Design of a transferrin–proteinase inhibitor conjugate to probe for active cysteine proteinases in endosomes

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A new technique has been developed to identify active proteinases in endosomes that does not require prior isolation of organelles and extraction of the active enzymes. [^{125}I]Iodotyrosylalanyldiazomethane was reversibly conjugated to transferrin to selectively deliver it to endosomes. The protein was conjugated to the inhibitor via a disulphide bond using *N*-succinimidyl 3-(2-pyridyldithio)propionate. The inhibitor portion of the conjugate bound irreversibly to active cathepsins B and L, and subsequently the reacted enzymes were separated from the transferrin after SDS/PAGE under reducing conditions. Uptake of the protein–inhibitor conjugate and incorporation of inhibitor into cathepsins was blocked at 4 °C, demonstrating that the conjugate enters cells by receptor-mediated endocytosis. Furthermore, endocytosed transferrin–inhibitor conjugate could be recycled back to the extracellular medium and binding to the transferrin receptor could be blocked by native transferrin. Labelling of the enzymes was not blocked by incubating cells at 16 °C, consistent with the majority of the reagent being targeted to endosomes. The inhibited enzymes remained conjugated to transferrin, showing that the disulphide bond between the transferrin and inhibitor was not reduced in the endosome. Results from these studies show that endosomes contain both intermediate and late biosynthetic forms of active cathepsin B, which are indistinguishable from those found in mature lysosomes. These results indicate that the active enzymes in endosomes are not early biosynthetic forms in transit to lysosomes but most probably enter the endosome via retrograde traffic from the lysosome.

INTRODUCTION

A range of extracellular proteins is taken up into cells by receptor-mediated endocytosis. The protein ligands bind to receptors at neutral pH and, after internalization into endosomes, acidification results in dissociation of the ligands from the receptors. The receptor can then recycle back to the plasma membrane, and the ligand can be delivered to the lysosome where it is degraded to small peptides and amino acids. The lysosomal proteinases cathepsins B and L are synthesized as inactive pro-enzymes and packaged into the endoplasmic reticulum by a signal peptide. After processing of carbohydrate residues in the Golgi complex to generate mannose 6-phosphate residues, the proteins bind to a receptor and are delivered to the endosomal/lysosomal compartment [1]. Acidification of the endosomal compartment causes the dissociation of the mannose 6-phosphate receptor (MPR) from the protein, and the receptor recycles back to the Golgi complex. The common features of acidification and subsequent delivery to lysosomes for both endocytosed proteins and newly synthesized lysosomal enzymes led to the hypothesis that the lysosomal and endosomal systems share a common location for uncoupling of proteins prior to delivery to lysosomes [1]. In support of this, immunological studies have shown that co-localization of endocytosed proteins, the lysosomal enzymes cathepsins B and D, and MPRs in low-density organelles occurs very rapidly [2]. Furthermore, endocytosis of a transferrin–antibody conjugate has been used to show that transferrin has access to a compartment to which a newly synthesized lysosomal enzyme, arylsulphatase, is delivered [3]. For the receptor to take part in further rounds of endocytosis, it is essential that the environment within the recycling endosome does not permit proteolysis of the receptor.

The mechanism by which proteolysis is regulated in endosomes is not clear. Acidification is the trigger for proteolytic activation of pro-forms of lysosomal proteinases, and active enzymes have been detected in isolated endosomes (e.g. [4–6]). Furthermore, in antigen-processing cells proteins are endocytosed, partially processed and then returned to the cell surface in association with class-II MHC molecules [7]. It therefore appears that at least some endocytosed proteins can be delivered to cellular compartments that contain active proteinases and escape delivery to mature lysosomes.

Conventional techniques used to detect proteinases in endosomes have several disadvantages. First, the endosomes have to be isolated free of other organelles that have similar biophysical characteristics. Secondly, the enzymes have to be detected by manipulation of incubation conditions that may not reflect conditions in the endosomes. Lack of stability of the isolated enzymes and presence of naturally occurring inhibitors can result in erroneous measurements of activity. We have devised a technique for identifying active proteinases in endosomes of intact cells by conjugating an active-site-directed inhibitor of cathepsins B and L to transferrin. The inhibitor is a peptidyl diazomethane that reacts covalently with the active-site cysteine of cysteine proteinases, but does not react with enzymes that are inactive, such as zymogens, or with denatured or inhibited enzymes [8–10].

EXPERIMENTAL

Materials

The human fibrosarcoma epithelial cell line, HT1080, was purchased from American Type Culture Collection (Manassas, VA, U.S.A.). Sheep anti-(human cathepsin B) IgG and rabbit anti-(bovine cathepsin S) IgG were generous gifts provided by

Abbreviations used: Fmoc, fluoren-9-ylmethoxycarbonyl; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; PDP, 2-pyridyldithiopropionate; DTT, dithiothreitol; MPR, mannose 6-phosphate receptor.

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D. J. Buttle (University of Sheffield, Sheffield, U.K.) and H. Kirschke (Halle University, Germany) respectively. Rabbit anti-(human cathepsin L) was obtained as described previously [11]. *N*-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP), Iodo-beads and Bicinchoninic Acid (BCA) Protein Assay Reagent were purchased from Pierce (Rockford, IL, U.S.A.). Fmoc-Tyr-Ala-OH (where Fmoc is fluoren-9-ylmethoxycarbonyl) was obtained from Bachem Feinchemikalien Inc. (Budendorf, Switzerland). $Na^{125}I$ was purchased from DuPont New England Nuclear Products (Boston, MA, U.S.A.). G-15 Sephadex and protein G–Sepharose 4 Fast Flow were obtained from Pharmacia Biotech (Uppsala, Sweden). Human transferrin was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals met American Chemical Society specifications for purity.

Synthesis of Fmoc-Tyr-Ala-CHN_2

Fmoc-Tyr-Ala-CHN_2 was synthesized based upon methods described previously [12]. Fmoc-Tyr-Ala-OH (1.0 mmol) was mixed with one equivalent of methyl morpholine in 5 ml of dry tetrahydrofuran and the reaction was cooled at −20 °C under N_2 gas protection. Isobutyl chloroformate (1.0 mmol) was then added dropwise and the reaction mixture was stirred at −20 °C for 15 min. Cold ethereal diazomethane (6 ml, 1.5 mmol) was then added. After incubation at 0 °C for 75 min, the reaction mixture was warmed to room temperature. Solvent was removed under reduced pressure and the crude product was recrystallized from methanol and diethyl ether. The product was dissolved in methanol and the Fmoc-Tyr-Ala-CHN_2 purified by silica-gel chromatography using an eluent of 80 % ethylacetate in hexane to give a white solid product after drying (22 % yield). Fmoc-Tyr-Ala-CHN_2 was iodinated as described previously [9]. The iodinated product was stored at −20 °C.

Synthesis of PDP (2-pyridyldithiopropionate)-Tyr[^{125}I]-Ala-CHN_2

NH_2-Tyr-Ala-CHN_2 was prepared by removal of the Fmoc-blocking group from Fmoc-Tyr-Ala-CHN_2. In this reaction, Fmoc-Tyr-Ala-CHN_2 (110 mg, 0.22 mmol) was dissolved in 1.0 ml of dimethylformamide and added to 1.0 ml of piperidine solution (dimethylformamide/toluene/piperidine, 7:7:6, by vol.). The reaction mixture was incubated at room temperature for 1 h and purified by silica-gel chromatography using 15 % methanol in chloroform as eluent. After rotary evaporation of the solvent, a white solid was obtained (41 mg, 66 % yield). The concentration of NH_2-Tyr-Ala-CHN_2 was determined by titration with papain as described previously [9].

A cross-linking reagent, SPDP, was then reacted with the NH_2-Tyr-Ala-CHN_2 [13]. NH_2-Tyr-Ala-CHN_2 (5.0 μmol) was dissolved in 500 μl of dry ethanol and mixed with one equivalent of SPDP dissolved in 500 μl of ethanol. The reaction mixture was incubated at room temperature for 60 min and the product was purified by HPLC at room temperature using a Waters Bonda-Pak C_{18} reverse-phase column (25 × 100 mm) attached to a Waters HPLC system. Product was allowed to elute at 3 ml/min with a linear gradient from 50 mM ammonium acetate, pH 6.5, to 100 % acetonitrile in 100 min. PDP-Tyr-Ala-CHN_2 was obtained as a white solid after removal of the solvent (12 % in yield). The concentration of inhibitor was determined by papain titration [9].

PDP-Tyr-Ala-CHN_2 was radio-iodinated using Iodo-beads. $Na^{125}I$ (1 mCi) and two Iodo-beads were added to a tube containing 180 μl of 50 mM phosphate buffer, pH 7.5. $^{125}I_2$ was generated by incubation at room temperature for 5 min. Then, 500 nmol of PDP-Tyr-Ala-CHN_2 in 200 μl of ethanol was added. After 20 min, radiolabelled PDP-Tyr[^{125}I]-Ala-CHN_2 was removed from Iodo-beads to stop the reaction and stored at −20 °C. The PDP-Tyr[^{125}I]-Ala-CHN_2 contained mostly mono- and non-iodinated inhibitors and these were not purified further.

Synthesis of transferrin–Tyr[^{125}I]-Ala-CHN_2

Human transferrin was linked to SPDP using a modification of a method described previously [14]. SPDP (2.6 μmol in 200 μl of dry ethanol) was mixed with a solution of transferrin (0.26 μmol in 2.0 ml of borate/saline buffer, pH 9.0). After reaction of SPDP with the primary amines in transferrin at room temperature for 30 min, the mixture was applied to a Sephadex G-15 column (16 × 35 cm) equilibrated with acetate/saline buffer, pH 4.5, and transferrin-PDP was eluted with same buffer. Transferrin-containing fractions were pooled and the concentration of transferrin was determined by protein assay. PDP concentration was determined by adding 10 mM dithiothreitol (DTT) to measure the absorbance of cleaved pyridine 2-thione at A_{343} and using a molar absorption coefficient of $8.08 \times 10^3\ M^{-1} \cdot cm^{-1}$ [15]. Transferrin-PDP (150 nmol of PDP) was reduced by adding DTT (final concentration, 10 mM) and incubating at room temperature for 30 min. DTT does not reduce disulphides in native proteins at pH 4.5 [13]. Transferrin-SH was separated from the low-M_r products by chromatography on a Sephadex G-15 column under anaerobic conditions. Pooled transferrin-SH was immediately added to radiolabelled PDP-Tyr[^{125}I]-Ala-CHN_2 (300 nmol) and incubated under N_2 gas protection at room temperature for 18 h before purification of the product by Sephadex G-15 chromatography. Transferrin–Tyr[^{125}I]-Ala-CHN_2 was collected and stored at −20 °C for labelling experiments. Transferrin–Tyr[^{125}I]-Ala-CHN_2 was evaluated for concentration of protein, inhibitor and radioactivity. Similarly, human IgG was conjugated to the inhibitor.

Analytical HPLC

Intermediates produced during the synthesis of PDP-Tyr-Ala-CHN_2 were analysed at room temperature using a Nova-Pak C_{18} reverse-phase column (Waters; 3.9 × 150 mm) monitored at A_{260}. Products were allowed to elute with a gradient from 50 mM ammonium acetate, pH 6.5, to 100 % acetonitrile (20 min) at a flow rate of 1.0 ml/min.

Reaction of cells with inhibitors

The human fibrosarcoma epithelial cell line, HT1080, was routinely cultured with 10 % fetal bovine serum in Dulbecco's modified Eagle's medium. Cells were cultured in a humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C. HT1080 cells were pre-cultured in serum-containing medium for 2–3 days, allowing cells to adhere to the bottom of the culture dishes. Cells were washed in serum-free medium and then incubated in serum-free medium containing radiolabelled inhibitors, unless otherwise stated. Cells were scraped and harvested by centrifugation (1000 ***g***, 5 min) followed by washing twice with cold PBS, pH 7.4, containing 10 μM non-radiolabelled Fmoc-Tyr-Ala-CHN_2 to prevent any post-processing reaction with labelled inhibitor.

Immunoprecipitation

Immunoprecipitation was performed as described previously [10] with slight modifications. For cathepsin B, cell pellets (1×10^6 cells) were re-suspended in 10 mM Tris/HCl, containing 1 mM EDTA, 0.1 % SDS and 0.05 % Brij-35, pH 7.5 (0.5 ml), and lysed by three rounds of freezing and thawing. The samples were boiled for 15 min and then cooled to room temperature. Affinity-

purified sheep anti-(human cathepsin B) IgG (10 μl, 1 mg/ml), 10 % protein G–Sepharose (30 μl) and 10 mM DTT were added and the mixture was incubated at room temperature for 4 h on a rotary mixer. For cathepsins L and S, cell pellets (1×10^6 cells) were re-suspended in 10 mM Tris/HCl containing 1.0 mM EDTA, pH 7.5 (0.5 ml), and lysed by three rounds of freezing and thawing. Homogenized cell extracts were pre-incubated with 10 mM DTT and 30 μl of 10 % protein G–Sepharose at room temperature for 4 h on rotary mixer. Samples were centrifuged at 1000 ***g*** for 10 min and the pellets were discarded. The supernatants were either incubated with 30 μl of 10 % protein G–Sepharose and 10 μl of rabbit anti-(human cathepsin L) antiserum, or 30 μl of 10 % protein G–Sepharose and 10 μl of rabbit anti-(bovine cathepsin S) IgG (1 mg/ml) at room temperature for 4 h on a rotary mixer. Following centrifugation of the reaction mixtures at 1000 ***g*** for 10 min, the pellets containing immunoprecipitated cathepsins were washed twice in 0.5 ml of 50 mM phosphate buffer, pH 7.0, and resuspended in SDS/PAGE sample buffer.

SDS/PAGE, autoradiography and Western blotting

For SDS/PAGE, slab gels (8×10 cm) containing 15 % polyacrylamide were prepared [16]. For some experiments, gradient gels (10–20 %) were used (Bio-Rad, Hercules, CA, U.S.A.). After electrophoresis, the protein was visualized by Coomassie Brilliant 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 erythrocyte carbonic anhydrase (M_r 29000), bovine pancreas trypsinogen (M_r 24000), soya bean trypsin inhibitor (M_r 20100) and bovine milk α-lactalbumin (M_r 14200). Autoradiography of the dried SDS/PAGE gel was performed by using DuPont autoradiography film, exposed at -80 °C with a phosphotungsten intensifying screen. Phosphorimager analysis was performed using a Storm phosphorimager (Molecular Dynamics) as described previously [17]. For Western blotting, proteins were transferred to a poly(vinylidene difluoride) membrane and proteins were identified using sheep anti-(cathepsin B), an avidin–biotin complex alkaline phosphatase kit and 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium as described by the manufacturers (Pierce, Rockford, IL, U.S.A.).

Quantification of reacted proteinases

Specific radioactivities of inhibitors were determined by titration with papain and sheep cathepsin L as described previously [17]. Protein concentrations were determined using the BCA protein assay.

RESULTS

Synthesis of transferrin–Tyr[^{125}I]-Ala-CHN$_2$

During the preparation of the conjugated inhibitor, the purity of the intermediates was confirmed by HPLC after each step. Fmoc-Tyr-Ala-CHN$_2$ eluted from the analytical HPLC column after 16.7 min. Upon removal of the Fmoc group, a new peak corresponding to the inhibitor with a free amino group eluted at 7.4 min. After reaction with SPDP, PDP-Tyr-Ala-CHN$_2$ eluted after 12.4 min. The yield of pure PDP-Tyr-Ala-CHN$_2$ was typically 10–15 %. When a 10 × molar excess of SPDP was reacted with transferrin, a ratio of 1:3 of protein to PDP was obtained in aqueous buffers with 90 % recovery of protein. After reduction of transferrin-PDP and purification of the transferrin-SH, a conjugate was produced by reaction with radio-iodinated PDP-Tyr-Ala-CHN$_2$. Excess low-M_r reagents and pyridine-2-thione were removed by chromatography on Sephadex G-15. Typically, the final yield of protein conjugate was 30 %. The ratio of protein to functional inhibitor was typically 1:1.3, and effectively linked transferrin to the inhibitor as follows: transferrin–CO-CH$_2$-CH$_2$-S-S-CH$_2$-CH$_2$-CO-Tyr[^{125}I]-Ala-CHN$_2$.

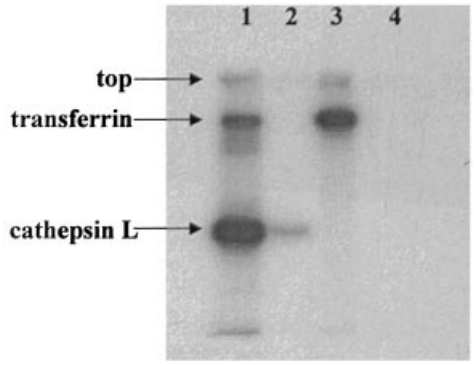

Figure 1 Reaction of cathepsin L with transferrin–Tyr[^{125}I]-Ala-CHN$_2$ and Fmoc-Tyr[^{125}I]-Ala-CHN$_2$

Purified sheep liver cathepsin L (0.2 pmol) was reacted with a 6-fold excess of radiolabelled inhibitor at 37 °C for 30 min. Samples were boiled in SDS/PAGE sample buffer, containing β-mercaptoethanol, and applied to SDS/PAGE followed by autoradiography. Lane 1, transferrin–Tyr[^{125}I]-Ala-CHN$_2$ and cathepsin L; lane 2, Fmoc-Tyr[^{125}I]-Ala-CHN$_2$ and cathepsin L; lane 3, transferrin–Tyr[^{125}I]-Ala-CHN$_2$ alone; lane 4, Fmoc-Tyr[^{125}I]-Ala-CHN$_2$ alone.

Both free and transferrin-conjugated inhibitor covalently bound to cathepsin L, and the reacted enzyme was visualized by SDS/PAGE and autoradiography (Figure 1). In the preparation of the protein–inhibitor conjugate, some non-reducible iodination of the transferrin occurred (Figure 1, lanes 1 and 3). Although the mixture containing the iodinated PDP-Tyr[^{125}I]-Ala-CHN$_2$ that was reacted with transferrin would have contained free Na^{125}I, and possibly ^{125}I$_2$, non-reducible iodination of the proteins could not be blocked by incubation with excess tyrosine during this reaction (results not shown). Furthermore, increased irreversible labelling of the protein was not seen on storage of the product, suggesting that non-specific reaction of the protein with the diazomethane group of the conjugated inhibitor was minimal. The cause of this non-specific labelling is not yet known. This labelling, however, did not interfere with the uptake of transferrin–Tyr[^{125}I]-Ala-CHN$_2$ into cells, and the labelling was used to trace uptake and release of the protein portion of the conjugate.

Identification of cathepsins B and L in HT1080 cells

Cathepsins B and L were identified and quantified in HT1080 cells using Fmoc-Tyr[^{125}I$_2$]-Ala-CHN$_2$ as described previously [17] (Figure 2). Two major bands of M_r 31000 and 5000 were labelled, corresponding to 84 ± 10 and 58 ± 9 pmol of active enzyme per mg of cell protein respectively. An additional minor band of M_r 39000 was also labelled, and the identity of this protein is not yet known [17]. Three molecular forms of cathepsin L (M_r 33000, 31000 and 25000) were detected by immunoprecipitation, and corresponded to two single-chain forms and the heavy chain of a two-chain form of the enzyme (Figure 2B) [11]. The cathepsin L bands were calculated to contribute less than 10 % of the labelling of the band of M_r 31000. The labelled single-chain form of cathepsin B was determined to be the major labelled protein in this band (Figure 2B). The unlabelled heavy

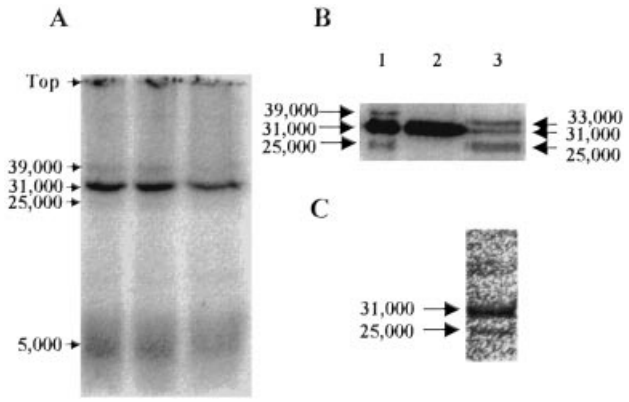

Figure 2 Detection of cathepsins B and L in HT1080 cells

HT1080 cells were incubated with Fmoc-Tyr[^{125}I]-Ala-CHN$_2$ for 3 h at 37 °C in triplicate and then labelled proteins were separated by SDS/PAGE and visualized by phosphorimager analysis (**A**). The quantities in the labelled bands were determined from the specific radioactivity of the inhibitor probe. The M_r values of the major labelled proteins are indicated with arrows. Total protein (27 μg, lane 1) or immunoprecipitates from 134 μg of HT1080 cell protein using anti-(cathepsin B) (lane 2) or anti-(cathepsin L) (lane 3) were loaded on to gels and the incorporated radioactivity determined by phosphorimager analysis as above (**B**). Western blotting was used to show the molecular forms of cathepsin B in HT1080 cells (**C**). The bands corresponding to migration positions of cathepsins are indicated with arrows.

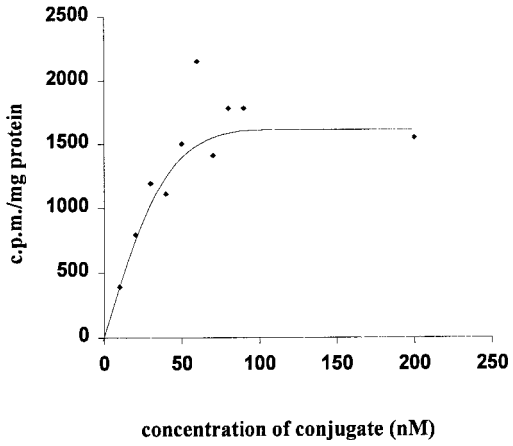

Figure 3 Binding of transferrin–Tyr[^{125}I]-Ala-CHN$_2$ to HT1080 cells

Cultured HT1080 cells were incubated at 4 °C for 10 min and then transferrin–Tyr[^{125}I]-Ala-CHN$_2$ (10–200 nM) was added. Cells were then incubated at 4 °C for 3 h, harvested, washed and the radioactivity bound to the cells and total cellular protein was determined.

chain of the two-chain form of cathepsin B was detected by Western-blot analysis (Figure 2C). These results show that the major active cysteine proteinases that react with Fmoc-Tyr[^{125}I$_2$]-Ala-CHN$_2$ in HT1080 cells are the single-chain form and the light chain of the two-chain form of cathepsin B. No labelled proteins were detected in the medium from these cells.

Characterization of transferrin receptor binding

HT1080 cells were incubated with varying concentrations of transferrin–Tyr[^{125}I]-Ala-CHN$_2$ at 4 °C and saturation of binding of the ligand to membrane receptors was seen, indicating that the modification of the transferrin still permits tight binding to the receptor (Figure 3). Treatment of the cells with PBS, adjusted to pH 2.5 with HCl, resulted in release of the bound radioactivity from the cells, and after SDS/PAGE and phosphorimager analysis no labelled proteinases were detected in cell extracts (results not shown). Binding of transferrin–Tyr[^{125}I]-Ala-CHN$_2$ to cells was inhibited by native transferrin (Table 1). However, the binding of transferrin–Tyr[^{125}I]-Ala-CHN$_2$ could not be blocked by free inhibitor. These results show that the conjugate was binding to cells via the transferrin receptor and not the inhibitor part of the molecule.

Table 1 Blocking of binding of transferrin–Tyr[^{125}I]-Ala-CHN$_2$ to HT1080 cells

Triplicate samples of cultured cells (10^6 per well) were incubated at 4 °C for 10 min. Then cells were incubated with 5 μM Fmoc-Tyr-Ala-CHN$_2$ or 5 μM human transferrin at 4 °C for 60 min. Thereafter, 50 nM transferrin–Tyr[^{125}I]-Ala-CHN$_2$ was added. After an additional 2 h incubation at 4 °C, cells were harvested, washed in cold PBS and the radioactivity remaining bound to the cells counted. Results are expressed as c.p.m. per mg of cell protein ± S.D.

Blocker	Radioactivity (c.p.m./mg of protein)	Uptake (%)
None	3540 ± 1670	100
Fmoc-Tyr-Ala-CHN$_2$	3010 ± 950	85
Transferrin	810 ± 380	23

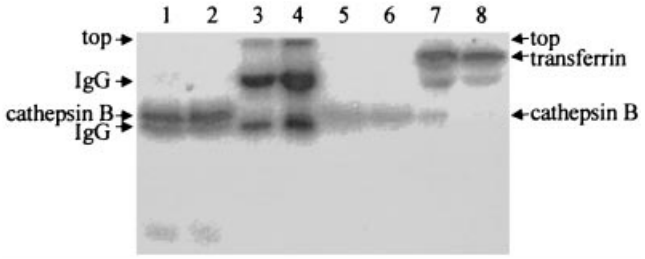

Figure 4 Recycling of conjugated inhibitors in HT1080 cells

Cultured HT1080 cells were incubated with 0.26 μM IgG–Tyr[^{125}I]-Ala-CHN$_2$ or 0.33 μM transferrin–Tyr[^{125}I]-Ala-CHN$_2$ respectively at 37 °C for 2 h. Media were removed and cells were washed with serum-free media twice. Cells were then incubated with serum-free media at 37 °C for an additional 2 h. Cells were harvested and media were centrifuged to remove cells and concentrated by filter centrifugation. Cells and concentrated media were boiled in SDS/PAGE sample buffer, containing β-mercaptoethanol, and applied to SDS/PAGE followed by autoradiography. Lanes 1 and 2, cells after IgG–Tyr[^{125}I]-Ala-CHN$_2$ labelling; lanes 3 and 4, media after IgG–Tyr[^{125}I]-Ala-CHN$_2$ labelling, lanes 5 and 6, cells after transferrin–Tyr[^{125}I]-Ala-CHN$_2$ labelling, lanes 7 and 8, media after transferrin–Tyr[^{125}I]-Ala-CHN$_2$ labelling.

Recycling of transferrin

Transferrin recycles back to the plasma membrane and is released into the medium after delivery of its cargo of iron to the cell. When HT1080 cells were cultured in the presence of the conjugate for 3 h and the excess conjugate removed by replacing the medium with inhibitor-free medium, it was found that the transferrin was released into the medium, whereas the bulk of the labelled proteinases were retained by the cells (Figure 4, lanes 5–8). Thus the transferrin–inhibitor conjugate entered cells and delivered inhibitor to endosomes. Human IgG conjugated to the inhibitor is also recycled back out of cells, presumably after binding to recycling Fc receptors [18]. Again, active proteinases were retained by the cells (Figure 4, lanes 1–4).

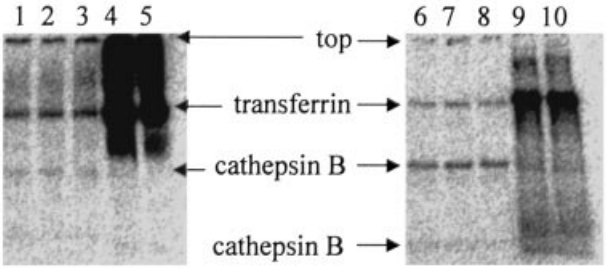

Figure 5 Effect of reduction on migration of conjugated-inhibitor-bound enzymes in SDS/PAGE

HT1080 cells were incubated with 100 nM transferrin–Tyr[^{125}I]-Ala-CHN$_2$ for 2 h. After this time cells were harvested and proteins boiled in SDS/PAGE sample buffer with or without reducing agent prior to separation by SDS/PAGE in 10–20% gradient gels. Lanes 1–5 are samples run under non-reducing conditions and lanes 6–10 are samples run under reducing conditions. Samples were extracts from cells after incubation with the conjugated inhibitor (lanes 1–3 and 6–8) or the conjugated inhibitor alone (lanes 4, 5, 9 and 10). Bands corresponding to the major labelled proteins are indicated with arrows.

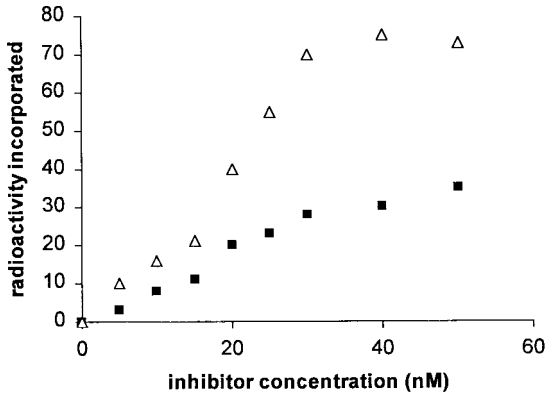

Figure 6 Concentration dependence of labelling of cellular proteinases with transferrin–Tyr[^{125}I]-Ala-CHN$_2$

HT1080 cells were incubated at 37 °C with the indicated concentration of transferrin–Tyr[^{125}I]-Ala-CHN$_2$ for 3 h and then cellular proteins were extracted and separated by SDS/PAGE. Single-chain (△) and light-chain (■) cathepsin B were detected by phosphorimager analysis. Radioactivity incorporated per mg of protein (arbitrary units) is plotted against concentration of transferrin (nM).

Stability of the disulphide bond of the conjugate in cells

Boiling of the transferrin–inhibitor conjugate in SDS/PAGE sample buffer containing mercaptoethanol (Figure 5, lanes 9 and 10) releases 90% of the radioactivity attached to the transferrin (Figure 5, lanes 4 and 5). Without reduction, very little cathepsin B can be separated from transferrin after reaction in cells (Figure 5, lanes 1–3) when compared with release after reduction (Figure 5, lanes 6–8). These results show that the majority of the proteinases that react with the inhibitor remain attached to the transferrin.

Concentration dependence of uptake of transferrin–inhibitor conjugate

The binding data above suggest that labelling of proteinases by the conjugate should be concentration-dependent but saturable. This was indeed the case (Figure 6). At very high concentrations of conjugate (above 400 nM), further labelling of the proteinases

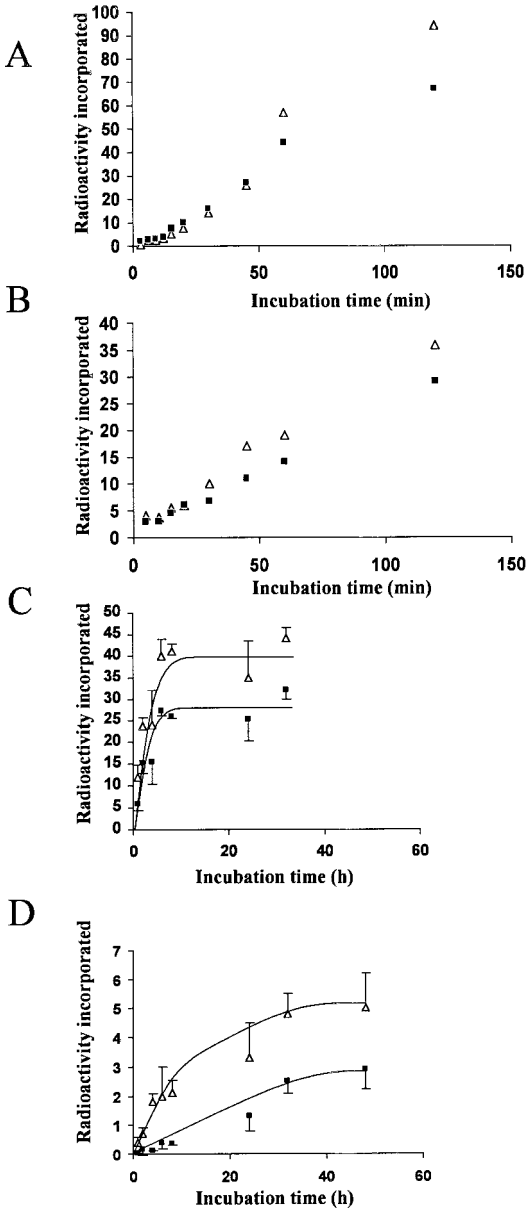

Figure 7 Time dependence of labelling of proteinases in HT1080 cells

HT1080 cells were incubated with transferrin–Tyr[^{125}I]-Ala-CHN$_2$ (**A**, **B** and **D**) or Fmoc-Tyr[^{125}I]-Ala-CHN$_2$ (**C**) for the indicated times, after which the cells were scraped, subjected to SDS/PAGE and single-chain (△) and light-chain (■) cathepsin B detected by phosphorimager analysis. Radioactivity incorporated per mg of protein (arbitrary units) is plotted against incubation time. Cells were incubated at 37 °C (**A**, **C** and **D**) or 16 °C (**B**) in serum-free medium (**A**, **B**) or medium containing 10% serum (**C**, **D**). (**A**) and (**B**) are representative examples of individual experiments; (**C**) and (**D**) show means ± S.D. of triplicate samples at each time point.

was seen, presumably due to fluid-phase endocytosis of the conjugate that would permit delivery to the lysosome (results not shown). Both the single chain and light chain of the two-chain form of cathepsin B were labelled.

Temperature- and time-dependent labelling of proteinases by inhibitors

Fmoc-Tyr[^{125}I$_2$]-Ala-CHN$_2$ rapidly diffused into cells and complete labelling of the proteinases was achieved within a few hours

(Figure 7C). Although the conjugated inhibitor was able to bind to HT1080 cells at 4 °C (Figure 3), no labelling of proteinases could be detected (results not shown). Uptake of the conjugate and labelling of proteinases at 37 °C increased with increasing incubation time (Figure 7A). Both molecular forms of cathepsin B were detected from the first time point (3 min). Saturation of labelling could not be demonstrated, even after 48 h incubation with the conjugate, although the amount of labelling at 48 h was not significantly different to that seen at 32 h (Figure 7D). By this time, the labelling of proteinases by the conjugated inhibitor was approximately 10 % of that obtained using the free inhibitor. Incubation of cells at 16 °C reduced labelling by approximately 50 % when compared with labelling at 37 °C, but the relative proportions of the two labelled molecular forms of cathepsin B were not significantly altered (Figures 7A and 7B). Incubation of cells at 16 °C has been shown to inhibit transfer of proteins from endosomes to lysosomes, but has little effect upon endocytic uptake [19].

DISCUSSION

A conjugate between transferrin and a peptidyl diazomethane (Tyr[^{125}I]-Ala-CHN$_2$) has been synthesized. The molecule retains all of the characteristics of native transferrin: it is taken up by cells by receptor-mediated endocytosis, its endocytosis is blocked by unmodified transferrin, it is efficiently delivered to endocytic compartments at 37 °C and 16 °C but is excluded from cells at 4 °C, and it recycles back out of the cell. The inhibitor is attached to the transferrin by a disulphide bond and this bond is stable in cells but can be reduced in order to identify reacted proteins after SDS/PAGE and autoradiography.

Both mature and intermediate molecular forms of cathepsin B are labelled by the reagent, showing that endosomes contain both forms of active proteinases. The different molecular forms of cathepsin B are indistinguishable from those found in the whole cell. Both forms are labelled when cells are incubated at 37 °C or 16 °C and both are labelled within 3 min of incubation of the conjugated inhibitor with the cells. Furthermore, the majority of the reacted proteinases remain bound to the transferrin via a reducible link, consistent with previous work that indicates that the environment within the endosome is non-reducing and that disulphide-bond reduction does not occur unless disulphides are delivered to the lysosome [20,21]. However, the active-site thiols of the proteinases are not oxidized and do not need the addition of thiol reagents in order to react with the inhibitor, indicating that they are active within the endosome. These results indicate that the reacted proteinases are in endosomes and do not reflect leakage of a significant portion of the reagent into the lysosomal compartment. The endosomal proteinases were calculated to comprise up to 10 % of the total active enzymes in the cell, and probably represent the active proteinases measured in subcellular fractions of endosomes seen by others (e.g. [4–6,22]).

The demonstration that the active forms of cathepsin B in endosomes are indistinguishable from those found in the whole cell indicates that they are essentially identical to lysosomal enzymes. Biosynthetic precursors of lysosomal proteins are trafficked to lysosomes by MPRs [1]. The initial cleavage to produce active enzymes from inactive precursors of the proteinases usually takes place 30–60 min after initial translation, and this time is approximately equivalent to the transit time from the endoplasmic reticulum to the endosome or early lysosome. Further processing is much slower, taking from 3 to 24 h, and is presumably due to the limited susceptibility of the active enzymes to other proteinases in the lysosome [23–26]. If the processing of pro-cathepsin L is blocked, the pro-enzyme is delivered to dense lysosomes within 60 min, showing that even though initial processing could occur in the endosome, the slower processing to mature forms must occur after delivery to the lysosome [27]. The simplest interpretation of the data is that the majority of the active proteinases in endosomes originate from lysosomes by retrograde traffic and that this permits an equilibrium to be set up between the two compartments. Retrograde traffic of endocytosed molecules from lysosomes to endosomes has been proposed by others [28,29]. We conclude that although the bulk flow of endocytosed molecules that are not rapidly recycled back to the plasma membrane is to the lysosome and that the majority of the lysosomal enzymes are also delivered to the lysosome, retrograde traffic does occur. This 'leakage' back into the endosomal system can account for the appearance of active lysosomal proteinases in isolated endosomes.

In a biosynthetic study of the processing of the lysosomal proteinase cathepsin D, it has been shown that organelles precipitated with an antibody to the cytoplasmic domain of the 300 kDa MPR contain mostly newly synthesized pro-cathepsin D [30]. A minor population of processed forms of the enzyme was also found in the MPR-positive organelles, and the authors suggested that this indicates that processing may be initiated in this MPR-positive organelle prior to trafficking to lysosomes. However, both intermediate and mature forms of cathepsin D were found in the MPR-positive organelles [30]. In addition, after long chase periods mature forms of cathepsin D were still found in the precipitated organelles. These results would be predicted if most of the proteinases were activated in the lysosome and returned to the endosome by retrograde traffic.

The appearance of active lysosomal proteinases in endosomes suggests that lysosomal enzyme packaging is poorly regulated in HT1080 cells. However, labelled proteinases were not released from HT1080 cells (this study), or from a range of breast cancer cells [31], and no cell-surface labelling of proteinases could be detected after incubation of cells at 4 °C. Only a small proportion of the total cellular active enzymes were found in endosomes, indicating that retrograde traffic is regulated in order to retain most of the enzymes in the lysosome. The mechanisms by which soluble proteins are retained by the endosomal/lysosomal system are not yet known, but it appears that retention of individual lysosomal proteinases may vary. Subcellular fractionation of J774 macrophages suggests that early endosomes preferentially contain cathepsin H, late endosomes preferentially contain cathepsin S and lysosomes preferentially contain cathepsin B [22]. Although the reason for this asymmetric distribution of three structurally related enzymes is not known, it could be due to an equilibrium between the different compartments, mediated by differential affinities for low-affinity receptor-like molecules. Weak retention signals for lysosomal and endosomal proteinases could also account for the release of active proteinases by other cell types (e.g. [32]). The efficiency of retention signals could therefore regulate activity of proteinases in endosomes and other cellular compartments. Proteolysis in endosomes is also likely to be regulated by other factors, including oxidation/reduction and pH. The non-reducing and moderate pH environment in the endosome will not be as denaturing to endocytosed proteins when compared with the environment in the lysosome, providing an environment for limited proteolysis.

The unique features of this new technique are that the active proteinases in endosomes are identified directly by a protein–inhibitor conjugate that is specifically targeted to recycling endosomes and that the molecular forms of the enzymes can be identified. Separation of individual cellular compartments is not

required and the inactivation of proteinases by pH or naturally occurring inhibitors after extraction is avoided. Furthermore, it is not necessary to add non-physiological concentrations of reducing agents and manipulate assay conditions in order to detect the active proteinases.

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