The identification of active forms of cysteine proteinases in Kirsten-virus-transformed mouse fibroblasts by use of a specific radiolabelled inhibitor

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The major active forms of cathepsins B and L were identified in Kirsten-virus-transformed mouse fibroblasts by the use of a specific radiolabelled inhibitor, benzyloxycarbonyl-Tyr(-^{125}I)-Ala-CHN$_2$. No other proteins were labelled, demonstrating the specificity of this inhibitor for cysteine proteinases. Cathepsins B and L were distinguished by the use of specific antibodies. One active form of cathepsin B, M_r 33000–35000, and two active forms of cathepsin L, M_r 30000 and 23000, were identified. The intracellular precursors of these proteins had higher M_r values of 39000 and 36000 for cathepsins B and L respectively, as shown by pulse–chase experiments with [^{35}S]methionine-labelled proteins. These did not react with the inhibitor under our culture conditions. The precursor of cathepsin L was secreted whereas the precursor of cathepsin B was not, demonstrating that secretions of the two enzymes are regulated differently. In contrast with results found previously for the purified protein [Mason, Gal & Gottesman (1987) Biochem. J. 248, 449–454], the secreted precursor form of cathepsin L did not react with the inhibitor either, indicating that it is not active and therefore, as such, cannot be directly involved in tumour invasion. The secreted protein did react with the inhibitor when incubated at pH 3.0, showing that the protein can be activated, although this did not occur under our culture conditions.

INTRODUCTION

The cysteine proteinases cathepsins B and L are synthesized as larger precursors and subsequently processed to their mature lysosomal forms. This has been shown by pulse–chase experiments and deduced by comparing primary protein sequences derived from cDNA sequences with the protein sequences of mature enzymes (Chan et al., 1986; Mason et al., 1986; Hanewinkel et al., 1987; Nishimura & Kato, 1987). The mature forms of the enzymes are found in lysosomes and have M_r values in the range 25000–33000 (Barrett & Kirschke, 1981; Mason, 1986a). These mature forms of the enzymes are active against a number of proteins, including components of extracellular matrix such as collagen and elastin. They have a general role in protein turnover in the lysosome, and in conjunction with lysosomal exopeptidases degrade proteins to small peptides and single amino acids.

Cathepsins B and L have also been reported to be found extracellularly (Baici & Knöpfel, 1986; Chang et al., 1986; Mort & Recklies, 1986). Some of these are active whereas others require activation. The extracellular role of these enzymes is less clear, however. They may be involved in degradation of extracellular-matrix components in diseases such as osteoporosis, rheumatoid arthritis or emphysema (Delaissé et al., 1984; Poole & Mort, 1981; Mason, 1988). Extracellular cathepsin B-like activity has been correlated with metastatic potential of tumour cells (Sloane & Honn, 1984).

Kirsten-virus-transformed mouse fibroblasts have been found to secrete the precursor of cathepsin L (Gottesman, 1978; Mason et al., 1987). Expression of the secreted protein correlates with the metastatic potential of ras-transformed mouse fibroblasts, and it has been proposed that the enzyme may be involved in tumour invasion (Denhardt et al., 1987). In support of this hypothesis we have previously demonstrated that the purified precursor of mouse cathepsin L is an active enzyme (Mason et al., 1987).

We have now taken an alternative approach in order to detect active forms of cysteine proteinases. The peptidyldiazomethane proteinase inhibitors have been demonstrated to be specific inhibitors of cysteine proteinases (Watanabe et al., 1979). They react covalently with the active-site cysteine residue and do not react with inactive or denatured enzymes. We have identified Z-Tyr-Ala-CHN$_2$ as being a good inhibitor of cathepsins B and L but ineffective against calpain (Crawford et al., 1988). Iodination of the tyrosine provides an even better inhibitor. The present paper describes the use of the radio-iodinated inhibitor to identify active forms of cathepsins B and L in cultures of Kirsten-virus-transformed mouse fibroblasts, enabling us to demonstrate the presence of active forms of these enzymes without using added activators or extracting and purifying the proteins. Specific antibodies were used to confirm the identity of these active enzymes and to demonstrate the biosynthetic processing of cathepsins B and L.

MATERIALS AND METHODS

Materials

Protein A–Sepharose CL-4B was purchased from Pharmacia. Radiochemicals were from Amersham, and

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Iodogen was from Pierce Chemical Co. Papain and E-64 were obtained from Sigma Chemical Co. Rabbit anti-(mouse cathepsin L) IgG preparations were gifts from Dr. M. M. Gottesman (National Cancer Institute, Bethesda, MD, U.S.A.) and Dr. D. A. Portnoy (The Rockefeller Institute, New York, NY, U.S.A.). Affinity-purified sheep anti-(human cathepsin B) IgG was a gift from Dr. D. J. Buttle of the Strangeways Research Laboratory. Rabbit anti-(human cathepsin L) serum was raised against purified human cathepsin L as described previously (Mason et al., 1985; Mason, 1986a).

Preparation of inhibitor

Z-Tyr-Ala-CHN$_2$ was prepared as described previously (Crawford et al., 1988) and iodinated by using the Iodogen method (Fraker & Speck, 1978). For this 10 μl of 50 mM-sodium phosphate buffer, pH 7.5, 10 μl of Na^{125}I (1 mCi) and 25 μl of Z-Tyr-Ala-CHN$_2$ (1 mM in 25% ethanol) were added to an Iodogen-coated glass tube and incubated at 0 °C for 10 min. Then 455 μl of 50 mM-sodium phosphate buffer, pH 7.5, was added and the reaction was stopped by removing the mixture from the Iodogen-coated tube.

The concentration of active inhibitor remaining was determined by titration with papain that had previously been titrated with E-64 (Barrett & Kirschke, 1981). The specific radioactivity of the iodinated inhibitor was determined by measuring trichloroacetic acid-precipitable radioactivity after reaction with papain. Typically, 90–95% inhibitory activity was recovered with a specific radioactivity of 6.0 mCi/μmol of inhibitor.

Cell-culture experiments

KNIH 3T3 cells were cultured with or without serum in Dulbecco's Modified Eagle's Medium (Imperial) as described previously (Gottesman, 1978). Pulse–chase experiments with [^{35}S]methionine were carried out in the absence of serum as described by Gal et al. (1985). Labelling of cysteine proteinases was performed by growing cells in medium containing 0.1 μM radiolabelled inhibitor.

Immunoprecipitation

Cathepsin B. Cell extracts were prepared by using a lysis medium of 20 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-EDTA, 0.2% SDS and 1% Triton X-100. A pre-clear was performed with normal sheep serum. Specific immunoprecipitation of cathepsin B was performed with affinity-purified sheep anti-(human cathepsin B) IgG (5 μl), affinity-purified rabbit anti-(sheep IgG) antibody (10 μl) and 10% (w/v) Protein A–Sepharose (30 μl) for 2 h at 20 °C. The resulting precipitate was washed three times in lysis buffer and then boiled for 5 min in SDS/polyacrylamide-gel-electrophoresis sample buffer.

Cathepsin L. Cell extracts were prepared as above, and subjected to reduction and carboxymethylation by first boiling for 15 min in 10 mM-dithiothreitol and then boiling for 15 min in 20 mM-iodoacetamide. Specific immunoprecipitation of cathepsin L was with rabbit anti-(mouse cathepsin L) IgG (10 μl) and 10% (w/v) Protein A–Sepharose (30 μl) for 2 h at 20 °C.

Gel electrophoresis and autoradiography

SDS/polyacrylamide-gel electrophoresis was performed as described by Bury (1981). Autoradiography of iodinated proteins was performed with pre-flashed X-ray film exposed at −70 °C with an intensifying screen. Fluorography of [^{35}S]methionine-labelled proteins was performed by autoradiography after impregnation of gels with 2,5-diphenyloxazole. M_r standards used were sheep IgG (M_r 50 000 and 25 000), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (M_r 36 000), bovine carbonic anhydrase (M_r 29 000), soya-bean trypsin inhibitor (M_r 21 000) and bovine β-lactoglobulin (M_r 18 000).

RESULTS

Biosynthesis and processing of cathepsins L and B

In order to determine which molecular forms of cathepsins B and L exist in cultures of Kirsten-virus-transformed fibroblasts, pulse–chase experiments were performed with specific antibodies. Three different molecular forms of cathepsin L were seen in cell extracts, with M_r 36 000, 30 000 and 23 000 (see Fig. 1a). Only the 36 000-M_r protein was detected in the culture medium.
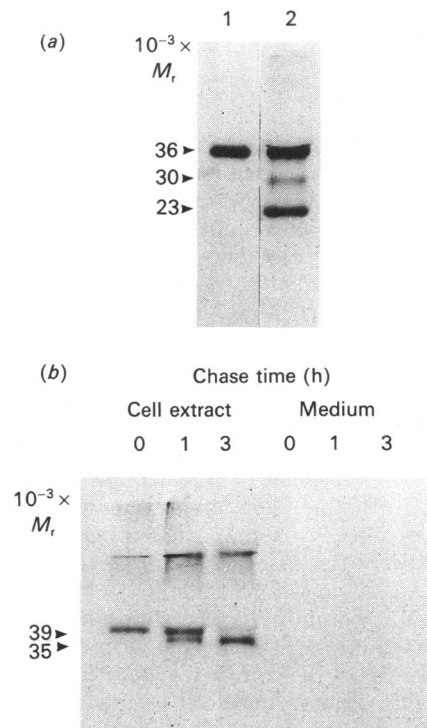

Fig. 1. [^{35}S]Methionine-labelled proteins from KNIH 3T3 cells

(a) Fluorogram of SDS/12.5%- polyacrylamide gel of anti-(cathepsin L) immunoprecipitates from KNIH 3T3 cell extracts. Cells were initially seeded at a density of 2 × 10^5/well in 24-well multiwell plates and after 2 days were pulsed for 15 min followed by a 3 h chase. Lane 1, medium; lane 2, cell extract. (b) Fluorogram of SDS/12.5% polyacrylamide gel of anti-(human cathepsin B) immunoprecipitates from KNIH 3T3 cell extract and medium. The cells were treated as above except that medium was made 75% saturated with (NH$_4$)$_2$SO$_4$ and the resulting precipitate was resuspended in 1 ml of cell lysis medium, pH 7.5, before immunoprecipitation.

This is consistent with results found previously with these cells by Gal *et al.* (1985), except that the M_r values differed slightly, most probably because of the different gel systems used. An antibody raised to human cathepsin L precipitated proteins of the same M_r (results not shown). The pattern of labelling was not significantly different when the cells were grown in the presence of 0.5 μM-Z-Tyr(-I)-Ala-CHN$_2$ (results not shown). Our results demonstrate that these cells also synthesize cathepsin B (Fig. 1*b*). The precursor has an M_r of 39000 and this is processed after 3 h to a form of M_r 35000. In contrast with cathepsin L, the cells did not secrete cathepsin B in detectable quantities.

Inhibitor-labelling

When cells were cultured in the presence of Z-Tyr(-^{125}I)-Ala-CHN$_2$ and then analysed by SDS/poly-acrylamide-gel electrophoresis and autoradiography, specific labelling of three bands, of M_r 35000, 30000 and 23000, was observed (Figs. 2*a* and 2*b*). The higher band seen on some autoradiographs represents protein that had not entered the gel. After 30 min and 1 h only two
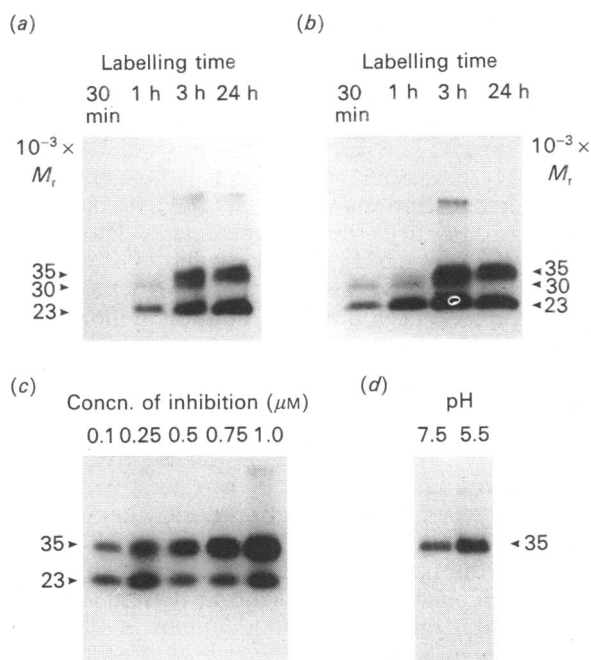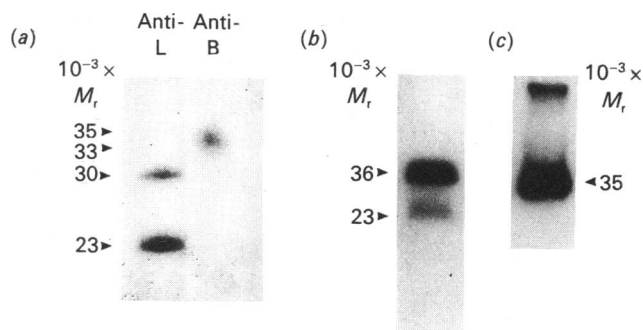

Fig. 3. Anti-(cathepsin L) and anti-(cathepsin B) immuno-precipitations of inhibitor-labelled KNIH 3T3 cell extracts and culture medium

(*a*) Autoradiograph of KNIH cell immunoprecipitates after 3 h inhibitor-labelling. A confluent layer of KNIH cells was labelled and immunoprecipitated with anti-(cathepsin B) IgG and anti-(cathepsin L) IgG as described in the Materials and methods section. (*b*) Immuno-precipitation of 24 h-conditioned medium from KNIH cells with anti-(cathepsin L) IgG. A confluent monolayer of cells was grown in serum-free medium overnight, and the medium was harvested and filter-concentrated. The pH of the medium was lowered to 3.0 with 5 M-sodium formate buffer, pH 3.0, in the presence of 1 μM radiolabelled inhibitor and labelled for 30 min at 30 °C. Immuno-precipitation was performed as described in the Materials and methods section after re-adjustment back to pH 7.5. (*c*) Cells were labelled as described in Fig. 2(*d*) at pH 5.5 and then immunoprecipitated with anti-(human cathepsin B) IgG as described in the Materials and methods section.

Fig. 2. Inhibitor-labelling patterns of KNIH 3T3 cells

(*a*) and (*b*) SDS/12.5%- polyacrylamide gel of trichloro-acetic acid precipitates of KNIH 3T3 cell extracts labelled during culture with radiolabelled inhibitor (as described in the Materials and methods section). The cells were labelled either in the presence (*a*) or in the absence (*b*) of serum. (*c*) Trichloroacetic acid precipitates of inhibitor-labelled KNIH 3T3 cell extract after 3 h labelling in the absence of serum at increasing inhibitor concentration. (*d*) Trichloro-acetic acid precipitates of KNIH 3T3 cell extract labelled with 0.1 μM inhibitor after lysis. Cells were harvested and lysed either at pH 7.5 (20 mM-Tris/HCl buffer containing 10 mM-EDTA, 0.2% SDS and 1% Triton X-100) or at pH 5.5 (50 mM-sodium acetate buffer containing 10 mM-EDTA, 0.2% SDS and 1% Triton X-100), then incubated at 30 °C for 30 min in the presence of 0.1 μM inhibitor. Protein from an equivalent number of cells was loaded per lane.

bands, of M_r 30000 and 23000, were labelled, but after 3 h and 24 h an additional band, of M_r 35 000, appeared. The relative intensity of labelling of the bands was similar at 24 h and 3 h, and therefore a 3 h incubation was chosen for routine labelling experiments. Cells labelled in medium containing serum showed a similar pattern of labelling, although the intensity was diminished and time course delayed, possibly owing to reversible binding of inhibitor by serum components. No labelling of proteins in the medium was observed.

Labelling of proteins was dependent on concentration of inhibitor used (Fig. 2*c*). This did not affect the pattern of labelling, however, and thus we decided to use a low concentration of inhibitor (0.1 μM) in order to conserve reagent and decrease the risk of toxicity.

Immunoprecipitation

The three different labelled proteins were identified by immunoprecipitation. The proteins of M_r 30000 and 23000 were precipitated by anti-(cathepsin L) IgG, and the protein of M_r 35000 was precipitated by anti-(cathepsin B) IgG (Fig. 3). The cathepsin B immuno-precipitate appeared as a diffuse band of M_r 33000–35000 when run on these larger gels. Precipitation of these bands was blocked when antibody was pre-incubated with its respective antigen (results not shown).

When cells were homogenized in either 20 mM-Tris/HCl buffer, pH 7.5, or 20 mM-sodium acetate buffer, pH 5.5, and then labelled in the presence of 1 μM inhibitor and 1 mM-dithiothreitol, only one band, of M_r 35000, was labelled (Fig. 2*d*). Immunoprecipitation showed that this was cathepsin B (Fig. 3*c*). When 24 h-conditioned

medium was incubated with 1 mM-dithiothreitol and 1 μM inhibitor at pH 7.5 or pH 5.0 for 1 h, no proteins were labelled. However, when the pH was lowered to 3.0, labelling was observed. Immunoprecipitation of these showed that there were two forms of cathepsin L, one of M_r 36000 and one of M_r 23000 (Fig. 3b). This is consistent with the precursor and mature forms of cathepsin L.

DISCUSSION

We have demonstrated that Kirsten-virus-transformed mouse fibroblasts synthesize cathepsins B and L. The pattern of processing of cathepsin L confirms the previous findings by Gal et al. (1985). We have shown that these cells also synthesize cathepsin B as a precursor of M_r 39000 and process it to an active form of M_r 33000–35000. In contrast, human skin fibroblast cathepsin B is synthesized as a protein of M_r 46000 and then processed to mature forms of M_r 33000 and 27000 (Hanewinkel et al., 1987), and rat hepatocyte cathepsin B is synthesized as a precursor of M_r 39000 and processed to a form of M_r 29000 (Nishimura & Kato, 1987).

Pulse–chase experiments do not show which forms of the enzymes are active. This can only be achieved by isolating the individual proteins or by taking an approach similar to ours. The active-site-directed inhibitors only react with active enzyme, and are thus able to distinguish between inactive precursors and degradation products and the functional proteinases. Docherty et al. (1984) have previously used a radiolabelled chloromethane to label cathepsin B in lysosomes and secretory granules from a rat insulinoma. Chloromethanes are reactive and may be toxic to cells. Furthermore they can also inhibit serine proteinases and react non-specifically with thiols. In contrast, the diazomethanes are specific for cysteine proteinases, and did not react covalently with any other proteins in our culture conditions; nor was cell growth affected at the inhibitor concentration used.

Cathepsin L was labelled within 30 min of addition of inhibitor whereas cathepsin B did not appear to become labelled until later. This time-dependent labelling was expected because of the lower rate of inactivation of cathepsin B by this inhibitor than of cathepsin L (Crawford et al., 1988). Another limitation on rate of reaction would be the rate of uptake of this inhibitor and hence effective concentration in the lysosome. Shaw & Dean (1980) have suggested that uptake of Z-Phe-Ala-CHN$_2$ could be by pinocytosis, although diffusion of the hydrophobic molecules across membranes could not be discounted as these authors found that significant amounts of inhibitor were removed from cells by washing. In our system, labelling of proteins increased with increased concentration of inhibitor, showing that not all of the active enzyme present was labelled and therefore our results are qualitative rather than quantitative. A quantitative assessment was not possible because we could not effectively determine the concentration of inhibitor in any specific compartment of the cell. Complete saturation would not be expected to occur with micromolar concentrations of inhibitor, as the concentration of cysteine proteinases in lysosomes in millimolar (Dean & Barrett, 1976).

Limitations on concentration of inhibitor by comparison with concentration of enzyme are not apparent with labelling of cell extracts. In this case the concentration of cysteine proteinases was diluted by approx.

10^5-fold, such that 1 μM inhibitor would be significantly higher than the concentration of enzymes. Addition of dithiothreitol and an incubation time of 30 min would be sufficient for complete reaction with active cathepsins B and L (Crawford et al., 1988). Only cathepsin B was labelled under these conditions. There are two possible explanations for this. Cathepsin L could have been denatured irreversibly during extraction, or labelling could be inhibited by interaction of the enzyme with intracellular inhibitors or substrates when cells are disrupted. The major cysteine-proteinase inhibitors have K_i values in the picomolar range for cathepsin L whereas they bind much less tightly to cathepsin B (for review see Barrett, 1987). This has been a major problem in the study of cathepsin L, which has no activity in tissue homogenates unless these inhibitors are destroyed by autolysis at acidic pH (Mason et al., 1985). Our procedure of labelling living cells overcomes this problem, allowing detection of active enzymes in situ. The M_r of active cathepsin B was the same when labelled in living cells (i.e. 33000–35000). This is somewhat larger than mature active forms purified from other species (Mason, 1986b; also see above). Some of these differences could be explained by species variations; bovine cathepsin B has been purified as two different molecular forms having similar catalytic activities (Mason, 1986b).

The predominant active form of cathepsin L in these cells has an M_r of 23000. This is consistent with it being the mature two-chain form of mouse cathepsin L (cf. Mason, 1986a). The small amount of label at M_r 30000 presumably represents single-chain cathepsin L. This is the first demonstration that the intermediate biosynthetic form of cathepsin L is an active enzyme. This indicates that further processing to the lower-M_r form is not necessary for activation of this enzyme, but more probably represents limited degradation in the hydrolytic environment of the lysosome.

Under culture conditions the precursor protein of M_r 36000 was not labelled at all. Nor were any proteins in the medium, even though this contains pro-cathepsin L. Even when the pH of the medium was lowered to 5.0 and 1 mM-dithiothreitol was added in order to mimic conditions in which the purified pro-enzyme was active (Mason et al., 1987), no labelling occurred. The potential activity of the precursor was demonstrated by reaction with the inhibitor at pH 3.0. Thus at present we have no direct demonstration that the precursor is active under normal cellular conditions. However, the purified protein and acid-treated enzyme do exhibit activity, presumably by undergoing unknown conformational changes (Mason et al., 1987). If the pro-enzyme is involved in the metastatic activity of transformed cells or degradation of extracellular matrix in diseases such as rheumatoid arthritis and emphysema, then the enzyme would have to either undergo a similar conformational change, or be proteolytically converted into lower-M_r active forms.

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