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A putative protein-sequestration site involving intermediate filaments for protein degradation by autophagy

Studies with microinjected purified glycolytic enzymes in 3T3-L1 cells

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Several glycolytic enzymes (lactate dehydrogenase, pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase) were radiolabelled by [^{125}I]iodination, conjugation with ^{125}I-labelled Bolton & Hunter reagent and reductive [^{3}H]methylation, and their degradative rates after microinjection into 3T3-L1 cells compared with that of the extracellular protein bovine serum albumin. Although the albumin remains largely cytosolic in recipient cells, the glycolytic enzymes rapidly (< 30 min) become insoluble, as measured by detergent and salt extractions. The microinjected glycolytic enzymes appear to form disulphide-linked aggregates, are found in a cell fraction rich in vimentin-containing intermediate filaments and histones (nuclear–intermediate-filament fraction), and are degraded slowly by a lysosomal mechanism, as judged by the effects of inhibitors (NH_4Cl, leupeptin, 3-methyladenine). ^{125}I-labelled bovine serum albumin appears to be degraded rapidly and non-lysosomally. Prolonged treatment (96 h) of cultured cells with leupeptin results in the accumulation of pulse-labelled ([^{35}S]methionine for 24 h) endogenous cell proteins in the detergent- and salt-non-extractable residue, but NH_4Cl and 3-methyladenine do not have this effect. The findings are discussed in terms of (1) the interpretation of experiments involving microinjection of proteins to study intracellular protein catabolism and (2) intermediate filaments as a protein-sequestration site for protein degradation by autophagy.

INTRODUCTION

Microinjection techniques are increasingly employed in the study of intracellular protein catabolism (Doherty & Mayer, 1985; Rogers & Rechsteiner, 1985; Dice *et al.*, 1985). It has been generally assumed that proteins microinjected into cells will simply act as probes for the degradation mechanisms responsible for the catabolism of endogenous proteins. However, in a previous study (Doherty & Mayer, 1985) we have shown that, despite microinjecting homologous cytosolic proteins into 3T3-L1 cells, and regardless of the labelling or microinjection procedures employed, a large proportion of the injected cytosolic proteins become sedimentable after microinjection. Similarly it has been demonstrated that many different proteins, all apparently 'soluble', become detergent-insoluble after microinjection (Rogers & Rechsteiner, 1985). Our previous work involved the transfer of a relatively complex mixture of cytosolic proteins between cells (Doherty & Mayer, 1985). In order to simplify the studies and in an attempt to identify the factors that determine the intracellular fate of microinjected proteins, we have now carried out microinjection studies with commercially available purified glycolytic enzymes in comparison with bovine serum albumin.

MATERIALS AND METHODS

Materials

Cells (3T3-L1) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) Culture medium, serum, tissue-culture dishes, radioisotopes and antibodies were obtained as described previously (Doherty & Mayer, 1985). 1,3,4,6-Tetrachloro-3,6-diphenylglycouril (Iodogen), rabbit muscle lactate dehydrogenase (freeze-dried), pyruvate kinase (freeze-dried) and glyceraldehyde-3-phosphate dehydrogenase (crystalline suspension) were obtained from Sigma (Poole, Dorset, U.K.). Nycodenz and Maxidenz were purchased from Nyegaard and Co. (Oslo, Norway).

Cell culture

3T3-L1 cells, a pre-adipocyte cell line, were maintained in culture and subcultured as described previously (Doherty & Mayer, 1985).

Labelling of proteins

Radioiodination of proteins was carried out either with Na^{125}I with chloramine-T (Hunter & Greenwood, 1962) or Iodogen (Pittman *et al.*, 1983) as oxidizing agents or with *N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate (Bolton & Hunter, 1973). Radioiodination with chloramine-T has been described previously (Doherty & Mayer, 1985). Proteins were radiolabelled with Iodogen as oxidizing agent essentially as described by Pittman *et al.* (1983), and unincorporated Na^{125}I was removed by centrifuge desalting on a column (1 ml) of Sephadex G-25 or G-50.

Reductive methylation of proteins with NaB^3H_4 was carried out as described previously (Doherty & Mayer, 1985).

Introduction of proteins into cells

Proteins were introduced into culture cells by either erythrocyte-mediated microinjection or scrape-loading as described previously (Doherty & Mayer, 1985), except that monolayers were washed twice in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (Doherty & Mayer, 1985) before scrape-loading.

Table 1. Effect of inhibitors on the degradation of proteins introduced into 3T3-L1 cells

Proteins were labelled with ^{125}I by using either $Na^{125}I$ and chloramine-T (†) or Iodogen (**) as oxidizing agents or with Bolton and Hunter reagent (††). Proteins were labelled with ^{3}H by reductive methylation with $NaB^{3}H_4$ (*).

Protein	Method of introduction into the cell	Addition...	$t_{\frac{1}{2}}$ (h) None	Inhibition (%) NH_4Cl	Leupeptin	3-Methyladenine
Lactate dehydrogenase	Erythrocyte microinjection		165†	71.4	—	—
	Erythrocyte microinjection		138*	91.0	—	—
	Scrape loading		182††	74.7	79.7	70.0
	Scrape loading		124††	—	72.1	—
	Scrape loading		143††	—	76.3	73.0
	Scrape loading		150**	66.6	47.4	—
Pyruvate kinase	Erythrocyte microinjection		217†	50.0	—	—
	Scrape loading		330**	—	62.0	62.0
Glyceraldehyde-3-phosphate dehydrogenase	Scrape loading		131**	—	64.2	66.0
Bovine serum albumin	Erythrocyte microinjection		17†	—	—	—
	Erythrocyte microinjection		20†	20	—	—

Cell fractionation

(1) Detergent and salt fractionation. Monolayers or cell pellets were treated for 4 min at 4 °C with 0.5 ml of digitonin (1 mg/ml) in 0.25 M-sucrose/3 mM-EGTA/17 mM-Mops, pH 7, to extract cytosolic proteins (Mackall *et al.*, 1979). After removal of the digitonin extract, in the case of cell pellets after centrifugation at 1000 *g* for 10 min, monolayers and cell pellets were suspended in 1% (w/v) Triton X-100 in 1.5 mM-$MgCl_2$/100 mM-Hepes, pH 6.9 (Heuser & Kirschner, 1980), and cells were incubated for 1 h at 4 °C, centrifuged as above and, after removal of the Triton X-100-soluble extract, the pellet was treated for 3 h with 0.3 M-KI at 4 °C to remove actin (Heuser & Kirschner, 1980). After centrifugation as above, the KI extract was removed, leaving an insoluble white fibrous residue. Extracts were precipitated with trichloroacetic acid (final concn. 10%, w/v), and acid-insoluble precipitates and the final cell residue were dissolved in 1 M-NaOH (1 ml) for the determination of radioactivity.

(2) Density-gradient fractionation of cell homogenates. Cell monolayers (confluent, from six to eight 90 mm culture dishes) were washed once with phosphate-buffered saline (Doherty & Mayer, 1985) and once with homogenization medium (218 mM-sucrose/3 mM-KCl/0.3 mM-EDTA/5 mM-Tris/HCl, pH 7.5) before being scraped in 4 ml of homogenization medium at 4 °C. Cells were homogenized by ten strokes in a Dounce tissue grinder (Wheaton Scientific, Millville, NJ, U.S.A.) with a clearance of 0.025–0.076 mm, and centrifuged at 1000 *g* for 10 min at 4 °C. The resulting supernatant was removed, and the pellet rehomogenized in homogenization medium with a further ten strokes and re-centrifuged as above. The supernatant was again removed and the pellet resuspended as above. The supernatants and the suspended final pellet were pooled, and a sample (4 ml) was applied to the top of a preformed density gradient of Nycodenz. Gradients were prepared by layering successively 2 ml solutions of 50% (w/v) Nycodenz in water and 27.6, 18.4, 13.5 and 9.2% (w/v) Nycodenz in homogenization medium into a 16 ml centrifuge tube. After capping the tube, gradients were left to diffuse on their sides for 60 min at room temperature before being cooled to 4 °C before use. Gradients with applied samples were centrifuged at 84000 *g* for 30 min in a swing-out rotor. Fractions (0.5 ml) were collected from the gradients by upward displacement with Maxidenz and stored at −20 °C.

Enzyme and other assays

Lactate dehydrogenase (EC 1.1.1.27), succinate dehydrogenase (EC 1.3.99.1) and protein were determined as described previously (Doherty & Mayer, 1985). Leucine aminopeptidase (EC 3.4.11.1) (Peters *et al.*, 1972), *N*-acetylhexosaminidase (EC 3.2.1.52), acid phosphatase (EC 3.1.3.2) and β-glucuronidase (EC 3.2.1.31) (Loffler *et al.*, 1984) activities were also determined. DNA was determined fluorimetrically (Labarca & Paigen, 1980).

SDS/polyacrylamide-gel electrophoresis

This was carried out as described by Doherty & Mayer (1985). Proteins were detected by staining with Coomassie Brilliant Blue, and radioactive proteins by autoradiography.

Measurement of protein degradation

The rates of degradation of radiolabelled proteins were determined as described previously (Doherty & Mayer, 1985).

RESULTS AND DISCUSSION

Radiolabelling of proteins

Proteins radioiodinated with $Na^{125}I$ were prepared with 0.25–2.00 atoms of iodine incorporated per

Table 2. Detergent and salt extraction of radioactive proteins introduced into 3T3-L1 cells

Proteins were labelled with ^{125}I by using chloramine-T or Iodogen (**). Detergent and salt extracts were obtained as described in the Materials and methods section. Results are means ± S.D. for four separate measurements.

Protein	Method of introduction into cell	Extracted protein (% of total acid-insoluble radioactivity)			
		Digitonin	Triton X-100	KI	Residue
Lactate dehydrogenase	Erythrocyte microinjection	15.1 − 5.0	3.5 ± 0.4	3.2 ± 0.6	78.2 ± 11.1
Pyruvate kinase	Erythrocyte microinjection	9.9 ± 0.4	4.3 ± 0.7	6.2 ± 2.6	79.6 ± 8.5
Glyceraldehyde-3-phosphate dehydrogenase**	Scrape loading	4.4 ± 0.1	17.6 ± 1.5	4.5 ± 0.1	73.4 ± 8.4
Bovine serum albumin	Erythrocyte microinjection	94.5 ± 5.0	3.1 ± 0.3	0.7 ± 0.2	1.7 ± 0.2

multimer of enzyme. Lactate dehydrogenase radioiodinated with Iodogen as the oxidizing agent could be prepared with 65–85% of the enzyme activity of the unlabelled enzyme. Lactate dehydrogenase radioiodinated with chloramine-T was inactive. However, inactive and active preparations of microinjected lactate dehydrogenase met the same fate with regard to subcellular distribution and rate of degradation.

Degradation of microinjected proteins

Proteins were introduced into confluent cultures of 3T3-L1 cells, which were maintained confluent throughout the course of the experiment. ^{125}I-labelled bovine serum albumin and ^{125}I-labelled lactate dehydrogenase were degraded to trichloroacetic acid-soluble products, which were released to the medium, after erythrocyte miroinjection, bovine serum albumin being degraded more rapidly (results not shown; Table 1). Small amounts (after 120 h, generally < 5% of total cell radioactivity at zero time) of acid-insoluble radioactivity were released to the medium from cells microinjected with ^{125}I-labelled lactate dehydrogenase (results not shown). In contrast, considerable amounts (10–20% after 20 h) of acid-insoluble material were released to the medium from cells microinjected with bovine serum albumin (results not shown).

The degradation of scrape-loaded ^{125}I-labelled lactate dehydrogenase appears to be biphasic, with an initial, more rapid, phase which is over after 20 h (results not shown). This more rapid initial phase of degradation may be due to the endocytosis of a small amount of protein during scrape-loading. Erythrocyte-microinjected ^{125}I-labelled lactate dehydrogenase, by contrast, is degraded in a monophasic manner, with a rate that is very similar to that of the second, slower, phase for scrape-loaded ^{125}I-labelled lactate dehydrogenase. Half-lives of scrape-loaded proteins are given for the second, slower, phase.

Neither the labelling procedure nor the method of introduction into the cell appears significantly to influence the half-life of injected lactate dehydrogenase (Table 1). This agrees with our previous studies with microinjected homologous cytosolic proteins (Doherty & Mayer, 1985). The proteins of intracellular origin introduced into cells in the present study, i.e. lactate dehydrogenase, pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase, have long half-lives in recipient cells (124–330 h). The degradation of these injected proteins is largely inhibited (50–90%; Table 1) by an inhibitor of lysosomal proteolysis (NH_4Cl), of thiol proteinases (leupeptin) and of autophagy (3-methyladenine), as has been found by others (Rogers & Rechsteiner, 1985). In view of the less than total disruption of lysosomal proteolysis by these drugs (Ahlberg *et al.*, 1985; Ahlberg & Glaumann, 1984), it is likely that the microinjected glycolytic enzymes tested are degraded exclusively lysosomally. In contrast, microinjected bovine serum albumin is degraded comparatively rapidly ($t_{\frac{1}{2}}$ 17, 20 h) by a mechanism which is much less affected by NH_4Cl (Table 1), agreeing with the work of others (Rote & Rechsteiner, 1983; Rechsteiner *et al.*, 1984).

The accumulation of injected [^3H]dextran in the lysosomes of BHK cells proceeds at rates equivalent to half-lives of 190–360 h (Hendil, 1981), which is in the range of half-lives of the microinjected glycolytic enzymes reported here (Table 1), which suggests that the degradation of these enzymes may be dependent on the rate of constitutive autophagy.

Fractionation of microinjected cells

The distribution of acid-insoluble radioactivity in the detergent (digitonin, Triton X-100) and salt (KI) extracts and non-extractable residue of microinjected cells is shown in Table 2. Digitonin extracts of cells contained 75–90% of the cytosolic marker lactate dehydrogenase activity and approx. 30% of the lysosomal marker acid phosphatase activity (results not shown), but only small amounts (< 15%) of the total radiolabelled microinjected glycolytic enzymes. Radiolabelled proteins were minimally extracted with either Triton X-100 (1%, w/v) or KI (Table 2) which solubilized most of the membrane-bound marker enzyme activities determined, i.e. aminopeptidase, succinate dehydrogenase, the remaining acid phosphatase and actin (results not shown). The microinjected ^{125}I-labelled lactate dehydrogenase was found predominantly in the final non-extractable cell residue throughout

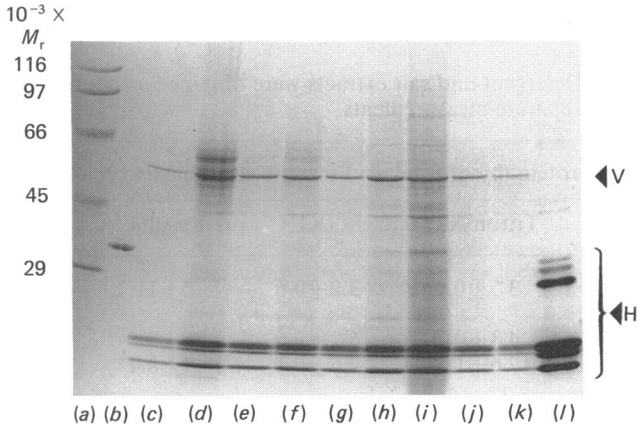

Fig. 1. SDS/polyacrylamide-gel electrophoresis of detergent- and salt-non-extractable cell residues obtained from cells cultured for 0 h (*c*), 24 h (*d, f, h, j*) and 120 h (*e, g, i, k*) in the presence of 10 mM-NH_4Cl (*f, g*), 0.5 mM-leupeptin (*h, i*) or 5 mM-3-methyladenine (*j, k*.)

The molecular lane (*a*) shows M_r standards ($\times 10^{-3}$). Lactate dehydrogenase was applied to lane (*b*), and histone standards (H) to lane (*l*). The position of the M_r-55000 protein identified as vimentin is indicated (V).

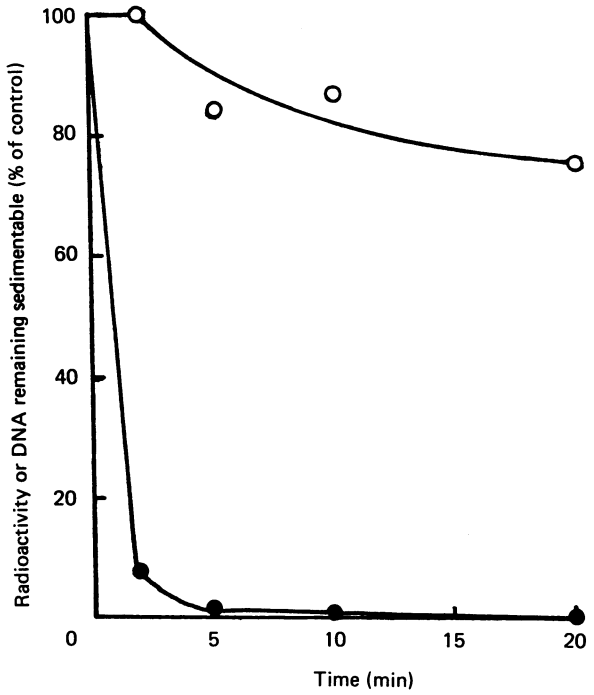

Fig. 2. Effect of deoxyribonuclease I treatment on the sedimentability of DNA (●) or radioactivity (○) in cell residues obtained from cells scrape-loaded with ^{125}I-labelled lactate dehydrogenase

Residues (obtained as described in the Materials and methods section) were suspended by sonication in phosphate-buffered saline, pH 7.4, and incubated at 37 °C with deoxyribonuclease I (50 μg/ml). Samples were removed at the times indicated, centrifuged at 12000 *g* for 1 min and assayed for DNA and radioactivity. Each point is the mean of duplicate determinations, which were within ±10%.

the experiment (120 h) in the presence or absence of inhibitors. Analysis of the non-extractable cell residue by polyacrylamide-gel electrophoresis in the presence of SDS and mercaptoethanol is shown in Fig. 1(*c*). Western blotting of such electrophoretograms, followed by probing with monoclonal antibody to vimentin, revealed the polypeptide of M_r approx. 55000 (V, Fig. 1) to be vimentin (result not shown), and the migration of the other major polypeptides with respect to histone standards suggests that they are histones (H, Fig. 1). Boiling of cell residues, obtained from cells labelled for 72 h with [^{3}H]methionine, in gel-electrophoresis buffer in the absence and presence of 2-mercaptoethanol solubilized 83 and 87% respectively of the total radiolabelled polypeptides. It is therefore conceivable that the cell residue contains polypeptides that do not appear in the electrophoretograms shown in Fig. 1. By including a KI extraction step in the sequential extraction procedure reported here, we have eliminated actin filaments (which are absent from the final residue; Fig. 1) as a site for sequestration of microinjected radiolabelled glycolytic enzymes. This is an important observation, as glycolytic enzymes are thought to associate with actin filaments in muscle cells (Arnold & Pette, 1968), and muscle was the source of the microinjected glycolytic enzymes.

The detegent- and salt-non-extractable residue from cells contains both vimentin and nuclear histones (Fig. 1), and therefore may contain DNA. Lactate dehydrogenase may bind single-stranded DNA (Cattaneo *et al.*, 1985). Non-extractable cell residue obtained from cells microinjected with ^{125}I-labelled lactate dehydrogenase was treated with deoxyribonuclease I (50 μg/ml). At intervals during the incubation with deoxyribonuclease samples were removed and centrifuged at 12000 *g* for 2 min and pellets were assayed for DNA and radioactivity (Fig. 2). Deoxyribonuclease I treatment rapidly removed DNA from the sedimentable residue, but the distribution of radioactivity was unaffected. Similar results were obtained by treatment of residues with ribonuclease (results not shown).

Density-gradient fractionation

Microinjected cells were homogenized, and the 10000 *g*-min pellet was homogenized, centrifuged and homogenized again to ensure complete cell breakage. Preliminary experiments revealed that further homogenization failed to release further enzymic activities from the 10000 *g*-min pellet. All cellular material was applied to density gradients and fractionated. The distribution of radioactivity in fractions from cells scrape-loaded with ^{125}I-labelled dehydrogenase is shown in Fig. 3(*a*), which shows that the enzyme is located in two regions of the gradient, immediately after scrape-loading, in a light peak (density 1.07 g/ml) and a dense peak (density 1.24 g/ml) near the bottom of the gradient. Although the light peak of ^{125}I-labelled lactate dehydrogenase radioactivity coincides with a peak of plasma membrane (aminopeptidase, Fig. 4*b*), and lysosomal (*N*-acetylhexosaminidase) markers (Fig. 4*a*), the dense peak is coincident with DNA and very little enzyme activity (Fig. 4*c*). The light peak of ^{125}I-labelled lactate dehydrogenase radioactivity has disappeared after 19 h (Fig. 3*c*), and the dense peak is decreased and is slowly lost over 67 h. Recoveries of radioactivity applied to the gradient were 70–75%. Dilution of fractions containing ^{125}I-labelled

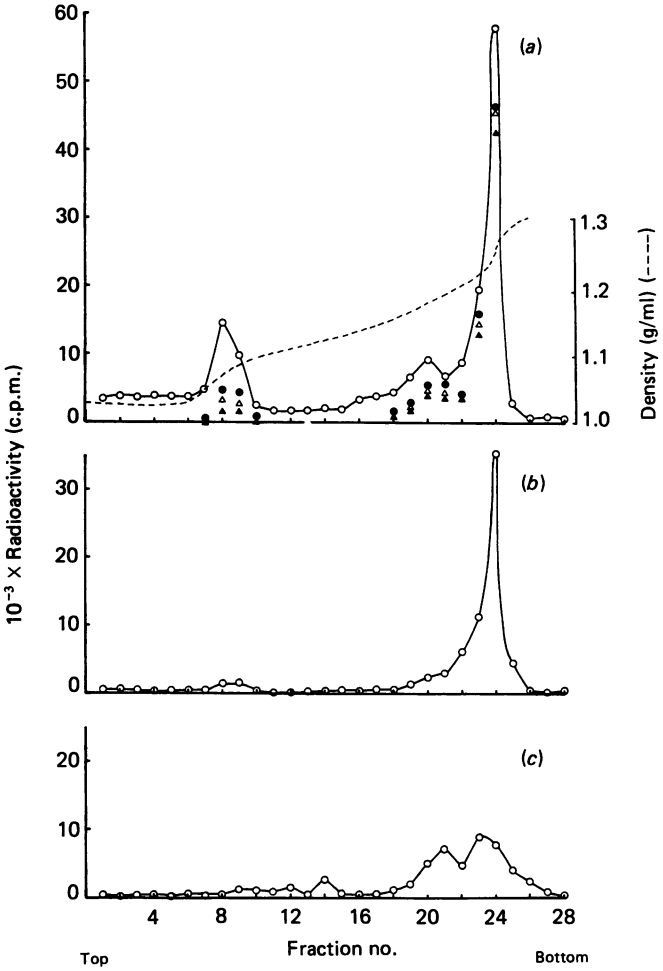

Fig. 3. Nycodenz-density-gradient fractionation of cells scrape-loaded with ^{125}I-labelled lactate dehydrogenase

Fractions were assayed for radioactivity (○), then diluted 10-fold with water (●), 1% Triton X-100 (see the Materials and methods section) (△) or 0.3 M-KI (▲), followed by centrifugation at 12000 *g* for 5 min, and the radioactivity in the resulting pellet was determined. Radioactivity applied to the gradient was > 95% acid-insoluble. Fractionation was carried out (*a*), 0 h, (*b*), 19 h or (*c*) 67 h after scrape-loading.

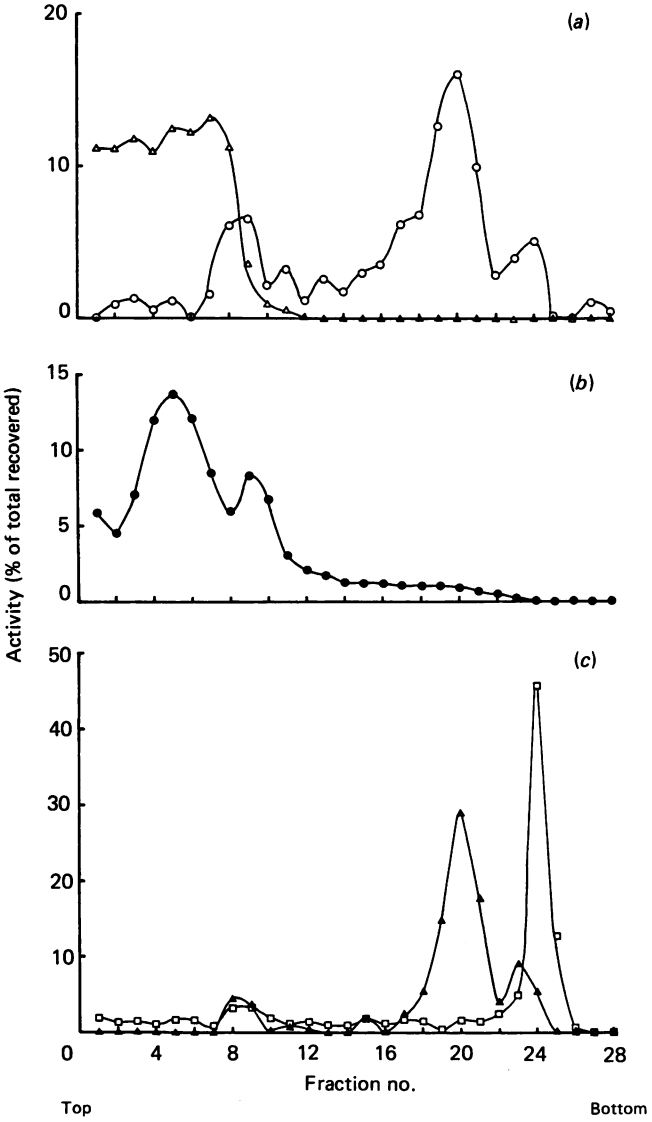

Fig. 4. Distribution of (*a*) lactate dehydrogenase (△) and *N*-acetylhexosaminidase (○), (*b*) leucine aminopeptidase (●), (*c*) succinate dehydrogenase (▲) and DNA (□) in fractions of Nycodenz density gradients of microinjected cells

Details of fractionation and assays are described in the Materials and methods section.

lactate dehydrogenase with water, followed by centrifugation, leads to solubilization of radioactivity in the more buoyant peak (Fig. 3*a*), but little change in the dense peak. We interpret this to mean that material in the more buoyant peak is trapped in vesicles which are lysed on water treatment. The radiolabelled protein in the light peak is probably due to the endocytosis of a small amount of ^{125}I-labelled lactate dehydrogenase during scrape-loading.

Treatment of fractions containing radioactivity from the dense peak (Fig. 3*a*) with Triton X-100 and KI failed to solubilize the radiolabelled protein, suggesting that these fractions correspond to the cell residue obtained by sequential detergent and salt extraction (Table 2, Fig. 1). ^{125}I-labelled lactate dehydrogenase remained in the dense peak and the insoluble cell residue throughout the experiment, suggesting that these fractions are closely related.

In a control experiment, when ^{125}I-labelled lactate dehydrogenase was mixed with cell homogenate and fractionated on Nycodenz density gradient, 90% of the ^{125}I-labelled enzyme was recovered in the lightest 11 fractions of the gradient, coincident with endogenous lactate dehydrogenase activity.

Polyacrylamide-gel electrophoresis and autoradiography

Cells scrape-loaded with ^{125}I-labelled lactate dehydrogenase were seeded into multi-well dishes and cultured in the absence (Fig. 5, lanes *a*, *b*, *f*, *g*) or the presence of drugs [NH_4Cl (lanes *c*, *h*), leupeptin (lanes *d*, *i*), 3-methyladenine (lanes *e*, *j*.)] for 4 h (lanes *a*, *f*) and 120 h (lanes *b*, *c*, *d*, *e*, *g*, *h*, *i*, *j*) before being solubilized in SDS-sample buffer by boiling in the absence or presence of 5% (w/v) 2-mercaptoethanol. After culture of scrape-loaded cells for 4 h or 120 h, autoradiography

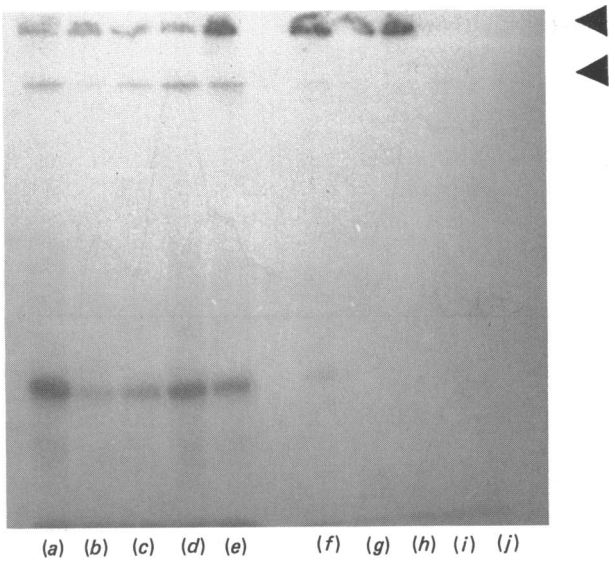

Fig. 5. Autoradiogram of polyacrylamide gels after electrophoresis of cells scrape-loaded with ^{125}I-labelled lactate dehydrogenase

Cells were cultured for 4 h (*a*, *f*) and 120 h (*b–e*, *g–j*) after scrape-loading in the absence of drugs (*a*, *b*, *f*, *g*) or in the presence of NH_4Cl (10 mM; *c*, *h*), leupeptin (0.5 mM; *d*, *i*) or 3-methyladenine (5 mM; *e*, *j*). Samples were electrophoresed after boiling in the presence of SDS and the presence (*a–e*) or absence (*f–j*) of 2-mercaptoethanol.

(Fig. 5) showed little or no ^{125}I-labelled lactate dehydrogenase in the resolving gel in the absence of 2-mercaptoethanol (lanes *f–j*), but ^{125}I-labelled enzyme was detectable in the expected positions in the presence of 2-mercaptoethanol (lanes *a–e*). In the absence of 2-mercaptoethanol, ^{125}I-labelled lactate dehydrogenase subunit was faintly observed in the resolving gel (Fig. 5, lane *f*) 4 h after scape-loading. This presumably represents endocytosed enzyme (Fig. 3*a*). Aggregates were observable on top of both the resolving and stacking gels (large arrows, Fig. 5) in the absence of previous reduction of samples (Fig. 5, lanes *f*, *g*, *i*) and after reduction (Fig. 5, lanes *a–e*), suggesting that dissolution of aggregates was not complete. The absence of aggregates above some lanes (*h*, *j*) is probably simply due to losses from above these lanes during handling after electrophoresis. When ^{125}I-labelled lactate dehydrogenase alone was electrophoresed, radioactive bands appeared at the expected position regardless of whether or not it was previously reduced (results not shown). ^{125}I-labelled bovine serum albumin migrated to the expected position on electrophoresis before and after introduction into cells, and regardless of whether the sample for electrophoresis was reduced (results not shown).

Intracellular proteins such as lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase are rich in free thiol groups (Holbrook *et al.*, 1977; Harris & Waters, 1977). Bovine serum albumin, an extracellular protein, has at most one free thiol group per molecule, but is rich in intramolecular disulphide bonds (Benesch *et al.*, 1955; Kalthoff *et al.*, 1958). The electrophoretic behaviour of microinjected ^{125}I-labelled lactate dehydrogenase in the absence of 2-mercaptoethanol suggests that disulphide-linked conjugates of the enzyme with macromolecules are formed.

Effect of leupeptin on the intracellular location of metabolically labelled cell proteins

Fig 1 (lanes *h* and *i*) shows that treatment of cells with 0.5 mM-leupeptin leads to the accumulation of numerous polypeptides in the detergent- and salt-insoluble cell residue not seen with the other inhibitors tested. Differential recoveries probably account for the small variations in protein amounts seen in some lanes (*d*, *e*, *g*, *k*, Fig. 1). In contrast, the effect of leupeptin, in resulting in the appearance of many polypeptides in the cell residue, is striking (Fig. 1, lanes *h* and *i*). Similarly, radiolabelled polypeptides accumulated in the detergent- and salt-insoluble cell fraction (Fig. 6*b*) in cells pulse-labelled with [^{3}H]methionine and chased in the presence of 0.5 mM leupeptin, in contrast with control cells chased in the absence of leupeptin, where there was no such accumulation (Fig. 6*a*). The loss of radioactivity from the detergent-soluble fractions of pulse-labelled cells was relatively little affected by the presence of leupeptin (Fig. 6). These results suggest that the thiol-proteinase inhibitor leupeptin, possibly in inhibiting intracellular proteolysis in the cells by 35%, causes the accumulation of proteins in the nuclear–intermediate-filament fraction (Fig. 6*b*; Table 2; Figs. 1–3). NH_4Cl and 3-methyladenine do not cause the accumulation of similarly radiolabelled proteins in this fraction (results not shown). The nuclear–intermediate-filament fraction is therefore either an intermediate in the degradation (Table 1) of some intracellular proteins or a site for intracellular proteins destined for degradation which cannot be immediately degraded by a thiol-proteinase-dependent system, e.g. after leupeptin treatment. Similarly, the nuclear–intermediate-filament fraction is the sequestration site for some microinjected proteins (Table 2, Figs. 1–3) and membrane glycoproteins (Earl *et al.*, 1987*a*,*b*), which are subsequently lysosomally degraded.

Implications for intracellular protein catabolism

Microinjection of some glycolytic enzymes into cells results in rapid sequestration into a cell fraction rich in vimentin and histones, the nuclear–intermediate-filament fraction, from which the proteins are slowly delivered to the lysosomal system, probably by a constitutive autophagic process. The glycolytic enzymes evaluated in this study rapidly form disulphide-linked insoluble aggregates after microinjection. These enzymes have abundant free thiol groups, whereas soluble proteins that escape disulphide cross-linking after microinjection, e.g. bovine serum albumin, carbonic anhydrase, superoxide dismutase and haemoglobin (Rogers & Rechsteiner, 1985; Gaskell *et al.*, 1987), are poor in cysteine (Croft, 1973). However, disulphide formation is not a prerequisite for sequestration in the nuclear–intermediate-filament fraction, since Sendai-viral HN and F proteins are sequestered without apparent disulphide cross-linking (Earl *et al.*, 1987*b*). Glycolytic enzymes may exist in the cytosol as loose functional complexes (Klyachko & Neifakh, 1985; Wood *et al.*, 1985; for an extensive review see Friedman, 1985). Microinjection of glycolytic enzymes surplus to requirement for the complex may result in enzyme inactivation, aggregation and sequestration into the nuclear–intermediate-filament fraction before autophagy. Similarly leupeptin treatment, by

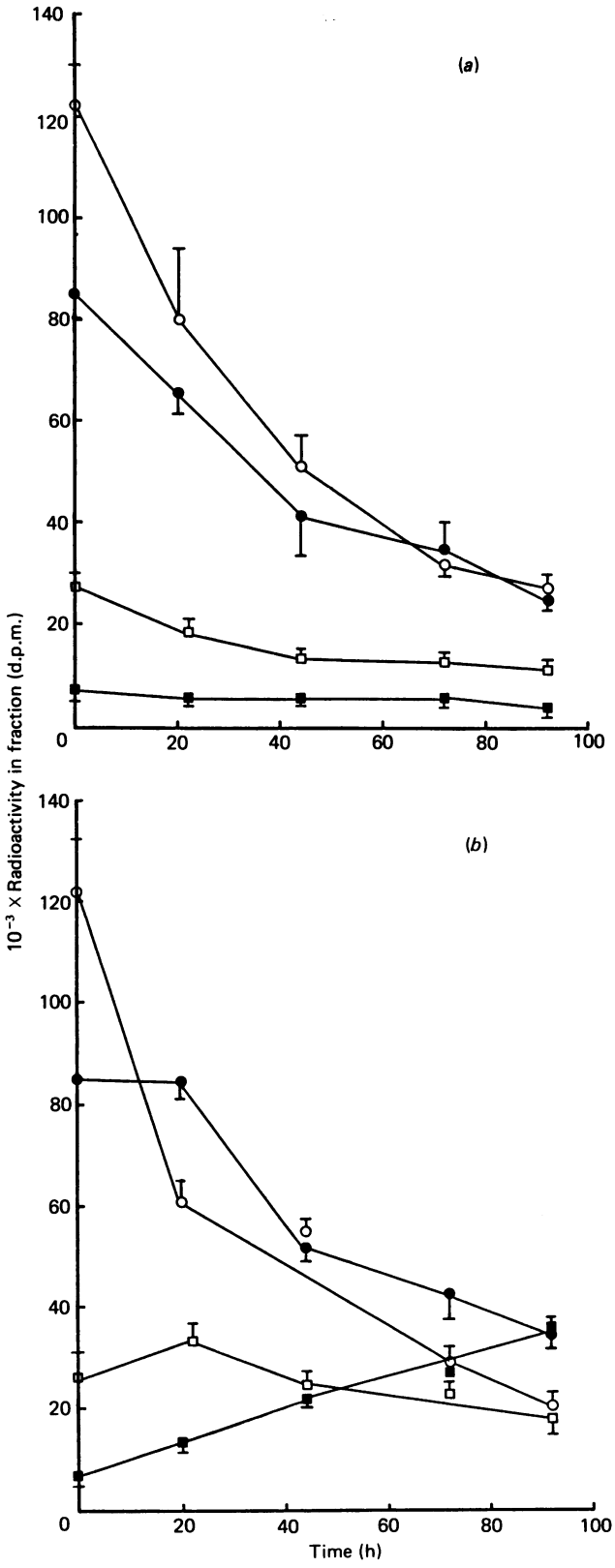

Fig. 6. Distribution of acid-insoluble radioactivity in digitonin (○), Triton X-100 (●) and KI extracts (□) and in non-extractable residue (■) (see the Materials and methods section) in cells pulse-labelled for 24 h in the presence of [^{3}H]methionine and chased in the presence of 10 mM unlabelled methionine in the absence (*a*) or presence (*b*) of 0.5 mM-leupeptin

Results are means ± S.D. for quadruplicate determinations.

inhibiting protein catabolism, may lead to the accumulation of endogenous proteins in excess of requirement for functional complex, which again are removed by an aggregation and sequestration process followed by autophagy. We estimate that only 1×10^5–10×10^5 molecules of lactate dehydrogenase are microinjected or scrape-loaded into a recipient cell, so that, if such control of intracellular enzyme content were to exist, it would have to be very sensitive. Sequestration of aggregated proteins into the nuclear–intermediate-filament fraction may occur continuously as part of a normal selective autophagic process, but aggregated proteins would be rapidly removed by autophagy and sequestration would therefore not be readily observed.

NH_4Cl, a lysosomotropic (acidotropic) agent, and 3-methyladenine, an inhibitor of autophagosome formation, fail to cause the accumulation of large amounts of polypeptides in the nuclear–intermediate-filament residue on prolonged culture with 3T3-L1 cells (Fig. 1). This may indicate that some property of leupeptin other than its effect as a lysosomal cathepsin inhibitor, e.g. inhibition of cytosolic calpain, which may digest intermediate filaments, or of other cytosolic thiol proteinases, is responsible for the accumulation of polypeptides in the residue in leupeptin-treated cells (Fig. 1, lanes *h* and *i*).

We have previously proposed an intracellular protein turnover cycle (Mayer *et al.*, 1984; Mayer & Doherty, 1986): the retrograde arm of the cycle requires protein removal from organelles and transfer to protein collection sites for autophagolysosomal degradation. The combined data (the present paper; Earl *et al.*, 1987*a*,*b*) suggest that the nuclear–intermediate-filament site is one such site in animal cells.

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