2 was purified by applying the crude product to a Waters RCM Bondapak C_{18} column (25 × 100 mm, pore size 30 nm, particle size 15–20 μm) and eluting at 5 ml/min with a gradient from 80 % 50 mM ammonium acetate, pH 6.5 and 20 % acetonitrile to 100 % acetonitrile from 5 to 45 min. Products were identified by absorbance at 260 nm, and analysed for ability to inhibit papain. Inhibitory fractions were re-analysed by analytical HPLC.

Analytical HPLC

Analytical HPLC of inhibitors was performed using a linear gradient from 80 % 50 mM ammonium acetate, pH 6.5 and 20 % acetonitrile to 100 % acetonitrile from 2 to 22 min on a NovaPak C_{18} column (3.9 × 150 mm) with a flow rate of 1 ml/min. Products were detected by monitoring absorbance at 260 nm. Homogeneous fractions were pooled, inhibitor concentration determined by titration with papain, and then freeze-dried.

MS

Jeff Shabanowitz, (University of Virginia, VA, U.S.A.) kindly performed MS analysis of the purified product on a Finnigan-MAT TSQ-70 triple quadrupole instrument using electrospray ionization and collision-activated dissociation, as described previously [16].

Iodination of inhibitor

Prior results had shown that Z-Tyr[I]-Ala-CHN_2 reacts faster with cathepsins B and L than Z-Tyr-Ala-CHN_2 [12]. In addition, in preliminary studies, we found that iodination of Fmoc-Tyr-Ala-CHN_2 altered its hydrophobicity. We rationalized that this would also alter its ability to penetrate cellular membranes, but we are not able to determine the relative rates of entry of these different species into lysosomes. These two effects indicate that a mixture of iodinated and non-iodinated inhibitor would not permit us to use incorporation of ^{125}I in trace-labelled inhibitor to determine quantities of the enzymes in cells. For these reasons, we developed a procedure to obtain a homogeneous form of the inhibitor. $Na^{125}I$ (10 μl, 1 mCi) was added to a glass tube containing 90 μl of 50 mM sodium phosphate buffer, pH 7.0, and 2 iodobeads. After incubating for 5 min at room temperature, Fmoc-Tyr-Ala-CHN_2 (50 nmol) dissolved in methanol (100 μl) was added. After a further 5 min, NaI (500 nmol) dissolved in water (10 μl) was added and incubated for a further 15 min. Finally, 290 μl of methanol/sodium phosphate buffer, pH 7.0 (1:1) was added, and the mixture removed from the iodobead to stop the reaction. Samples (20 μl) were separated into aliquots into microfuge tubes, and frozen for future use. It was found that the labelled inhibitor could be separated from the non-incorporated iodide and iodine by HPLC on a C_{18} column, but, owing to the small quantities of sample, yields were typically less than 20 %. During the development of this protocol, different incubation times and NaI concentrations were used. Products were analysed by HPLC and papain inhibition.

Kinetic analyses

Rates of inactivation of enzymes by peptidyl diazomethanes were determined as described previously [12]. Cathepsins B, L and S were assayed at pH values of 6.0, 5.5 and 7.0 respectively, using conditions described previously [13].

Titration of inhibitor

Fmoc-[$^{125}I_2$]Tyr-Ala-CHN_2 concentration, and incorporation of ^{125}I, were determined by a modified titration assay. Briefly, increasing concentrations of Fmoc-[$^{125}I_2$]Tyr-Ala-CHN_2 were added to a fixed concentration of papain (3–5 μM), determined by titration with L-3-carboxy-*trans*-2,3-epoxypropionyl-L-leucine 4-guanidinobutylamide (E-64) [17]. A portion of this solution was then added to SDS/PAGE sample buffer, boiled and applied to an SDS/polyacrylamide gel (12.5 %) [18]. The gel was then dried, and radiolabelled papain identified by autoradiography. Incorporated radioactivity was determined by cutting out labelled bands and counting. The point of maximal label incorporation was used to determine inhibitor concentration and specific radioactivity. Additional confirmation of the quantity of inhibitor was obtained by measuring inhibition of papain activity, as described for E-64 titration.

Cell culture

A range of cell lines was chosen in order to determine the concentrations of enzymes in cells with different proteolytic requirements. KNIH 3T3 cells were chosen for study because these transformed mouse fibroblasts have been shown to overexpress cathepsin L [19,20]. THP-1 cells are human monocytic cells that can be differentiated into macrophage-like cells and are phagocytic. A range of human breast tumour cell lines was studied to determine whether expression of cathepsins B and L correlated with invasive properties of cells [21]. KNIH 3T3, MCF7 and Hs 578T cells were grown in Dulbecco's modified Eagle's medium; THP1, BT-549 and BT-474 cells in RPMI 1640; SK-BR-3 cells in McCoy's 5A; and the Mcell lines in Leibovitz's L-15. Unless otherwise stated, media were supplemented with 10 % (v/v) fetal bovine serum. The Mcell lines were cultured in air instead of 95 % air/5 % CO_2. THP1 cells were differentiated into macrophage-like cells by addition of PMA to a final concentration of 500 nM.

Protein determination

Cell pellets were resuspended in 1 % (w/v) SDS and 8 M urea, and protein concentraiton was then determined using the BCA protein assay (Pierce). Samples were then prepared for SDS/PAGE by addition of SDS/PAGE sample buffer containing 2-mercaptoethanol, and boiled for 5 min.

Cell dimensions

Cell volumes were determined by measuring the volume of 10^9 cells after centrifugation. The volume occupied by lysosomes was assumed to be 2.5 % of the total cell volume, as calculated for

mouse macrophages and fibroblasts [22]. Lysosomal protein concentration was assumed to be 312.5 mg/ml, as calculated by Henning [23].

Immunoprecipitation of cathepsins B and L

Immunoprecipitation was performed as described previously, with a slight modification [10]. Cell pellets (10^6) were resuspended in 0.5 ml of 10 mM Tris/HCl containing 1.0 mM EDTA, pH 7.5, and lysed by three rounds of freezing and thawing. For cathepsin B, the pH 7.5 buffer contained 0.1% (w/v) SDS and 0.05% (w/v) Brij-35, samples were boiled for 15 min and cooled to room temperature. Affinity-purified sheep anti-(human cathepsin B) IgG (10 μl of 1 mg/ml), 10% protein G–Sepharose (30 μl) and dithiothreitol (to a final concentration of 10 mM) were added, and the mixture was incubated at room temperature for 4 h on a rotary mixer. For cathepsin L, homogenized cell extracts were incubated with 10 mM dithiothreitol and 30 μl of 10% protein G–Sepharose at room temperature for 4 h on a rotary mixer. Following centrifugation at 1000 ***g*** for 10 min, the pellets were discarded and the supernatants were incubated with 30 μl of 10 % protein G–Sepharose, and 10 μl of rabbit anti-(human cathepsin L) serum at room temperature for 4 h on a rotary mixer. Following centrifugation of the reaction mixture at 1000 ***g*** for 10 min, the pellets containing immunoprecipitated cathepsin L were washed twice in 0.5 ml of 50 mM phosphate buffer, pH 7.0, and resuspended in SDS/PAGE sample buffer.

SDS/PAGE and phosphorimager analysis

For SDS/PAGE, slab gels (8×10 cm) containing 12% polyacrylamide were prepared [18]. After electrophoresis, the protein was revealed by Coomassie Blue staining. The standard proteins were BSA (M_r 66000), egg albumin (M_r 45000), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (M_r 36000), bovine carbonic anhydrase (M_r 29000), bovine pancreatic trypsinogen (M_r 24000), soybean trypsin inhibitor (M_r 20100), and bovine milk α-lactalbumin (M_r 14200). Radioactivity was recorded using a Molecular Dynamics Storm phosphorimager. Data were collected on a storage phosphor screen for measured times of up to 14 days. The phosphorimager was calibrated by exposing the screen to a range of known quantities of ^{125}I for a fixed period of time.

Labelling of cells and quantification of individual molecular forms of cathepsins B and L

To label intracellular proteinases, cells were incubated with 0.5 μM Fmoc-[$^{125}I_2$]Tyr-Ala-CHN_2 for 3 h in serum-free medium. This concentration of inhibitor and this incubation time gave maximal labelling of active proteins. Cells were then harvested, counted and analysed using a calibrated phosphorimager after SDS/PAGE. In some cases, individual bands were excised and counted for total radioactivity. Quantities of inhibitor in the individual bands were calculated from the specific radioactivity of the inhibitor probe, calculated as described above. The individual bands were identified by immunoprecipitation, as described above. When more than one protein contributed to radioactivity in an individual band (i.e. the 'M_r 33000' band), the relative contribution of each protein was determined from immunoprecipitation data. For cathepsin L, the ratios of intensities of the precipitated molecular forms of M_r 31000, 33000 and 36000 compared with the unique M_r 25000 form were multiplied by the quantities of the M_r 25000 band determined directly. For cell lines that did not express the band of M_r 25000, the immunoprecipitation data alone were used, taking into account efficiency of precipitation and recovery. The contribution of cathepsin B to the 'M_r 33000' band in the direct labelling experiments was determined either by subtracting the contribution of cathepsin L to the band, or by calculating the amount of protein recovered by immunoprecipitation.

RESULTS

Preparation of Fmoc-Tyr-Ala-CHN_2

The major product of the diazomethylation reaction eluted at 43 min from the preparative HPLC column. This fraction contained the papain-inhibitory activity, and corresponded to a peak that eluted at 11.4 min from the analytical column. Yield of pure inhibitor was approx. 15%. The product was titrated with papain and freeze-dried. The inhibitor was stable when stored at -20 °C for at least 6 months. The purified inhibitor was further characterized by electrospray ionization in MS. A major peak of mass 499.4 Da corresponded to the M–H^+ ion of the parent compound, $C_{28}H_{26}N_4O_5$. Fragmentation of this peak gave masses of 179 Da ($C_{14}H_{11}$), 249 Da ($C_{13}H_{17}N_2O_3$), 293 Da ($C_{14}H_{17}N_2O_5$) and 471 Da ($C_{28}H_{27}N_2O_5$). This fragmentation pattern is consistent with the structure of Fmoc-Tyr-Ala-CHN_2, and the proposed structures of the fragments are shown in Figure 1.

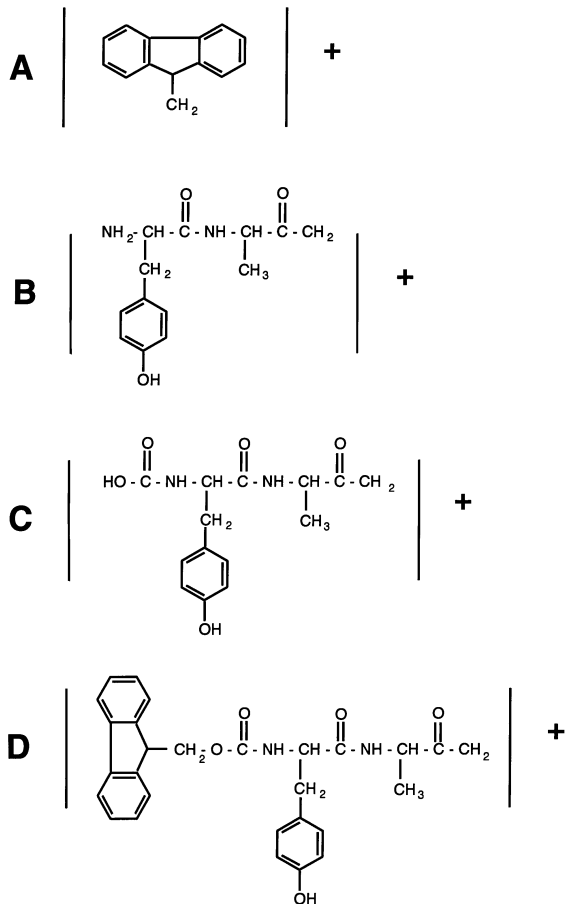

Figure 1 MS analysis of Fmoc-Tyr-Ala-CHN_2

Fmoc-Tyr-Ala-CHN_2 was subjected to MS analysis as described in the text. Fragmentation of the parent ion (M–H^+ 499.4) gave fragments of m/z of 179, 249, 293 and 471. The structures in **A**, **B**, **C** and **D** have m/z values that correspond to each of these values respectively.

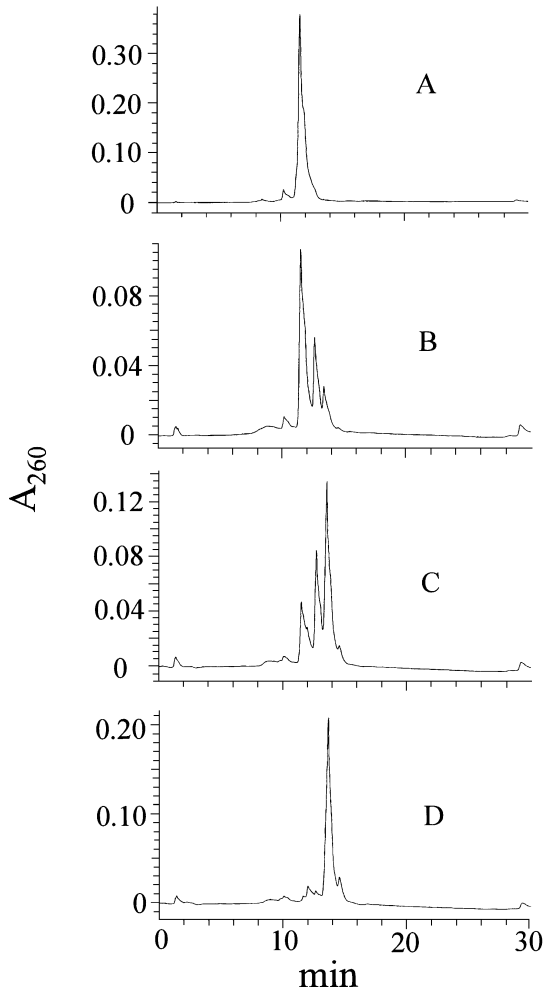

Figure 2 Iodination of Fmoc-Tyr-Ala-CHN_2

Fmoc-Tyr-Ala-CHN_2 was incubated with NaI and iodobeads, as described in the text. Chromatogram **A** was derived from a sample taken at the start of the reaction, chromatogram **B** after 5 min, chromatogram **C** after 10 min and chromatogram **D** after 15 min.

Iodination of inhibitor

In order to determine optimal conditions for iodination of Fmoc-Tyr-Ala-CHN_2, 50 nmol of inhibitor was incubated with varying amounts of sodium iodide for up to 30 min. Using 500 nmol of sodium iodide, HPLC analysis showed the time-dependent appearance of two new peaks (Figure 2). The two new peaks were both effective inhibitors of papain, and when tracer levels of ^{125}I were used, both of these peaks incorporated radioactivity. The characteristic progression from the early peak to the later peak with increased incubation time indicates that the compounds of increasing hydrophobicities eluted at 11.4, 12.6 and 13.4 min represent the non-, mono- and di-iodo forms of the inhibitor respectively. Incubation with 500 nmol of sodium iodide for 15 min gave optimal conversion to Fmoc-[I_2]Tyr-Ala-CHN_2. The slight modification of this procedure for incorporation of ^{125}I as described in the Experimental section gave only the di-iodo product. Titration of Fmoc-Tyr[$^{125}I_2$]-Ala-CHN_2 showed that a typical preparation of the inhibitor had a specific radioactivity of 500 d.p.m. per pmol.

Table 1 Inhibition of cathepsins B, L and S by iodinated forms of Fmoc-Tyr-Ala-CHN_2

Fmoc-Tyr-Ala-CHN_2 was iodinated with equimolar NaI using iodobeads. Non-, mono- and di-iodo species were then purified by HPLC. The concentrations of each form of the inhibitor were determined by titration with papain, and rates of inactivation of cathepsins B, L and S determined at least three times for each inhibitor. Rates of inactivation are corrected for competition with substrate and rates shown are means ± S.D. [12].

	Inhibitor	$k_{+2'}$ (M^{-1} s^{-1})
Cathepsin B	Fmoc-Tyr-Ala-CHN_2	1660 ± 220
	Fmoc-[I]Tyr-Ala-CHN_2	10200 ± 2300
	Fmoc-[I_2]Tyr-Ala-CHN_2	25300 ± 10000
Cathepsin L	Fmoc-Tyr-Ala-CHN_2	194000 ± 28000
	Fmoc-[I]Tyr-Ala-CHN_2	240000 ± 90000
	Fmoc-[I_2]Tyr-Ala-CHN_2	60900 ± 12000
Cathepsin S	Fmoc-Tyr-Ala-CHN_2	576 ± 147
	Fmoc-[I]Tyr-Ala-CHN_2	770 ± 176
	Fmoc-[I_2]Tyr-Ala-CHN_2	Not irreversible

Inhibition of cathepsins B, L and S by iodinated forms of Fmoc-Tyr-Ala-CHN_2

The individual peaks obtained by separation on HPLC were quantified and evaluated for inhibition of cathepsins B, L and S (Table 1). Fmoc-Tyr-Ala-CHN_2 inhibited the kinetics of cathepsins B, L and S similarly, as seen for Z-Tyr-Ala-CHN_2 [12,13]. The mono-iodo compound was a significantly better inhibitor of cathepsin B than the non-iodo form, again consistent with results seen previously for Z-Tyr-Ala-CHN_2 and its related mono-iodo form. In contrast, there was little difference in inhibition of cathepsins L and S between the mono- and non-iodo forms. The di-iodo form proved to be the best inhibitor of cathepsin B, but reacted more slowly than the mono-iodo form with cathepsin L, and did not react at all with cathepsin S.

Quantities of different molecular forms of cathepsins B and L in cells

Labelling and immunoprecipitation showed that breast tumour cells contain up to four different molecular forms of cathepsin L and two different molecular forms of cathepsin B (Figures 2 and 3, Tables 2 and 3). For the mouse cells, there were two forms of cathepsin L with M_r values of 31000 and 23000, and only one form of cathepsin B, of M_r 35000 [9]. Recoveries of cathepsins B and L after immunoprecipitation were 51 ± 23 % and 13 ± 5 % respectively (data given as means ± S.D.). Recovery of the light chain of cathepsin B was less than 5 %. Although this extent of recovery was sufficient to enable identification of the light chain as cathepsin B, it was not reliable for quantification. Data calculated from immunoprecipitation were more variable than from the direct labelling, presumably due to the additional steps in the procedure (standard deviation for immunoprecipitation of cathepsin L was 38 % versus only 24 % for the direct labelling of bands). For these reasons, immunoprecipitation was primarily used for identification and not quantification. However, both techniques gave similar quantities of enzymes in the 25000–33000 molecular mass range (Table 3).

The quantity of cathepsin L was lower than cathepsin B in all of the breast tumour cells. Two additional proteins of molecular masses 65000 and 39000 were also labelled (Figure 3). The M_r 65000 protein was found in all of the cell lines, but the M_r 39000 protein was not detected in either the BT-549 or MCF7 cells.

Table 2 Quantities of reactive bands in breast tumour cells

The intensities of individually labelled bands from total cell extracts were determined by quantitative analysis using a phosphorImager as described in the Experimental section. Values are in pmol/mg cell protein, and results are the means ± S.D. of four determinations. N.D., band not detectable.

Reactive band	SK-BR-3	MDA-MB-453	MDA-MB-435S	MDA-MB-231	BT-549	BT-474	HS 578T	MCF7
65000	5.5 ± 1.6	5.7 ± 1.1	5.1 ± 2.2	1.3 ± 0.5	1.8 ± 0.5	3.7 ± 0.4	1.5 ± 0.7	2.2 ± 0.7
39000	5.4 ± 0.6	1.8 ± 0.6	77 ± 9	8.4 ± 1.1	N.D.	2.1 ± 0.3	7.2 ± 2.8	N.D.
33000	6.6 ± 0.7	6.4 ± 1.0	139 ± 24	23 ± 6	36 ± 4	4.0 ± 0.4	68 ± 9	9.7 ± 1.0
25000	1.7 ± 0.7	N.D.	N.D.	2.2 ± 1.5	0.4 ± 0.05	0.8 ± 0.3	4.8 ± 1.1	1.4 ± 0.2
5000	7.5 ± 1.6	16.9 ± 4.6	94 ± 13	18.6 ± 2.2	4.9 ± 2.8	12.9 ± 0.7	74 ± 17	15 ± 2.5

Table 3 Quantities of cathepsins B and L in the 'M_r 33000' band

The immunoprecipitation data shown in Figure 3 were analysed using a phosphorimager as described in the Experimental section. From these data, it was possible to calculate the relative contributions of 2 or 3 molecular forms of cathepsin L and the single-chain form of cathepsin B in the band identified as M_r 33000 in Table 2. The values for totals are taken from Table 2. Values shown in parentheses were determined directly from the immunoprecipitation data. Calculated values are in pmol/mg cell protein. N.D., band not detectable.

Protein	SK-BR-3	MDA-MB-453	MDA-MB-435S	MDA-MB-231	BT-549	BT-474	HS 578T	MCF7
Total	6.6 ± 0.7	6.4 ± 1.0	139 ± 24	23 ± 6	36 ± 4	4.0 ± 0.4	68 ± 9	9.7 ± 1.0
Cathepsin B: M_r 31000	3.9 (2.8)	(5.9)	(173)	12.7 (8.9)	35 (26)	1.9 (1.3)	62 (70)	5.5 (5.0)
Cathepsin L: M_r 36000	N.D.	N.D.	N.D.	1.7	N.D.	N.D.	N.D.	N.D.
Cathepsin L: M_r 33000	1.6	N.D.	N.D.	2.2	0.9	0.8	3.0	2.0
Cathepsin L: M_r 31000	1.1	(1.0)	(30)	6.6	0.8	1.3	2.9	2.2

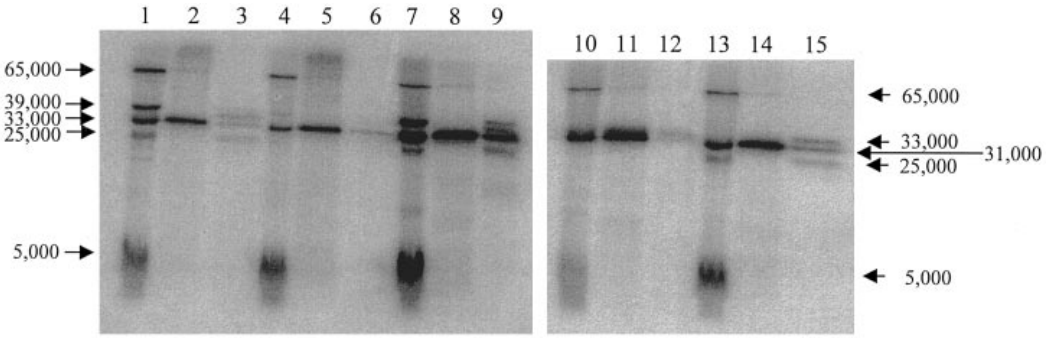

Figure 3 Analysis of proteins from breast tumour cells after reaction with Fmoc-Tyr[$^{125}I_2$]-Ala-CHN_2

Breast tumour cells were labelled with 0.5 μM Fmoc-Tyr[$^{125}I_2$]-Ala-CHN_2 for 3 h as described in the Experimental section. Cells were harvested, and then either total cell protein or proteins immunoprecipitated with antibodies to cathepsins B and L were loaded on to the gels. Incorporated radioactivity was detected by phosphorimager analysis after SDS/PAGE. For lanes 1, 4, 7, 10 and 13, 48 μg of cell protein was loaded directly on to the gel. For the other lanes, 240 μg of cell protein was used for immunoprecipitation, and the precipitated proteins were loaded on to the gel. Lanes 2, 5, 8, 11 and 14 were precipitated using anti-(cathepsin B) and lanes 3, 6, 9, 12 and 15 were precipitated using anti-(cathepsin L). The cell lines shown are SK-BR-3 (lanes 1–3); MDA-MB-453 (lanes 4–6); MDA-MB-231 (lanes 7–9); BT-549 (lanes 10–12); and MCF7 (lanes 13–15). Molecular masses of identified proteins are shown.

These proteins were not identified, but may represent novel cysteine proteinases. There appeared to be some correlation between expression of the M_r 39000 protein and cathepsin L with cathepsin B (r^2 values of 0.78 and 0.80), but these correlations were dependent upon the high levels of expression of all three proteins in the MDA-MB-435S cells (when this sample was omitted, r^2 values dropped to 0.27 and 0.19 respectively). However, there was a weak correlation between expression of cathepsin L and the M_r 39000 protein in the breast tumour cells (r^2 value of 0.88). Two of the breast tumour cell lines, however, do not express any detectable M_r 39000 protein, and this protein is also not expressed by the THP1 cells or a range of other human cell lines (unpublished work).

Active cathepsin L is expressed at high levels in KNIH 3T3 cells (82 ± 24 pmol/mg protein for the M_r 31000 form and 130 ± 20 pmol/mg protein for the M_r 25000 form) and in differentiated THP1 cells (73 ± 8 pmol/mg for only one form, M_r 32000), similarly to levels of cathepsin B (177 ± 25 pmol/mg protein for a M_r 35000 form in KNIH 3T3 cells and 88 ± 9 pmol/mg protein for only one form, M_r 31000, in THP1 cells). Neither cell line processes cathepsin B to a two-chain form. Before differentiation of THP1 cells, the same forms of cathepsins B and L are expressed, but much less abundantly (2.9 ± 1.2 and 0.29 ± 0.11 pmol/mg protein respectively).

KNIH 3T3 cells (10^6) and differentiated THP1 cells were determined to occupy 1.5 μl and 2.5 μl of space and contain

254 μg and 480 μg of protein respectively. Lysosomes have been calculated to occupy 2.5% of the cellular volume of fibroblasts and macrophages [22], and the concentration of protein in lysosomes has been calculated to be 312.5 mg/ml [23]. Using these data, the concentrations of cathepsins B and L in KNIH 3T3 cells were calculated to be 1.2 and 1.4 mM and, in THP1 cells, 0.7 and 0.6 mM, respectively. The individual enzymes were calculated to comprise between 9% and 25% of the total lysosomal protein.

When the culture media from the cells were analysed by SDS/PAGE and autoradiography, negligible quantities of reactive proteins were found, indicating that none of these cells secrete significant levels of active enzymes during the course of these experiments.

DISCUSSION

Peptidyl diazomethanes have been developed as specific inhibitors of cysteine proteinases, although a few have been demonstrated to react slowly with serine proteinases [24,25]. The advantage of these inhibitors is that their specificity can be demonstrated by incorporation of a radioactive tag into the compound, which becomes covalently bound to target enzymes. Results from this study show that iodination of Fmoc-Tyr-Ala-CHN_2 to form mono- and di-iodo species results in molecules with increased hydrophobicities. Iodination also alters the specificity of the inhibitor. The di-iodo inhibitor did not react irreversibly with cathepsin S, consistent with the restricted reactivity of cathepsin S with reagents that have bulky side chains in P_2 [3,26]. All molecular forms of the inhibitor reacted rapidly with cathepsin L, an enzyme with a large S_2 pocket [26], and the di-iodo inhibitor was the best inhibitor of cathepsin B. The increased hydrophobicity of the iodinated inhibitors is likely to increase their ability to penetrate cell membranes whilst their increased size could decrease their ability to penetrate membranes [27]. This would result in different molecular forms of inhibitors reaching intracellular enzymes at different rates. Thus although trace labelling of the inhibitor with ^{125}I provides a sensitive reagent for detecting small quantities of reacted proteins in cells, the differing hydrophobicities and specificities of the iodinated species would make it difficult to use trace labelling for accurate quantitative analyses.

Several different techniques are available to determine the quantities of proteins in cells and tissues. The original amounts of proteinases in tissues have been calculated from purification data [2]. However, lack of specificity of the substrate would result in an overestimation, whereas inhibition by endogenous inhibitors would result in an underestimation of the quantities of enzyme in the original extract from tissues [1]. Experiments using incorporation of Z-[^{125}I]Tyr-Ala-CHN_2 into active proteinases have actually shown that homogenization of whole human liver fails to yield any active cathepsin L, and only subsequent treatments to disrupt enzyme–inhibitor complexes are able to release active enzyme [11]. Immunological techniques are widely used to measure protein quantities in crude extracts because these can detect proteins even after they have been denatured. These techniques do, however, depend upon the availability of antibodies that recognize all molecular forms of the proteins equally, and they do not distinguish between active and inactive proteins. We were able to identify active-site-labelled cathepsins B and L using our antibodies after denaturation of the proteins, but the efficiency of precipitation of individual proteins was variable. Furthermore, we have shown that human lung macrophages contain inactive forms of cathepsin L, which were presumed to be taken up from serum, and thus immunological quantification of the enzyme in these cells would be unrelated to the ability of cells to digest protein [28].

The technique that we have devised to quantify cathepsins B and L using Fmoc-[$^{125}I_2$]Tyr-Ala-CHN_2 avoids the problem of inhibition or inactivation of proteinases by allowing the inhibitor to react with enzyme in the live cells before homogenization. This has permitted the quantification of active enzymes in cells, thus demonstrating the proteolytic potential of the cells. Despite the quite different techniques used to estimate cellular quantities of cathepsin B, our calculations of the lysosomal concentrations of cathepsin B in cultured cells give similar values to those calculated from yields of activity after purification of the enzyme from human liver [2]. If the volume of the cell occupied by the lysosomal systems of the cells studied is higher than the 2.5% calculated for mouse fibroblasts and macrophages [22], then the concentration of the enzymes will be less than 1 mM. Conversely, if the enzymes are unevenly distributed through the endosomal–lysosomal system, the concentration in individual lysosomes could be even higher. Such studies are beyond the scope of the current paper, but if used in conjunction with the radiolabelled inhibitor, more accurate concentrations of enzymes in the lysosomes of discrete cells could be determined. The concentrations that we have estimated emphasize that cathepsin B exists at very high concentrations in the lysosomes of many cells, and constitutes a major portion of the total lysosomal protein. An enzyme concentration of 1 mM is very high when compared with other intracellular enzymes. However, the concentration of peptide bonds, i.e. potential substrates, in the lysosome is 1 M, and therefore such high concentrations of enzyme may be necessary for efficient proteolysis. The high concentration of proteinases in lysosomes suggests that these organelles may also serve as storage granules for delivery to sites in endosomes or extracellular compartments for proteolysis [29]. Our results show that the concentration of cathepsin L is as high as that of cathepsin B in THP1 macrophages. The proteolytic efficiency of cathepsin L has been shown to be much greater than that of cathepsin B [17], and thus it is likely that the former enzyme plays a major role in proteolysis by phagocytic macrophages. The very high lysosomal concentrations of proteinases explain, in part, the relatively slow kinetics of enzyme labelling in cells when compared with the free enzyme. We consistently find that 0.2 μM inhibitor takes 3 h to react with all active forms of cathepsins B and L in cells, whereas only a few minutes are needed for typical *in vitro* experiments. The concentration of the enzyme in the lysosome is over 10000-fold higher than the free inhibitor concentration, which must accumulate by diffusion across cellular membranes.

Elevated levels of cathepsin L mRNA have been detected in several human tumours, including those from breast tissues [30]. This enzyme is proposed to play a role in tumour cell invasion and may also play a role in tumour cell growth [30,31]. Six of the breast tumour cell lines used in this study have also been evaluated for invasiveness [21]. Cell lines that expressed the highest levels of cathepsins B and L and the M_r 39000 protein (Hs 578T, MDA-MB-231 and MDA-MB-435S) have been reported to be invasive after injection into nude mice, whereas cell lines that express low levels of these proteins (MCF7 and SK-BR-3) are not invasive [21]. BT-549 cells that were reported to be non-tumorigenic in nude mice [21] express high levels of cathepsin B, but low levels of cathepsin L, and no M_r 39000 protein. Although all of the cells that are invasive in nude mice expressed the largest amounts of cathepsins B and L and the M_r 39000 protein, the correlations between expression of each protein are weak, and thus the expression of each active protein is probably regulated independently. Results of this study support the

proposal that lysosomal proteinases are involved in tumour cell growth and/or tumour cell invasion, and levels of active proteinases in tumour cells could have prognostic value for the invasiveness of tumours.

KNIH 3T3 cells overexpress cathepsin L [19] but the intracellular concentration of active cathepsin L is not significantly higher than that of cathepsin B. These cells also secrete cathepsin L as an inactive proform, and although we and others have shown that secreted proforms of cysteine proteinases can react with peptidyl diazomethanes when activated [32,33], we were unable to demonstrate any significant reaction with our inhibitor under normal culture conditions. Furthermore, we were unable to detect labelled proteins in the medium from any of the cells tested. It thus seems that the cells studied retain the majority of their lysosomal proteinases after inhibition, and do not release significant quantities of active cysteine proteinases. However, human blood monocytes cultured for a long time overexpress cysteine proteinases and when these are labelled with peptidyl diazomethanes they are found to be secreted as mature forms [29], indicating that release of active proteinases is possible. Although we could find no evidence for secretion of active forms of the proteinases in our cell culture system, other factors could permit release of active enzymes by the cells *in vivo* to aid tumour cell invasion.

The M_r 65000 protein was found at low levels in all cells studied, indicating that it is constitutively expressed. We originally thought that this represented low-level reaction with albumin used in culture, but the inhibitor does not react with serum proteins to this extent (results not shown). Peptidyl diazomethanes will react very slowly with any free thiols, but they must be unusually reactive to label in preference to the thiols in other proteins. To date, the only cellular proteins that have been identified which react with peptidyl diazomethanes are proteinases, raising the likelihood that this is a novel cysteine proteinase. The M_r 39000 protein shows a more discrete cellular distribution than any of the other labelled proteins. This protein is rather large for a typical active lysosomal cysteine proteinase, and its identification also requires further characterization.

In this study, we have demonstrated that a specific membrane-permeant inhibitor can be used to quantify active cathepsins B and L in cultured cells. Provided that the required specificity and cellular penetration can be demonstrated, other inhibitors of the enzymes could be used in the described procedures, enabling identification of active proteinases with different specificities. This technique is unique in that it only recognizes active enzymes, and thus can be used to determine both the cellular location and quantities of proteinases that are capable of hydrolysing proteins, distinguishing them from denatured, oxidized and inhibited enzymes and inactive pro-enzymes.

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