Degradation of erythrocyte-microinjected and scrape-loaded homologous cytosolic proteins by 3T3-L1 cells

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Homologous cytosol was introduced into 3T3-L1 cells by two different methods. Erythrocytes loaded with radiolabelled cytosolic proteins extracted from 3T3-L1 cells were fused with the aid of Sendai virus to 3T3-L1 cells, which were then seeded to confluent and non-confluent cultures. Cytosolic proteins were also introduced into cells by the technique of scrape-loading. In confluent cells, injected cytosolic proteins were recovered largely (54-93%) in a sedimentable ($6 \times 10^6 g_{av. min}$) fraction from recipient cells irrespective of the method of introduction or of radiolabelling of the injected proteins ($[^{125}I]$ iodination, reductive methylation with NaB^3H_4 and backbone labelling with L-[4,5- 3H]leucine). The degradation of microinjected cytosolic proteins was in all cases inhibited by the lysosomotropic agent NH_4Cl to a greater extent (32-75%) than that observed for endogenous cytosolic ($\leq 19\%$) proteins (labelled with L-[4,5- 3H]leucine). In growing cells both endogenous total cell proteins and microinjected proteins were degraded at a slower rate than in confluent cell monolayers. The inhibition by NH_4Cl of the degradation of both the endogenous and microinjected proteins is decreased compared with the inhibition observed in confluent monolayers. The results are discussed in terms of the cytoplasmic capacity to segregate microinjected homologous proteins before protein degradation can take place.

Until recently the study of protein degradation in cultured cells has been restricted to the use of pulse-chase techniques. Such studies precipitate interpretative problems because of reutilization of labelled precursor amino acids. Also labelling of all cellular proteins makes identification of the sub-cellular site(s) of protein destruction extremely difficult. Consequently, the erythrocyte-mediated microinjection of prelabelled proteins is being increasingly employed to study mechanisms of intracellular protein degradation (Zavortink *et al.*, 1979; Hendil, 1980; Neff *et al.*, 1981). Microinjection permits the introduction of proteins with non-reutilizable labels. Labelling of proteins with lysosomal membrane-impermeant markers, i.e. $[^{14}C]$ sucrose, facilitates the determination of the intracellular sites of protein destruction (Bigelow *et al.*, 1981).

Previous studies employing the erythrocyte-mediated microinjection technique have all involved the transfer of heterologous proteins. Pure proteins, usually obtained commercially, have been introduced into a variety of cell lines

(Zavortink *et al.*, 1979; Hendil, 1980; Neff *et al.*, 1981; McElligott & Dice, 1983). It has been argued that the degradation characteristics of such heterologously transferred proteins reflect those of endogenous soluble proteins (Rote & Rechsteiner, 1983; McElligott & Dice, 1983). No direct comparison has been made, however, between the degradation of microinjected homologous protein(s) and their endogenous counterparts. We report here experiments carried out with soluble proteins extracted from 3T3-L1 cells after erythrocyte microinjection or scrape-loading into 3T3-L1 cells. The degradation of such homologously transferred proteins has been compared with that of endogenous total cell and cytosol proteins.

Materials and methods

Materials

Cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Dulbecco's modified Eagle's medium and culture dishes were supplied by Gibco Bio-Cult (Paisley,

Scotland, U.K.), and newborn-calf serum was from Imperial Laboratories (Salisbury, Wilts., U.K.). All radioisotopes were obtained from Amersham International (Amersham, Bucks., U.K.), and penicillin G, streptomycin, trypsin, chloramine-T and horseradish peroxidase were from Sigma Chemical Co. (Poole, Dorset, U.K.). Fiso fluor was obtained from Fisons (Loughborough, Leics., U.K.) and Sepharose 6B from Pharmacia Fine Chemicals (Hounslow, Middx., U.K.).

Cell culture

3T3-L1 cells, a pre-adipocyte cell line, were maintained with fibroblast morphology in Dulbecco's medium containing 10% (v/v) newborn-calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) in an atmosphere of air/CO₂ (9:1) at 37°C. Cells were removed from their substrata for subcultivation with a solution of 0.5% (w/v) trypsin and 0.2% (w/v) EDTA in Ca²⁺- and Mg²⁺-free phosphate-buffered saline (i.e. 137 mM-NaCl, 2.7 mM-KCl, 81 mM-Na₂HPO₄, 1.46 mM-KH₂PO₄).

Metabolic labelling of cell proteins

Cell proteins were metabolically labelled by culture of monolayers (70% confluent) for the times indicated in the Results section with 0.1 µCi of L-[4,5-³H]leucine or [U-¹⁴C]leucine/ml in Dulbecco's medium containing 0.1 mM-leucine and 10% newborn-calf serum. Cells were then washed with Ca²⁺- and Mg²⁺-free phosphate-buffered saline, removed for subcultivation by incubation with trypsin, and resuspended in Dulbecco's medium containing serum, antibiotics and 10 mM-leucine.

Preparation of radiolabelled cytosolic proteins from 3T3-L1 cells

Cells grown to confluence were washed with phosphate-buffered saline (i.e. as described above, plus 0.9 mM-CaCl₂ and 0.5 mM-MgCl₂) and collected by scraping in 0.15 M-sodium phosphate, pH 7.5. After sonication of the cell suspension (2 × 10 s, 7 µm amplitude, 21 kHz; MSE sonicator), the resulting homogenate was centrifuged at 100 000 g for 60 min, and the supernatants (cytosol), after filtration through a 0.22 µm filter (Millipore, Molsheim, France) and concentration by ultrafiltration (stirred cell with filter of 10 000 Da cut-off; Amicon, Stonehouse, Gloucs., U.K.), was assayed for protein (Geiger & Bessman, 1972) and stored at -20°C until used.

Cytosolic proteins were radioiodinated by the chloramine-T method (Hunter & Greenwood, 1962). Radioactive cytosol was applied to a column of Sephadex G-25 (1.5 cm diameter × 25 cm) equilibrated with 10 mM-sodium phosphate, pH 7.4, and

eluted with the same buffer. Protein peaks were pooled, concentrated to 0.1 ml and kept at 4°C. Cytosol was used to load erythrocytes within a few hours of iodination.

Cytosol proteins were reductively methylated with NaB³H₄ essentially by the method of Tack *et al.* (1980). Cytosol was prepared as described above, except that sodium borate, pH 9, replaced sodium phosphate. Radioactive cytosol was eluted from a column of Sephadex G-25 as described above.

Microinjection

(a) *Erythrocyte-mediated.* Proteins were microinjected into culture cells essentially by the erythrocyte-mediated technique of Schlegel & Rechsteiner (1975). Human erythrocytes loaded with radioactive proteins were either used immediately or stored at 4°C in phosphate-buffered saline containing penicillin (100 units/ml) and streptomycin (100 µg/ml) for not more than 24 h. Culture cells were removed from Petri dishes by incubation with 0.05% (w/v) trypsin and 0.2% (w/v) EDTA in Ca²⁺- and Mg²⁺-free phosphate-buffered saline. Fusion incubations contained 50–200 haemagglutinating units/ml of u.v.-inactivated Sendai virus (Harris & Watkins, 1966). Sendai virus was kindly given by the Sir William Dunn School of Pathology, University of Oxford. After incubation at 4°C for 10 min, followed by gentle shaking at 37°C for 25 min, fusion mixtures were centrifuged (400 g-min), then washed once with Dulbecco's medium containing 10% (v/v) serum, followed by three washes with warm (37°C) 0.83% (w/v) NH₄Cl, to lyse unfused erythrocytes. Washed cells were suspended in Dulbecco's medium containing serum and antibiotics.

(b) *Scrape-loading.* Proteins were introduced into cells by scrape-loading (McNeil *et al.*, 1984). Cell monolayers on Petri dishes (90 mm diameter) were washed once with warm (37°C) Dulbecco's medium without serum and then rapidly scraped in warm medium with serum (2 ml) but containing the cytosol proteins to be loaded. Scraped cells were added to 8 ml of ice-cold medium containing serum, and the cells collected by centrifugation (400 g-min). After a further wash in 10 ml of medium containing serum, cells were suspended in medium and seeded into culture dishes.

Measurement of protein degradation

Metabolically labelled or microinjected cells were seeded into six multi-well plates at a density of 5 × 10⁴ cells/cm² for confluent cultures and 5 × 10³ cells/cm² for growing cultures. If any contaminating erythrocytes were found, the adherent cells were washed with phosphate-buffered saline containing 5% (v/v) newborn-calf serum

until monolayers were free of unfused erythrocytes. Fresh medium (2ml) containing antibiotics, serum and, for metabolically labelled cells, 10mM-leucine, was added and measurements commenced. From this point, medium was removed from monolayers and cells were washed once with 1ml of phosphate-buffered saline, and medium and washing were combined. Monolayers were scraped in 2ml of phosphate-buffered saline, the well was washed with a further 1ml of phosphate-buffered saline and scrapings and washing were combined. After sonication the resulting homogenates were stored at -20°C .

Proteins were precipitated from the medium and from cell homogenates by addition of ice-cold trichloroacetic acid [final concn. 10% (w/v), containing 10mM-leucine] and incubation at 4°C for 10min. After centrifugation (10000g-min), final precipitates were either dissolved in phosphate-buffered saline (^{125}I) or in 0.3ml of 30% (v/v) formic acid (^3H , ^{14}C). Samples containing ^{125}I were counted for radioactivity in an Intertechnique Gamma Counter. Samples containing ^3H or ^{14}C were mixed with Fisofluor scintillation fluid (3ml) and their radioactivity was determined in a Packard Tri-Carb 460CD liquid-scintillation counter.

Measurements made on cells microinjected with ^{125}I -labelled cytosolic proteins indicated that the total radioactivity released to the medium over the course of the experiment was equal to the loss of acid-insoluble radioactivity from the cell monolayers. For the metabolically labelled cells, however, it was found that total radioactivity in the medium at the end of each experiment exceeded the loss of cell acid-insoluble radioactivity from the cells (Silverman & Amenta, 1984). Protein degradation was therefore estimated by the following calculation.

Cell acid-insoluble radioactivity at zero time (t_0) = total acid-soluble radioactivity at end of experiment + cell acid-insoluble radioactivity at end of experiment (1)

$[(\text{Cell acid-insoluble radioactivity at } t_0 - \text{acid-soluble radioactivity at time } t) / \text{cell acid-insoluble radioactivity at } t_0] \times 100 = \text{percentage of radioactivity remaining in cell protein at time } t$ (McElligott & Dice, 1983) (2)

Owing to the small release of trichloroacetic acid-insoluble radioactivity to the medium with time, this calculation of degradation rate may not give an accurate estimate. This point is discussed further in the Results and discussion section.

Protein degradation results in the loss of radiolabel from protein, which approximates to a first-order exponential-decay process. Therefore degradation results were plotted with a logarithmic ordinate for the percentage of radioactivity remaining in cell protein, and from the resulting straight lines protein half-lives were determined.

Subcellular fractionation of 3T3-L1 cells

Cell monolayers were washed with phosphate-buffered saline containing Ca^{2+} and Mg^{2+} , and then with homogenization medium (0.25M-sucrose containing 10mM-Tris/HCl, pH 7.4, and 3mM-EGTA) before being scraped in homogenization medium (5ml). Microinjected cells from one Petri dish (90mm diameter) were mixed with unlabelled cells to give approx. 3×10^6 cells/ml. After sonication as described previously, samples (4ml) of the resulting homogenates were centrifuged at $6 \times 10^3 g_{\text{av}}$ -min, $30 \times 10^3 g_{\text{av}}$ -min, $250 \times 10^3 g_{\text{av}}$ -min and $6 \times 10^6 g_{\text{av}}$ -min to give five successive fractions, crude nuclei (N), heavy mitochondria (M), light mitochondria and lysosomes (L), microsomes (P) and cytosol (S), i.e. supernatant of final centrifugation (de Duve *et al.*, 1955). All pellets were resuspended by sonication at the same amplitude and frequency, but only for 10s, and assayed for enzymic activities and trichloroacetic acid-insoluble radioactivity.

Enzyme activities

Subcellular fractions were assayed for the following enzyme activities: lactate dehydrogenase (EC 1.1.1.27) (Johnson, 1960), alkaline phosphodiesterase I (EC 3.1.4.1) (Touster *et al.*, 1970), acid phosphatase (EC 3.1.3.2) (de Duve *et al.*, 1955) and succinate dehydrogenase (EC 1.3.99.1) (Pennington, 1961). Protein was determined as described by Geiger & Bessman (1972).

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate

Electrophoresis was carried out as described by Laemmli (1970). Proteins were separated in linear gradients of 7.5–15% (w/v) polyacrylamide. Densitometry of Coomassie-Blue-stained protein bands was carried out with a Helena Quick-scan instru-

ment before soaking of gels for 1h in sodium salicylate. Fluor-impregnated gels were dried down (Bio-Rad Gel Drier 1125) and exposed to Fuji R-X X-ray film at -70°C . Densitometry of developed X-ray plates was carried out as described above.

Results and discussion

Microinjection

Preliminary studies (results not shown) with microinjection and scrape-loading of horseradish

peroxidase into 3T3-L1 cells showed that peroxidase reaction product was distributed in the nucleus and throughout the cytoplasm of the cells.

Calculation of degradation rates

Calculation of degradation rates for both metabolically labelled and microinjected proteins is complicated by the appearance of acid-insoluble radioactivity in the medium with time (Fig. 1). If the released radioactive protein (acid-insoluble) represents a pool that is not available for degradation, the calculations described in eqns. (1) and (2) will yield a true degradation rate. However, if the acid-insoluble radioactivity recovered in the medium at the end of the experiment represents radioactive protein that was available for degradation at the start of the experiment, but which was gradually removed from the pool available for degradation during the course of the experiment, it then becomes difficult to establish the true size of the pool of radioactive protein available for degradation. A solution to this problem has been suggested (McGarry *et al.*, 1983) which relies on the observed constant ratio of acid-soluble to acid-insoluble radioactivity in the medium throughout the course of the experiment. Although this relationship was observed for ^{125}I -IgG microinjected into HeLa cells (McGarry *et al.*, 1983), it is

Fig. 1. Appearance of trichloroacetic acid-soluble ($\square$, $\circ$) and insoluble ($\blacksquare$, $\bullet$) radioactivity in the medium of cells either microinjected with ^{125}I -labelled cytosolic proteins ($\circ$, $\bullet$) or metabolically labelled with L-[4,5- ^3H]leucine for 16 h ($\square$, $\blacksquare$).

Each point is the mean of quadruplicate ($\circ$, $\bullet$) or duplicate ($\square$, $\blacksquare$) determinations, as described in the Materials and methods section. Error bars represent $\pm$ S.D. Duplicate determinations were within $\pm 5\%$.

not true for microinjected ^{125}I -labelled cytosolic proteins and metabolically labelled endogenous proteins in 3T3-L1 cells. We have adopted the method for calculating degradation rates described in eqns. (1) and (2), since the errors will be small, owing to the small proportion of acid-insoluble radioactivity released into the medium (i.e. $<10\%$ at the end of the experiment; Fig. 1) for both microinjected and endogenous proteins. It is not clear why the fraction of microinjected proteins released intact into the medium is so small compared with that observed by others (McGarry *et al.*, 1983; Rechsteiner *et al.*, 1984), but this may be a consequence of the homologous nature of the proteins microinjected in our experiments.

Degradation studies

Comparison of endogenous and injected cytosol.

Cells metabolically labelled for 16 h were homogenized and fractionated into cytosolic and particulate fractions at intervals during the chase period, and the degradation of each fraction was determined (Table 1, Ai). Proteins in the cytosolic fraction were degraded more rapidly (half-life, $t_{1/2}$, 31 h) than particulate proteins ($t_{1/2}$ 42 h). NH_4Cl inhibited the degradation of particulate proteins to a much greater extent (46%) than that of cytosolic proteins (15%), but to a similar extent to that of microinjected proteins (42%, 56%; Table 1, Aii). A large inhibition of the degradation of scrape-loaded cytosolic proteins was also observed (Table 1, Aiii). Labelling cells for a longer period (48 h) resulted in essentially the same half-life for endogenous cytosolic proteins (34 h) and particulate proteins (42 h) and the same effects of NH_4Cl as seen with labelling for the shorter period (Table 1, Ai).

We carried out a double-label experiment (Arias *et al.*, 1969; with [^3H]leucine as decaying label and [^{14}C]leucine as non-decaying label) to determine whether endogenous 3T3-L1-cell cytosol contains proteins whose degradation is very sensitive to NH_4Cl (results not shown). The results suggest that the NH_4Cl -sensitivity of microinjected cytosolic proteins is not due to the presence in the injected mixture of proteins whose degradation rate is particularly susceptible to inhibition by NH_4Cl .

Sedimentability of injected cytosolic proteins. The data presented here suggest that microinjected proteins are being treated differently from endogenous cytosol proteins, with more lysosomal involvement in the degradation of the injected proteins. When 3T3-L1 cells that had been injected with ^{125}I -labelled cytosolic proteins were homogenized by sonication and subfractionated by differential centrifugation, it was found that the trichloroacetic acid-insoluble radioactivity did not

Table 1. *Degradation of endogenous and microinjected proteins by 3T3-L1 cells*

Cells were labelled for 16 h or 48 h (*) with L-[4,5-³H]leucine for experiments to determine endogenous half-lives. [³H]Leucine-labelled cytosolic proteins for scrape-loading were prepared by incubating confluent cells for 72 h with medium containing serum and 0.1 mM-L-[4,5-³H]leucine (500 Ci/mol), followed by homogenization and fractionation as described in the Materials and methods section and dialysis against Dulbecco's medium at 4°C for 24 h. Each reported half-life was measured in a separate experiment. In each experiment, the trichloroacetic acid-soluble radioactivity in the medium above cells (see eqn. 2) was measured in quadruplicate wells at five separate time points. At each time point the quadruplicate determinations were within $\pm 10\%$. Abbreviation: n.d., not determined.

	Half-life (h)			
	Control	+ NH ₄ Cl	Inhibition (%)	Sedimentable (%)†
(A) Confluent cells				
(i) Endogenous [³ H]leucine-labelled proteins				
Total	40	73	44	—
	42*	80*	47*	—
Cytosolic	31	37	15	—
	34*	42*	19*	—
Particulate	42	78	46	—
	42*	107*	61*	—
(ii) Erythrocyte-microinjected				
¹²⁵ I-labelled cytosolic proteins	48	83	42	54
	44‡	100‡	56‡	n.d.‡
[³ H]Methyl-labelled cytosolic proteins	52	76	32	93
(iii) Scrape-loaded				
¹²⁵ I-labelled cytosolic proteins	42	129	68	83
[³ H]Methyl-labelled cytosolic proteins	38	140	75	89
[³ H]Leucine-labelled cytosolic proteins	37	60	38	83
(B) Growing cells				
(i) Erythrocyte-microinjected				
¹²⁵ I-labelled cytosolic proteins	103	130	21	n.d.
(ii) Endogenous [³ H]leucine-labelled proteins				
Total cell	66	95	30	—

† Percentage of trichloroacetic acid insoluble radioactivity sedimentable at 6 × 10⁶g·min.

‡ Experiment with a different preparation of ¹²⁵I-labelled cytosolic proteins.

† Percentage of trichloroacetic acid insoluble radioactivity sedimentable at 6×10^6 g·min.

‡ Experiment with a different preparation of ¹²⁵I-labelled cytosolic proteins.

co-distribute with the soluble enzyme lactate dehydrogenase (Fig. 2).

(i) *Cytosol proteins do not bind to the cell surface.* It is not obvious why proteins extracted from the soluble fraction of cells should subsequently become sedimentable with intracellular particulate fractions when microinjected into 3T3-L1 cells with the erythrocyte-mediated technique. As some erythrocyte lysis occurs during incubation of loaded erythrocytes with Sendai virus, it is conceivable that some of the released protein may bind to the cell surface. Erythrocytes loaded with ¹²⁵I-labelled cytosol were incubated with Sendai virus for 10 min at 4°C and then for 20 min at 37°C. After removal of intact erythrocytes by centrifugation (400g·min), the supernatant was incubated with 3T3-L1 cells for a further 10 min at 4°C and 20 min at 37°C. Analysis revealed that the amount of trichloroacetic acid-insoluble ¹²⁵I radioactivity associated with the 3T3-L1 cells was less than 10%

of that associated with 3T3-L1 cells in a parallel incubation with loaded erythrocytes and Sendai virus. This suggests that only a small proportion of the acid-insoluble radioactivity associated with microinjected erythrocytes is the result of the uptake of radiolabelled proteins released from loaded erythrocytes during the fusion incubation. Furthermore, subcellular fractionation of erythrocyte-microinjected 3T3-L1 cells (Fig. 2) showed that trichloroacetic acid-insoluble radioactivity did not co-fractionate with the plasma-membrane marker alkaline phosphodiesterase I.

(ii) *Injected cytosol is free of membrane proteins.* Trichloroacetic acid-insoluble ¹²⁵I associated with the particulate fractions could be derived from membrane proteins extracted from cells along with cytosolic proteins by the sonication extraction procedure used (see the Materials and methods section). Such proteins may re-associate with the particulate fraction after microinjection. However,

Fig. 2. Subcellular fractionation of 3T3-L1 cells erythrocyte-microinjected with ^{125}I -labelled cytosol. Fractions were assayed for alkaline phosphodiesterase (a), acid phosphatase (b), succinate dehydrogenase (c), lactate dehydrogenase (d), trichloroacetic acid-insoluble ^{125}I radioactivity (e) and protein. Results are presented as described by Lopez-Saura *et al.* (1978). Enzyme and radioactivity determinations were in triplicate and were within $\pm 10\%$.

as cells were homogenized and fractionated in exactly the same way in order to prepare cytosolic proteins for microinjection and to analyse the distribution of microinjected acid-insoluble ^{125}I radioactivity, proteins would be expected to distribute in the same way on both occasions. It seems unlikely therefore that the association of acid-insoluble ^{125}I radioactivity with the $6 \times 10^6 g_{av.}$ -min pellet of microinjected cells is due to the presence of contaminating membrane proteins in the cytosol preparations injected. In order to determine whether the cytosol fraction used for microinjection did contain contaminating

membrane proteins, several fractions were prepared from cells (homogenized by sonication) which were then radioiodinated with ^{125}I and analysed by polyacrylamide-gel electrophoresis under denaturing conditions. Autoradiography followed by densitometry revealed that few (i.e. at 36 and 46 kDa) of the radiolabelled polypeptides present in the cytosol fraction (Fig. 3d) also were present in the $6 \times 10^6 g_{av.}$ -min pellet of the water-treated membrane fraction (Fig. 3b). The densitometer was optimized for detecting the number of bands in each fraction. Radiolabelling of the water-treated membrane fraction (Fig. 3b) was

Fig. 3. Densitometric profiles after denaturing polyacrylamide-gel electrophoresis of ^{125}I -labelled homogenate (a), pellet of water-treated fraction (b), supernatant of water-treated membrane (c) and cytosolic proteins (d)

Gels were subjected to autoradiography, and the developed X-ray film was analysed densitometrically. Numbers (K = kDa) and arrows refer to the position of M_r standards

approximately one-quarter (specific radioactivity) of that of the cytosol fraction (Fig. 3d). The $6 \times 10^6 g_{av.}$ -min supernatant of water-treated membranes (Fig. 3c) contained a radioactive subunit of 46 kDa which migrated the same distance into the gel as a radiolabelled subunit in the cytosol fraction. It is possible therefore that the cytosol fraction contains polypeptides that may be derived from membrane vesicles or subcellular organelles. The 46 kDa subunit containing protein, however, is not loaded into erythrocytes (Fig. 4c) and is not present in microinjected cells (Fig. 4d). The electrophoretic profile of polypeptides scraped into cells is the same as the profile for erythrocyte-microinjected polypeptides (results not shown).

During the incubation of erythrocytes with ^{125}I -labelled cytosol at 37°C to promote erythrocyte resealing, there was an increase in trichloroacetic acid-soluble radioactivity of only 3%. This suggests that the disappearance of the 46 kDa subunit on erythrocyte loading is not due to degradation by the erythrocyte. Larger proteins are known to be trapped less efficiently by erythrocytes than are smaller proteins (Rechsteiner, 1978). Gel chromatography of the soluble fraction of lysed loaded erythrocytes suggests that some larger proteins are excluded (Fig. 5). It may be that the 46 kDa subunit(s) is part of an oligomeric protein(s) of much larger size, which is therefore excluded from the erythrocyte during loading. Actin obtained from the 3T3-C2 cell line, a mouse pre-adipocyte cell similar to 3T3-L1, has a subunit of 46 kDa (Spiegelman & Green, 1980). The exclusion of the 46 kDa subunit from erythrocytes may therefore be due to this subunit being part of the actin filaments of the 3T3-L1 cytoskeleton.

Gel chromatography did not show any appreciable amount of very-low- M_r material in the ^{125}I -labelled cytosol preparation or the loaded erythrocytes. These observations suggest that the band seen at the very bottom of the polyacrylamide gels was either a small subunit of an oligomeric protein (Figs. 4b, 4c and 4d) or was due to artifactual blackening of the X-ray film, and represents a well-known artifact with radiolabelled proteins in fluorography after polyacrylamide-gel electrophoresis in sodium dodecyl sulphate (Gasser *et al.*, 1982).

(iii) *Sedimentability of injected cytosol proteins is not due to radioiodination.* Radioiodination of proteins could lead to alteration of protein structure, resulting in increasing binding to membranes. Cells were therefore mixed with ^{125}I -labelled cytosolic proteins, sonicated and fractionated. A proportion (10 and 20% in two separate experiments) of acid-insoluble radioactivity was sedimentable. Part of the association of ^{125}I -labelled

Fig. 4. Densitometric profiles after polyacrylamide-gel electrophoresis of unlabelled cytosol (a), ^{125}I -labelled cytosolic proteins (b), erythrocytes loaded with ^{125}I -labelled cytosolic proteins (c) and 3T3-L1 cells microinjected with ^{125}I -labelled cytosolic proteins (d)

The lane containing unlabelled cytosol proteins was stained with Coomassie Brilliant Blue and analysed densitometrically. Lanes containing radioactive proteins (b, c, d) were subjected to autoradiography and the developed X-ray plate was analysed densitometrically. Numbers (K = kDa) and arrows refer to M_r standards.

cytosolic proteins with particulate fractions of microinjected cells could therefore be due to increased binding of the ^{125}I -labelled cytosolic proteins to 3T3-L1 cell membranes. However, in cells microinjected with ^{125}I -labelled cytosolic proteins by either the erythrocyte-mediated or the scrape-loading methods, between 54 and 83% of the acid-insoluble radioactivity became sedimentable (Table 1, Ai and Aii). It seems therefore that the ^{125}I -labelled cytosolic proteins are associating with intracellular particulate structures after microinjection.

Radiolabelling of cytosolic proteins with ^{125}I by using lactoperoxidase (David & Reisfeld, 1974) generated the same pattern of radiolabelled polypeptides on denaturing electrophoresis as seen with chloramine-T-labelled proteins (results not shown). After microinjection the lactoperoxidase-iodinated proteins (83% sedimentable) demonstrated similar subcellular distribution and inhibition of degradation by NH_4Cl (46%) as did chloramine-T-radioiodinated cytosol proteins.

Abnormal intracellular proteins (i.e. containing amino acid analogues) are degraded at a much faster rate than for their normal counterparts (Capecchi *et al.*, 1974; Knowles & Ballard, 1976; Hendil, 1981). Injected cytosolic proteins, however, were degraded at slower rates than their endogenous counterparts, suggesting that they are not recognized as being abnormal in the same sense as proteins containing amino acid analogues. Iodination of proteins could lead to dissociation of oligomeric proteins. However, it has been observed that subunits of ribulose-1,5-bisphosphate carboxylase from *Chlamydomonas* that are not incorporated into intact proteins are destroyed with half-lives of a few minutes (Schmidt & Mishkind, 1983). In contrast, the injected ^{125}I -labelled cytosolic proteins had an average half-life of days, which suggests that the protein dissociation had not occurred.

It may be the presence of the ^{125}I atom itself or some feature of the labelling process common to both iodination methods that leads to the behaviour of the microinjected proteins. In order to test this, cytosolic proteins were labelled with NaB^3H_4 and introduced into cells by either erythrocyte-microinjection or scrape-loading. In both cases the introduced ^3H -labelled cytosolic proteins became sedimentable (Table 1, Aii and Aiii). Although NH_4Cl had a greater effect on the degradation of scrape-loaded erythrocyte-injected ^3H -labelled cytosolic proteins, in both cases the effect was greater than for endogenous cytosolic proteins (Table 1, Ai). In order to overcome any effects of modification of amino acid side chains (i.e. with ^{125}I or ^3H methylated), cytosol proteins were extracted from cells labelled with $[4,5\text{-}^3\text{H}]\text{leucine}$ and intro-

Fig. 5. Chromatography of cytosolic proteins (----), ^{125}I -labelled cytosolic proteins (O) and the lysate obtained from erythrocytes loaded with ^{125}I -labelled cytosolic proteins (●) on Sepharose 6B

The column (1.6 cm diam. $\times$ 54 cm) was equilibrated with 0.15 M-KCl/10 mM-sodium phosphate, pH 7.4, and samples were eluted in the same buffer. Each fraction contained 1.5 ml and was assayed for either A_{280} (----) or radioactivity (O, ●). Numbers (K = kDa) and arrows refer to the fractions containing M_r standards.

duced into cells by scrape-loading (in order to inject sufficient labelled protein for accurate radioactivity measurements). Such backbone-labelled cytosol proteins were degraded at a rate similar to that for ^{125}I - and ^3H -methyl-labelled cytosolic proteins (Table 1, Aiii), and labelled proteins were sedimentable after scrape-loading. NH_4Cl had a greater effect on the degradation of scrape-loaded cytosol backbone-labelled proteins than on their endogenous counterparts (38% versus $\leq 19\%$).

Previous workers have observed that the rate of degradation of heterologous proteins microinjected into culture cells was either unaffected (bovine serum albumin) or decreased by not more than 35% (pyruvate kinase) by NH_4Cl (Rote & Rechsteiner, 1983). When bovine serum albumin and pyruvate kinase labelled with membrane-impermeant ^{14}C -sucrose were microinjected into L929 cells and the recipient cells fractionated 20 h (bovine serum albumin) and 44 h (pyruvate kinase) later, 31% of the bovine serum albumin and 65% of pyruvate kinase (trichloroacetic acid-insoluble radioactivity) were found associated with particulate fractions (Bigelow *et al.*, 1981). The greater association of injected pyruvate kinase with particulate fractions, compared with bovine serum albumin, and the greater inhibition of pyruvate kinase degradation by NH_4Cl would support the results reported here, which suggest that the association of soluble proteins with cell particulate

fractions leads to a greater involvement of lysosomes in their destruction.

It has also been observed that ^{125}I -labelled glutamine synthetase when introduced into hepatoma cells by erythrocyte-mediated microinjection associates largely (70–90%) with the particulate fraction of the recipient cells, compared with bovine serum albumin, which is recovered almost entirely in the soluble fraction (Freikopf-Cassel & Kulka, 1981).

Erythrocyte-mediated microinjection affects endogenous protein degradation. It is possible that the microinjection process itself may influence intracellular protein degradation. When cells, metabolically labelled with ^{14}C -leucine, were injected with bovine serum albumin, there was a small but discernible decrease (15%) in the degradation of endogenous total cell proteins.

Growing cells degrade microinjected proteins more slowly than do confluent cells. Cells seeded at densities low enough to permit cell division throughout the course of the experiment degraded both erythrocyte-injected ^{125}I -labelled cytosolic proteins ($t_{1/2}$ 103 h) and total endogenous proteins ($t_{1/2}$ 66 h) more slowly than in non-growing cells (Table 1, B). Cytosol proteins used in injection experiments were extracted from density-inhibited cultures. However, after proteins were injected into cells that were allowed to divide, their degradation rate decreased compared with that for density-

inhibited cultures, suggesting that density-inhibition does not lead to the synthesis of a population of shorter-lived proteins, but rather to more rapid degradation of proteins that are relatively stable under growing conditions. This is true of metabolically labelled total cell proteins, since all metabolic labelling was carried out with cultures that became density-inhibited during the labelling period. This confirms for 3T3-L1 cells the published observations (Hendil, 1977) that density-inhibition of cell division results in an increase in intracellular protein degradation (Hendil, 1977; Cockle & Dean, 1982). Interestingly, it was found that the addition of NH_4Cl to confluent cultures led to an increase in cell protein compared with control cells of 38% and 87% (two separate experiments; results not shown). This would agree with the observation that NH_4Cl at a concentration of 10mM does not inhibit protein synthesis (Amenta & Brocher, 1980; Jessup *et al.*, 1983).

Implication of results for mechanisms of intracellular protein catabolism

The data show that, irrespective of labelling procedure (two types of iodination, reductive methylation, and backbone labelling with [^3H]leucine) or mode of introduction into the target cell (erythrocyte-mediated microinjection or scrape-loading), homologous cytosolic proteins do not assume a cytoplasmic distribution analogous to that of endogenous cytosolic proteins, but are recovered in a sedimentable fraction from target cells (Fig. 2).

Sedimentable injected cytosolic proteins (Fig. 2) were recovered in several subcellular fractions, with the largest proportion (41%) in the crude nuclear fraction. This could mean that injected cytosolic proteins have accumulated in the nucleus, or more likely that they have associated with structure(s) which co-sediment with nuclei. Microinjected heterologous soluble proteins are not extractable with Triton X-100 from recipient cells (Rechsteiner *et al.*, 1984), i.e. indicating a non-vesicular intracellular distribution. The combined data can be most easily explained by the model shown in Fig. 6. Microinjected proteins may enter directly into the sedimentable structure and be degraded largely by the lysosomal mechanism.

In contrast, endogenous cytosolic proteins may be degraded by an NH_4Cl -insensitive (non-lysosomal) route (Rechsteiner *et al.*, 1984; Ciechanover *et al.*, 1984) or, after association with a sedimentable structure, by an NH_4Cl -sensitive (lysosomal) mechanism.

The availability of a non-lysosomal degradative mechanism to endogenous cytosol proteins along with a lysosomal mechanism may explain the

Fig. 6. *Proposed model for the degradation of endogenous and microinjected cytosolic proteins*

Endogenous cytosol proteins (A), i.e. labelled with an amino acid precursor, are either degraded by a non-lysosomal mechanism (3) to amino acids (C) or deposited (1) in a sedimentable pool (B) before degradation by an NH_4Cl -sensitive (lysosomal) mechanism (2). Microinjected cytosolic proteins enter directly into B. This model is an adaptation of a principle previously proposed for intracellular protein turnover (Amenta & Brocher, 1981; Mayer *et al.*, 1984).

generally faster degradation of endogenous compared to microinjected cytosol proteins (Table 1).

The cytosol is known to have an ordered structure (Fulton, 1982), and many cytosolic, in particular glycolytic, enzymes are known to be associated with cytoskeletal elements (Hübscher *et al.*, 1971; Masters, 1978). It may be that microinjected cytosolic proteins cannot enter into these functional relationships and are consequently taken into some currently unknown sedimentable structure before delivery to the lysosome. Results from several laboratories agree with such a possibility. The diffusion of bovine serum albumin microinjected into human fibroblasts has been suggested to involve the cytoskeleton (Wojcieszyn *et al.*, 1981). The data of Rechsteiner *et al.* (1984) show that lysosomal degradation is inversely proportional to the degree of Triton X-100 extractability from cells after microinjection, proteins which are not readily extracted being degraded more obviously in lysosomes. This suggests that some close association of microinjected proteins with the cytoskeleton may precede their degradation by lysosomes. After microinjection, ribonuclease is rapidly associated with a sedimentable structure, which in this case is postulated as being vesicular (McElligott & Dice, 1984). A prelysosomal fraction has been suggested as being involved in the degradation of membrane proteins (Mayer *et al.*, 1984; Evans & Mayer, 1984; Russell *et al.*, 1984). Together the data from several laboratories provide an argument in support of the notion that some microinjected proteins, unable to take up the organization of their endogenous counterparts, enter a prelysosomal, sedimentable, possibly cytoskeletal-associated, structure before delivery to the

lysosomal system. This route may be available to endogenous proteins after their exit from their functional sites.

Clearly, microinjection or scrape-loading of soluble proteins, or transplantaion of membrane proteins (Evans & Mayer, 1983, 1984; Russell & Mayer, 1983; Russell *et al.*, 1984), challenges a currently little understood cytoplasmic system which can segregate and process the incoming material. Characterization of the system which 'recognizes' microinjected proteins is an essential requisite to understanding protein catabolism.

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