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Ubiquitin–protein conjugates accumulate in the lysosomal system of fibroblasts treated with cysteine proteinase inhibitors

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Mouse fibroblasts (3T3-L1 cells) accumulate detergent- and salt-insoluble aggregates of proteins conjugated to ubiquitin when incubated in the presence of inhibitors of lysosomal cysteine cathepsins, including E-64. These ubiquitin–protein conjugates co-fractionate with lysosomes on density gradients and are found in multivesicular dense bodies which by electron microscopy appear to be engaged in microautophagy. Both E-64 and ammonium chloride increase the intracellular concentration of free ubiquitin, but only E-64 leads to the formation of insoluble lysosomal ubiquitin–protein conjugates. The results are discussed in relation to the possible intracellular roles of ubiquitin conjugation.

INTRODUCTION

Ubiquitin is a small protein which has been implicated as a cofactor in the non-lysosomal rapid turnover of some short-lived and abnormal proteins by a soluble proteinase of high molecular mass (Ciechanover *et al.*, 1984; Hough *et al.*, 1987). Some ubiquitin–protein conjugates, however, have been found to be relatively stable in human lung fibroblasts (Haas & Bright, 1987) and may not be good substrates for the ATP-dependent proteinase of high molecular mass which degrades the substrate protein of ubiquitin conjugates in the cytosol (Hough *et al.*, 1987; McGuire *et al.*, 1988). Ubiquitin is also found conjugated to various plasma membrane proteins (Siegelman *et al.*, 1986; Yarden *et al.*, 1986; Meyer *et al.*, 1986) and the turnover of plasma membrane proteins is thought to occur in the lysosome (Tweto & Doyle, 1976; Fernig & Mayer, 1987). In addition, we have found that ubiquitin immunoreactivity coincides with areas of granulovacuolar degeneration in Alzheimer's disease (Lowe *et al.*, 1988*a*). Ubiquitin has been found to be released into the medium from cells in tissue culture (Carlson & Rechsteiner, 1987; Haas & Bright, 1987) and recently free ubiquitin has been located in lysosomes and autophagic vacuoles of hepatoma cells by immunogold electron microscopy (Schwartz *et al.*, 1988). These observations suggest that ubiquitin and ubiquitin–protein conjugates may be found in the intravesicular spaces of the cell as well as in the cytosol. We report here that treatment of cells with inhibitors of lysosomal cathepsins, in particular the membrane-impermeant (Shoji-Kasai *et al.*, 1988) inhibitor of cysteine cathepsins, E-64, leads to the accumulation of multivesicular bodies containing proteins conjugated to ubiquitin.

MATERIALS AND METHODS

Materials

Epoxysuccinyl-leucylamido-(4-guanidino)butane (E-64), antipain, leupeptin and Z-Phe-Arg-7-aminomethylcoumarin were obtained from Cambridge Research Biochemicals (Cambridge, U.K.). Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2 were the generous gift of Dr. E. Shaw, Friedrich Miescher-Institut, Basle, Switzerland. Nitrocellulose (0.45 μm) was obtained from Schleicher and Schuell (Dassel, Germany) and 0.2 μm from Sigma (Poole, U.K.). Biotinylated antibodies, avidin and biotinylated alkaline phosphatase were supplied by Vector Laboratories (Peterborough, U.K.). Radiochemicals were supplied by Amersham International plc (Amersham, U.K.). Scintillation fluid was LKB 'Optiphase X' manufactured by FSA Laboratory Supplies (Loughborough, U.K.). Affi-Sep affinity matrix was supplied by Anachem (Luton, Beds., U.K.). Bovine erythrocyte ubiquitin, streptavidin–gold and hydroxysuccinimide-activated Sepharose were from Sigma. Other chemicals were obtained as described previously (Doherty *et al.*, 1987). Lowicryl was supplied by TAAB Laboratories (Aldermaston, U.K.).

Protein degradation

3T3-L1 fibroblasts were cultured and used at confluency as described previously (Doherty *et al.*, 1987), except that stock cultures were grown in antibiotic-free medium. Cells were pulse-labelled for 48 h with either [^{3}H]methionine or [^{3}H]leucine (1–5 μCi/ml) in Dulbecco's modification of Eagle's medium containing either 50 μM-methionine or 100 μM-leucine, depending on the radiolabelled amino acid employed, as well as new-born calf serum (10 %, v/v), gentamycin (50 mg/l) and amphotericin (2.5 mg/l). After the labelling period, cells were subcultured into multiwell plates and chased for 16 h in chase medium containing either 10 mM-leucine or 10 mM-methionine, depending on the radiolabelled amino acid used, to remove short-lived labelled polypeptides, and then chased again (48 h) in fresh chase medium in the presence or absence of proteolytic inhibitors. Controls for the effects of these agents consisted of cells chased with the appropriate concentration of solvent diluted in chase medium. Protein degradation

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; e.l.i.s.a., enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

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rates were calculated as described previously (Doherty *et al.*, 1987). Inhibition of protein degradation was calculated from the rate constant of degradation (k_d) for control and inhibitor-treated cells, i.e. inhibition (%) = $[(k_{d,\text{control}} - k_{d,\text{inhibitor}})/k_{d,\text{control}}] \times 100$.

Detergent and salt extraction of cells

Sequential extraction of 3T3-L1 cells with detergent and salt was carried out as described previously (Doherty *et al.*, 1987), except that the Triton extraction medium [Triton X-100 (1%, v/v) in 100 mM-Hepes, 2 mM-magnesium chloride, pH 6.9] and salt extraction medium (0.3 M-KI) contained deoxyribonuclease 1 (10 μg/ml), 1 mM-phenylmethanesulphonyl fluoride (PMSF) and 50 μM-leupeptin. The final insoluble pellet was collected by centrifugation as before and washed briefly with distilled water.

Density gradient fractionation of cells

Confluent monolayers of cells grown on three to nine Petri dishes (100 mm diameter) were washed in phosphate-buffered saline (PBS) followed by a wash in homogenization medium (0.25 M-sucrose in 10 mM-Tris/HCl, pH 7.4, containing 1 mM-PMSF, 1 mM-EGTA, 10 mM-iodoacetamide and 10 μM-haemin) at 4 °C. Cells were then scraped into 4 ml of homogenization medium at 4 °C and homogenized as described previously (Doherty *et al.*, 1987), and a sample (4 ml) of the entire homogenate was applied to a preformed Nycodenz gradient and centrifuged (Doherty *et al.*, 1987). Fractions (0.5 ml) of the centrifuged gradient were collected and stored at −20 °C until use.

Enzyme assays

Acid phosphatase (EC 3.1.3.2) and lactate dehydrogenase (EC 1.1.1.27) were assayed as described previously (Doherty *et al.*, 1987), except the volumes were reduced to permit enzyme reactions to be carried out in 96-well microtitre plates. Absorbances were determined in an EAR 340 enzyme-linked immunosorbent assay (e.l.i.s.a.) plate reader (SLT-Labinstruments, Austria). DNA was determined as described previously (Doherty *et al.*, 1987).

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of SDS was carried out with large-pore (acrylamide/bisacrylamide, 100:1) 7.5–15% (w/v) linear gradients (Doucet & Trifaro, 1988). Samples were briefly sonicated in sample buffer [2.3% (w/v) SDS, 10% (v/v) glycerol in 62.5 mM-Tris/HCl, pH 6.8]. To reduce samples, 2-mercaptoethanol was added to 5% (v/v), and all samples were heated in a boiling water bath for 3 min.

Antibodies

Rabbit and sheep antiserum to SDS-denatured ubiquitin–γ-globulin conjugates were raised by the method of Haas & Bright (1985). Antiserum was tested against ubiquitin–haemocyanin conjugates by e.l.i.s.a. Affinity-purified anti-ubiquitin was isolated with a 1 ml column of ubiquitin coupled to Affi-Sep or with a 5 ml column of ubiquitin–Sepharose (Haas & Bright, 1985) Affinity-purified antibody recognized ubiquitin–protein conjugates generated by incubation of Fraction II of reticulocyte lysate with ATP and ubiquitin on Western blots (Haas & Bright, 1985), and both ubiquitin–protein conjugates and free ubiquitin after autoclaving of the nitrocellulose replicas (Swerdlow *et al.*, 1986).

Immunoblotting

After electrophoresis nitrocellulose (0.2 or 0.45 μm pore size) replicas of gels were obtained and allowed to dry. They were autoclaved for 20 min at 120 °C (Swerdlow *et al.*, 1986), then incubated with blocking solution [10% (w/v) milk powder or non-immune rabbit serum (sheep primary antibody) in 0.2 M-NaCl/0.05 M-Tris/HCl, pH 7.5] before incubation with affinity-purified rabbit or sheep antibody to ubiquitin–protein conjugates (2–5 μg/ml, in blocking solution). After washing, bound primary antibody was detected by incubation with biotin-conjugated second antibody to rabbit or sheep IgG (0.1 μg/ml), followed, after washing, with avidin–biotin-conjugated alkaline phosphatase complex (prepared according to the manufacturer's recommendations and diluted 50-fold before use), before localization of alkaline phosphatase (Blake *et al.*, 1984).

Fractions from density gradients were assayed for the presence of antibody-immunoreactive material by dot-blotting. An aliquot of each fraction was heated for 3 min in a boiling water bath, after mixing with an equal volume of 1% (w/v) SDS and 10 mM-dithiothreitol in PBS, and portions of this were applied to nitrocellulose with a 96-well manifold apparatus. Probing with antibody was carried out as described above and immunoblots or electrotransfers were scanned at 600 nm with a Quickscan R&D (Helena Laboratories, TX, U.S.A.) in reflectance mode.

Immunohistochemistry

Cells were grown on glass coverslips and fixed for 10 min with 2.5% (v/v) formaldehyde in PBS. They were permeabilized with 0.1% (w/v) Triton X-100 in PBS containing glycine (100 mg/ml), and blocked for 1 h in 10% (v/v) non-immune goat serum in PBS before incubation with affinity-purified rabbit antibody to ubiquitin–protein conjugates [1:20 in PBS containing non-immune goat serum and 0.1% (w/v) Tween 20] for 1 h. After washing off excess antibody, bound antibody was detected as described for Western blots. Cells were lightly counterstained with haematoxylin.

Immunogold electron microscopy

Cells were removed from plastic Petri dishes with trypsin, diluted with medium containing serum, then washed with PBS before fixation in 1.5% (w/v) paraformaldehyde/1% (v/v) glutaraldehyde containing 1% (w/v) sucrose and 2 mM-calcium chloride. Post-fixation was by standard methods in osmium tetroxide and uranyl acetate. For morphological examination, cells were embedded in epoxy resin. For immunogold electron microscopy, cells were embedded in Lowicryl K4M. Sections in Lowicryl were blocked with non-immune goat serum (3%, v/v) in PBS, then probed with affinity-purified rabbit antibody to ubiquitin–protein conjugates [diluted 1:20 in PBS containing 0.1% (v/v) Tween 20], washed and then incubated with biotinylated goat anti-(rabbit IgG) (1:50), diluted as above, followed by washing and incubation with streptavidin–gold (15 nm, 1:20). Sections were examined with a Philips 410 electron microscope at 80 kV.

RESULTS

Effects of proteolytic inhibitors on the degradation of long-lived polypeptides

The effects of various cysteine proteinase inhibitors on the degradation of long-lived proteins in 3T3-L1 cells either alone or in combination with ammonium chloride are shown in Table 1. The concentrations employed in Table 1 were above those required to give maximal inhibition of protein degradation (Doherty *et al.* 1989). Z-Phe-Phe-CHN_2 formed an insoluble precipitate above 20 μM in the medium. All the inhibitors tested in Table 1 produced inhibition of degradation of 30–40%. Addition of ammonium chloride along with inhibitor produced very small increases in inhibition, suggesting that the action of these inhibitors is on an ammonium chloride-susceptible, i.e. lysosomal, mechanism. Rates of protein turnover in control cells were 1.20–1.35%/h.

The inhibitors described above were tested for their ability to inhibit the action of the lysosomal cathepsins B and L. In agreement with others (Shaw & Dean, 1980), extracts of cells cultured for 24 h with E-64 (500 μM) and Z-Phe-Ala-CHN_2 (100 μM) showed little or no ability to hydrolyse Z-Phe-Arg-7-aminomethylcoumarin (< 5% of control cells; Doherty *et al.*, 1989), a substrate for cathepsins B and L. A small amount (25%) of activity was left in extracts of Z-Phe-Phe-CHN_2-treated cells and even less in extracts of chloroquine-treated cells (17.5%). The effects of E-64 and the diazomethanes were only observable after several hours (Doherty *et al.*, 1989; Shaw & Dean, 1980) and is consistent with their entry into the cell by pinocytosis where they accumulate in lysosomes (Shaw & Dean, 1980).

Ubiquitin–protein conjugates in inhibitor-treated and untreated cells

Autoclaving of nitrocellulose replicas enabled the detection of free ubiquitin as well as conjugated ubiquitin in cell extracts (Fig. 1; Swerdlow *et al.*, 1986). Detection of bound antibody with alkaline phosphatase revealed the presence of a variety of ubiquitin–polypeptide conjugates, as well as free ubiquitin, in whole-cell extracts of treated and untreated cells (Fig. 1, lanes *a*, *c* and *e*). Discrete antibody-positive bands can be identified corresponding to ubiquitin monomer (Ub, Fig. 1, lanes *a*, *c* and *e*); a doublet around 15 kDa (clearly seen in lane *e*, but also visible in lanes *a* and *c*); an intense band of approximately 23 kDa, and a doublet around 29 kDa (Fig. 1, lanes *a*, *c* and *e*). In addition, there is a large smear of antibody-positive reaction product from the 40 kDa region of the gel to the top of the resolving gel (HMW Ub conj.; Fig. 1, lanes *a*, *c* and *e*). This pattern of ubiquitin-containing proteins is similar to that obtained by others when ^{125}I-labelled ubiquitin was introduced into permeabilized HTC cells (Raboy *et al.*, 1986) and microinjected into HeLa cells (Carlson *et al.*, 1987; Carlson & Rechsteiner, 1987). The antibody-positive band at 23 kDa has been suggested to contain the ubiquitinated histone H_2A (Fig. 1, u-H_2A), while that at 29 kDa may contain the di-ubiquitinated variant (Raboy *et al.*, 1986). The intensity of the 23 kDa (u-H_2A) band was unchanged in E-64- and ammonium chloride-treated cells as compared with control cells (Fig. 1, lane *c* and *e* versus *a*). Cells treated with E-64 showed greater antibody staining of the smear of larger molecular mass conjugates than untreated cells or cells treated 48 h with ammonium chloride; reflectance densitometry revealed a 74% increase in antibody staining of high molecular mass conjugates (Fig. 1, lane *e* versus lanes *a* and *c*).

It was of interest to see if ubiquitin–protein conjugates would accumulate in a detergent- and salt-insoluble cell fraction, as we have previously shown that polypeptides destined for degradation in the lysosomal system accumulate in a Triton X-100- and KI-insoluble form in the

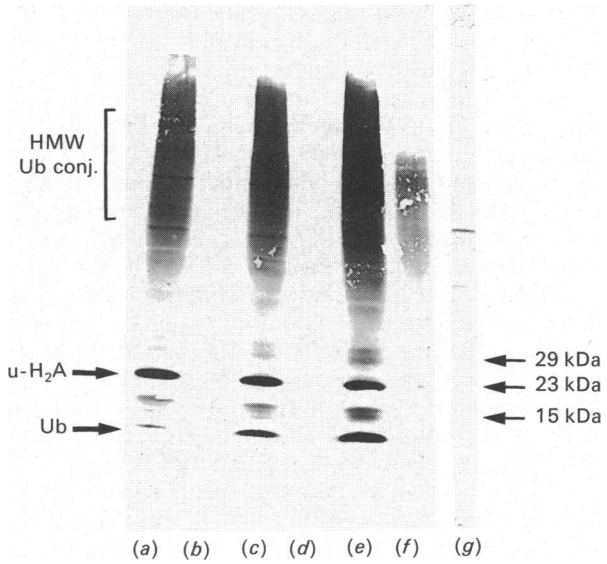


Fig. 1. Immunoblot analysis of SDS electrophoretograms of 3T3-L1 cells chased in the presence and absence of proteolytic inhibitors with a specific antibody to ubiquitin–protein conjugates

Cells were treated for 48 h in the absence of inhibitors (*a* and *b*) or in the presence of ammonium chloride (15 mM; *c* and *d*) or E-64 (0.5 mM; *e*, *f* and *g*). Whole cells (*a*, *c*, *e* and *g*) or Triton X-100- (1%, w/v) and salt- (0.3 M-KI) insoluble cell extracts (*b*, *d* and *f*) were boiled in electrophoresis buffer containing SDS and 2-mercaptoethanol. A nitrocellulose replica of the gel was autoclaved and probed with an affinity-purified rabbit antibody against ubiquitin–protein conjugates. u-H_2A (23 kDa) indicates the ubiquitinated histone H_2A. Ub, a ubiquitin standard and HMW Ub conj., ubiquitin–protein conjugates of high molecular mass. (*g*) E-64-treated cells incubated with affinity-purified ubiquitin–protein conjugate specific antibody pre-absorbed with ubiquitin–γ-globulin.

Table 1. Inhibition of degradation of intracellular long-lived proteins in 3T3-L1 cells by proteinase inhibitors and ammonium chloride

Inhibitor	Concentration (mM)	Inhibition ± S.D. (%)	
		$-NH_4Cl$	$+NH_4Cl$
E-64	1.00	31±4	38±3
Z-Phe-Ala-CHN_2	0.10	34±1	41±3
Z-Phe-Phe-CHN_2	0.02	31±2	34±2
Chloroquine	0.10	36±1	41±4
NH_4Cl	15.00	35±2	

presence of inhibitors such as leupeptin (Doherty *et al.* 1987). The detergent- and salt-insoluble fractions contain little antibody-positive staining material (Fig. 1, lanes *b* and *d*), except in the case of cells treated for 48 h with E-64 (Fig. 1, lane *f*), where a smear of higher molecular mass material (35–200 kDa, HMW Ub conj.) was present. Staining of the ubiquitin monomer band increased in both E-64- and ammonium chloride-treated cells (Fig. 1, lanes *c* and *e* versus *a*; 3-fold in both cases when normalized with respect to u-H_2A). Incubation of the affinity-purified antibody to ubiquitin–protein conjugates with ubiquitin–γ-globulin conjugate (10 μg/ml), before incubation with the nitrocellulose replica, abolished subsequent staining in E-64-treated cells (Fig. 1, lane *g* versus *e*), indicating that antibody staining was specific for ubiquitin and ubiquitin–protein conjugates. These results suggest that incubation of cells with E-64 leads to an increase in ubiquitin–protein conjugates of high molecular mass, some of which are detergent and salt insoluble.

Distribution of ubiquitin–protein conjugates

Dot-immunoblotting of density gradient fractions of cells incubated for 48 h with E-64 demonstrated a dramatic increase in ubiquitin-containing material (Fig. 2, filled circles in *b* versus *a*) in fractions containing acid phosphatase activity (Fig. 2, open circles). This confirms the suggestion that ubiquitin and ubiquitin–protein conjugates (Fig. 1) are found in the lysosomal fraction of inhibitor-treated cells. There was also an increase in immunoreactive material at the top of the gradient, comprising the soluble and plasma membrane cell components (Fig. 2*b*, filled circles). This may reflect an increase of cytosolic ubiquitin–protein conjugates or free ubiquitin (which does occur; Fig. 1, lane *e*). Acid phosphatase activity increased on treatment of cells with E-64 (Fig. 2, open circles); this is probably due to an increase in the volume of the acidic vesicular compartment in inhibitor-treated cells (Fig. 5). Lysosomally related multivesicular dense bodies were found to increase from less than 0.5 to 6% of the fractional volume of cytoplasm after only 12 h treatment with E-64 (Doherty *et al.*, 1989). In previous experiments we have reported that the lysosomal enzymes *N*-acetylhexosaminidase and β-galactosidase co-distribute with acid phosphatase on Nycodenz gradients of 3T3-L1 cells (Doherty *et al.*, 1987).

That there was an increase in conjugated ubiquitin in the gradient fractions containing lysosomal enzyme activity was confirmed by analysis of density gradient fractions by gel electrophoresis followed by immunoblotting. The soluble fractions of untreated cells contained ubiquitin–protein conjugates (Fig. 3*a*, fractions 1–8) and ubiquitinated histone (u-H_2A) was present in the fractions containing nuclei (N, Fig. 3*a*, fractions 23–24). The apparent increase in ubiquitinated histone seen in E-64-treated cells in this experiment is in contrast to the lack of an increase in this conjugate seen in Fig. 1. Several other experiments similar to that reported in Fig. 1 demonstrated a lack of increase of ubiquitinated histone H_2A in E-64-treated cells. The apparent increase seen in Fig. 3 could therefore be an artefact owing to incomplete electrotransfer from the resolving acrylamide gel or insufficient mixing with the primary antibody; however, we cannot rule out an E-64-induced increase in histone ubiquitination.

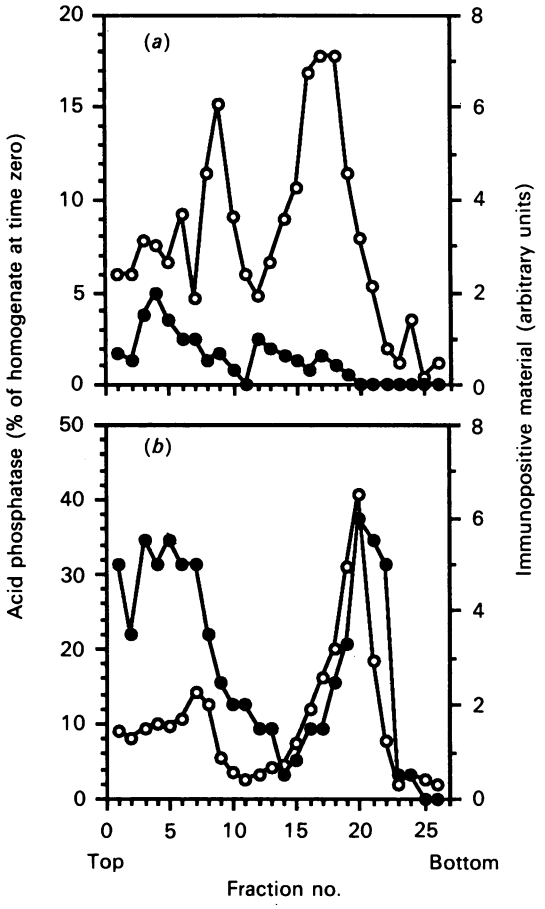


Fig. 2. Density gradient distribution of material reactive with the antibody specific for ubiquitin–protein conjugate

Aliquots of density gradient fractions from 3T3-L1 cells incubated 48 h in the absence (*a*) and presence (*b*) of E-64 were applied to nitrocellulose and after autoclaving probed with the affinity-purified antibody to ubiquitin–protein conjugates. After visualization, dots were scanned by reflectance densitometry to determine antibody-reactive material (●). Acid phosphatase (○) was determined in each fraction and is expressed as a percentage of the total activity in the homogenate at the start of the 48 h chase period.

Cells incubated for 48 h with E-64 were found to contain increased levels of ubiquitin–protein conjugates in fractions containing cytosolic proteins and fractions containing lysosomal enzyme activity (Fig. 3*b*, fractions 1–8 and fractions 15–22), while conjugates were almost absent from the denser lysosomal fractions of control cells (Fig. 3*a*, fractions 15–22). Free ubiquitin could be found in the denser lysosome fractions of control cells which increased substantially when cells were treated with E-64 (Fig. 3*a* versus *b*, fractions 15–22).

Light microscopy and ubiquitin–protein conjugate immunocytochemistry

Antibody reaction product was apparent in the nucleus of fixed and permeabilized 3T3-L1 cells (Fig. 4*a*), while the cytoplasm contained only very low levels of reaction product. The lack of uniform staining of the cytoplasm suggests that cytosolic ubiquitin monomer is not detected under these conditions (Riley *et al.*, 1988). Treatment of

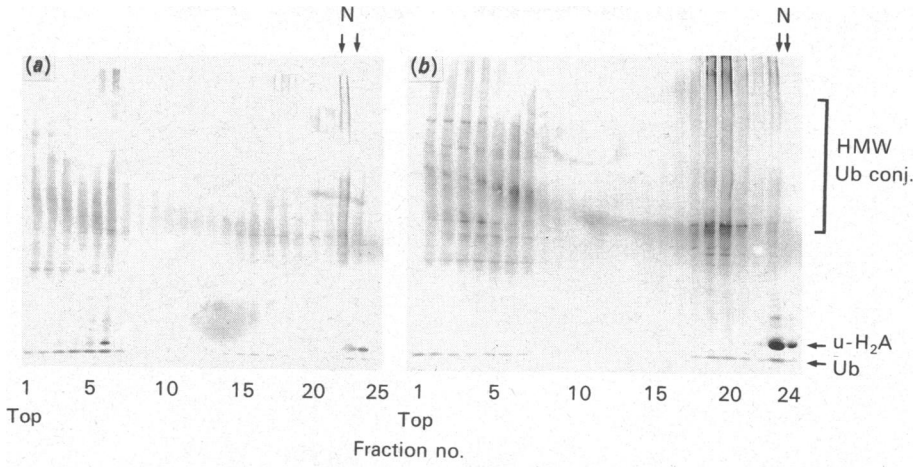


Fig. 3. Distribution of ubiquitin and ubiquitin–protein conjugates in density gradient fractions of E-64-treated and untreated fibroblasts

Cells were incubated for 48 h in the absence (*a*) or presence (*b*) of E-64 (0.5 mM) and after homogenization in medium containing iodoacetamide (10 mM) and haemin (100 μM) fractionated on a Nycodenz density gradient. Aliquots of each fraction were subjected to polyacrylamide-gel electrophoresis and after autoclaving each nitrocellulose replica was probed with sheep antibody specific for ubiquitin–protein conjugates. Ub, ubiquitin; HMW-Ub conj., ubiquitin conjugates of high molecular mass; u-H_2A, ubiquitinated histone H_2A. N: fractions containing nuclei.

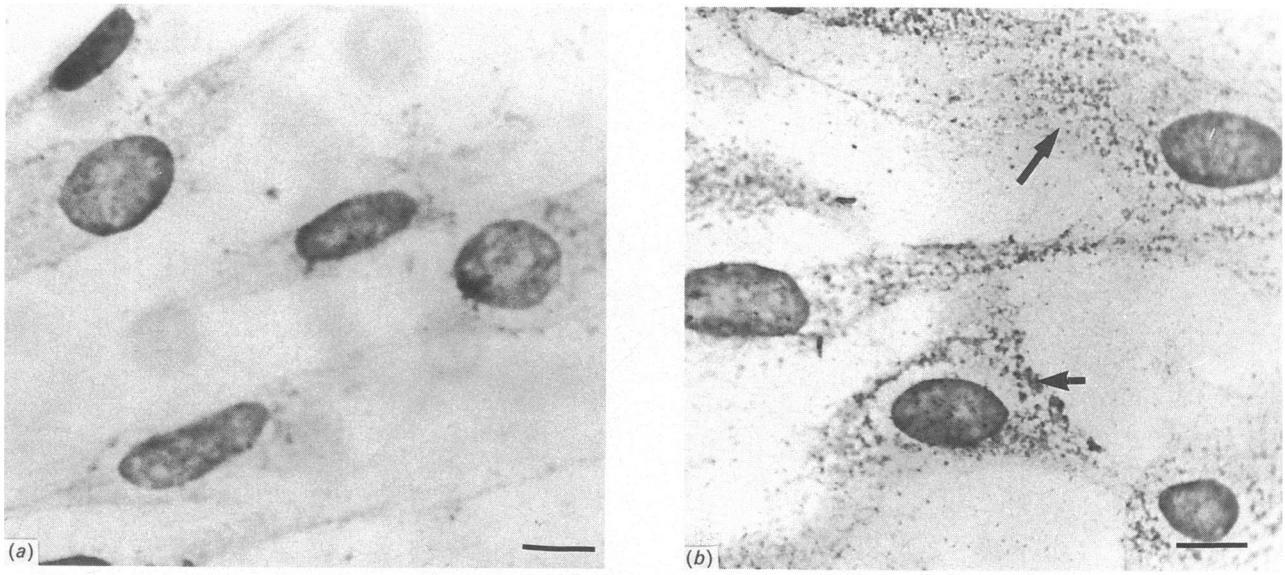


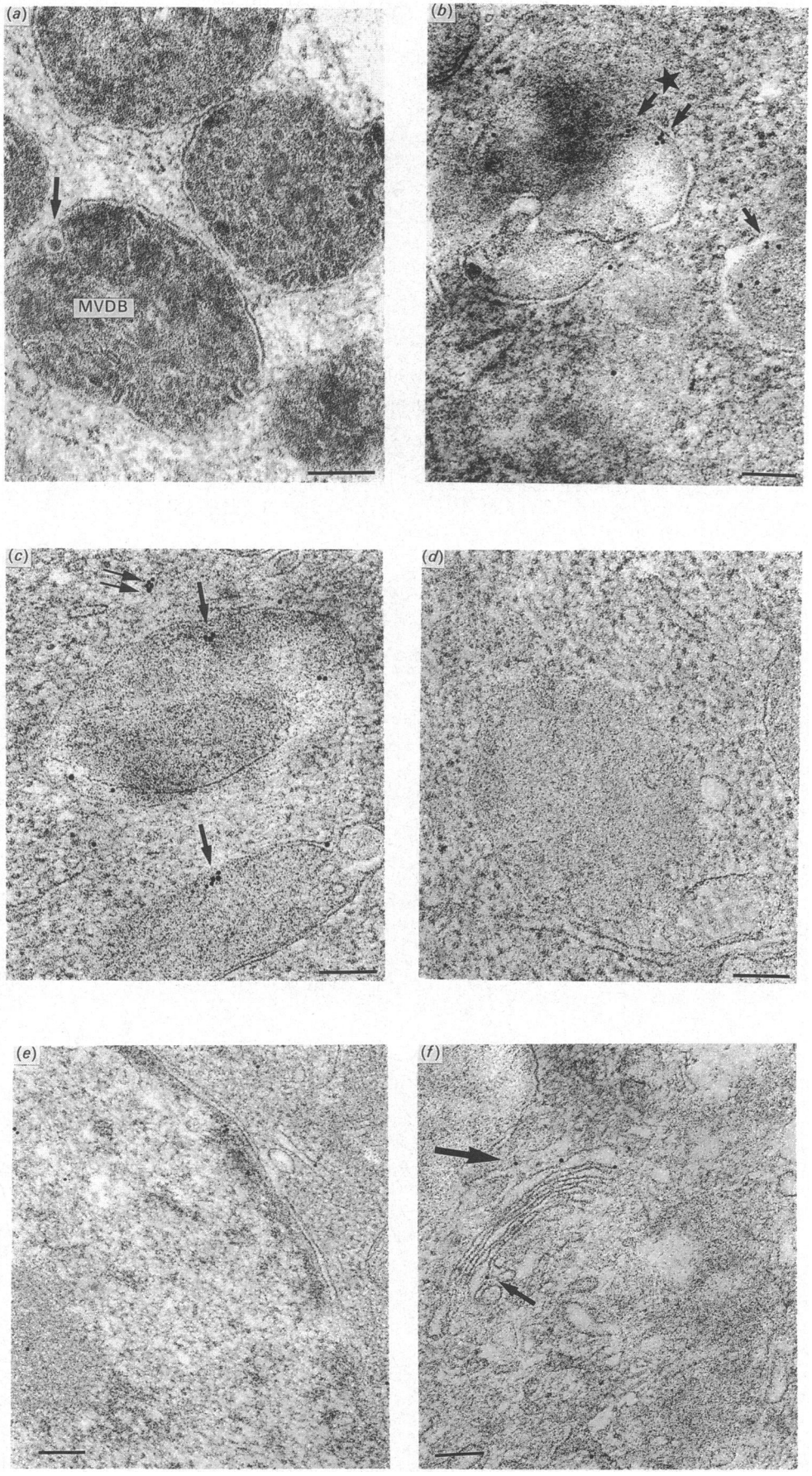
Fig. 4. Distribution of ubiquitin–protein conjugates in 3T3-L1 cells

Cells were treated for 48 h (*a*) in the absence of inhibitors or (*b*) in the presence of E-64 (0.5 mM). Ubiquitin–protein conjugates were detected in fixed cells with rabbit antibody specific for ubiquitin–protein conjugates and visualized as described in Materials and methods. When rabbit anti-(muscle LDH) replaced anti-ubiquitin as a control for the specificity of antibody, there was little or no staining (not shown). Bar, 10 μm. Arrows, punctate cytoplasmic reaction product.

cells for 48 h with E-64 leads to the appearance of numerous aggregates of antibody reaction product in the cytoplasm (Fig. 4*b*, arrows). Cells were found to contain a similar distribution of large amounts of ubiquitin-positive material when treated with the diazomethane proteinase inhibitors Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2 (Doherty *et al.*, 1989) and also when cells were treated with chloroquine (Doherty *et al.*, 1989). In contrast, ammonium chloride-treated cells were found to contain lower levels of ubiquitin conjugates (Doherty *et al.*, 1989).

Immunogold electron microscopy of ubiquitin–protein conjugates

Electron micrographs of E-64-treated cells revealed the presence of numerous multivesicular dense bodies (Fig. 5*a*) which were absent from untreated cells (not shown). Some of the many individual vesicles which are found inside the enveloping membrane of these bodies appear to originate from invaginations of this membrane (Fig. 5*a*, arrow) and are strongly suggestive of the involvement of these bodies in microautophagy (Ahlberg


(a)
MVDB
(b)
(c)
(d)
(e)
(f)

& Glaumann, 1985; Dice, 1987) and contrast with the formation of autophagic vacuoles seen in macroautophagy (Seglen *et al.*, 1985). E-64-generated dense bodies were also found to contain ubiquitin–protein conjugates as determined by immunogold electron microscopy (Fig. 5*b* and 5*c*), thus confirming the biochemical observations, indicated in Figs. 1–3, that ubiquitin–protein conjugates accumulate in the lysosomal system. Gold particles were seen close to the invaginating membrane of dense bodies, and even in an invagination (Fig. 5*b*, arrow with star), again suggesting that uptake into these bodies of ubiquitin–protein conjugates is by microautophagy. Ubiquitin–protein conjugates were also detected in the nucleus, Golgi and diffusely in the cytoplasm by this method (Fig. 5*e* and 5*f*). Immunogold electron microscopy of reticulocytes demonstrated that the antibody specific for ubiquitin–protein conjugates bound to aggregates in the reticulocyte cytoplasm (Laszlo *et al.*, 1989, unpublished work), while the rest of the cytoplasm, which contains large amounts of free ubiquitin, remained unlabelled confirming that free ubiquitin is not detected under these conditions (Riley *et al.*, 1988).

DISCUSSION

Mode of action of inhibitors

In this study we have shown that inhibitors of cysteine cathepsins inhibit lysosomal proteolysis in confluent mouse fibroblasts. There is no evidence that any of these membrane-impermeant inhibitors (Shoji-Kasai *et al.*, 1988) are acting on extra-lysosomal proteinases, as judged by the small additive effects of ammonium chloride when added alongside these inhibitors (Table 1). Polypeptides destined for lysosomal degradation are sequestered in a detergent- and salt-insoluble form, and continue to accumulate in the lysosomal system in the presence of cysteine proteinase inhibitors (Doherty *et al.*, 1987, 1989); therefore, sequestration can occur even when subsequent proteolysis is prevented. Cysteine proteinases would appear to have a very important role in the degradation of proteins in the lysosome (Grinde & Seglen, 1980; Shaw & Dean, 1980; Grinde, 1983).

Ubiquitin conjugation and lysosomal proteolysis

Incubation of cells for a relatively long period with E-64 resulted in the accumulation of ubiquitin–protein conjugates of high molecular mass, some of which were detergent and salt insoluble (Fig. 1), which co-fractionated with acid phosphatase-positive lysosome-related vesicles on density gradient centrifugation (Figs. 2 and 3). The presence of ubiquitin–protein conjugates in these lysosomal fractions is surprising as ubiquitin conjugation is not thought to be involved in lysosomal proteolysis, and the inhibitors used appear to act only on the lysosomal system.

Bovine thymus isopeptidase is unaffected by E-64 (Kanda *et al.*, 1986) and we have found that the disassembly of ^{125}I-labelled ubiquitin–protein conjugates by isopeptidase action in a Fraction II (McGuire *et al.*, 1988) from 3T3-L1 cytosol is not prevented by E-64 (Doherty *et al.*, 1989). Ubiquitin–protein conjugates in reticulocytes can be degraded by a soluble proteinase of large molecular mass, which although thiol dependent, is not inhibitable by leupeptin or antipain (Hough *et al.*, 1987), both of which lead to the accumulation of conjugates in 3T3-L1 cells (Doherty *et al.*, 1989). A similar enzyme from BHK 21/C13 fibroblasts is inhibitable by 50–80% by E-64 or leupeptin, but only at concentrations of 5 mM (McGuire *et al.*, 1988), 10-fold higher than employed in these studies. Therefore, it would seem that the accumulation of ubiquitin–protein conjugates seen in E-64-treated 3T3-L1 cells is not due to the inhibition of either cytosolic deconjugating activity or a soluble ubiquitin–protein conjugate ATP-dependent proteinase activity. The failure of ammonium chloride to cause the same degree of accumulation of ubiquitin–protein conjugates (Fig. 1 and Doherty *et al.*, 1989), may be due to incomplete inhibition by ammonium chloride of a lysosomal activity capable of degradation of the non-ubiquitin moiety of ubiquitin–protein conjugates or of disassembly of conjugates.

We have looked at the cellular localization of aggregates containing ubiquitin–protein conjugates using immunohistochemistry and immunogold electron microscopy. E-64-treated cells (Fig. 4*b*) accumulate multivesicular dense bodies (Fig. 5*a–c*) which are membrane-bordered organelles related to lysosomes (Ahlberg & Glaumann, 1985), and which appear to contain ubiquitin–protein conjugates (Fig. 5*b* and *c*). The interesting question is how ubiquitin–protein conjugates can enter this cell compartment. Ubiquitin is found at the cell surface conjugated to several receptor proteins (Siegelman *et al.*, 1986; Yarden *et al.*, 1986; Meyer *et al.*, 1986) and plasma membrane proteins are thought to be degraded largely by lysosomal mechanisms (Tweto & Doyle, 1976); therefore, these ubiquitin–protein conjugates may be degraded lysosomally and would accumulate when lysosomal proteolysis of the plasma membrane is inhibited. Alternatively, ubiquitin–protein conjugates

Fig. 5. Electron micrographs of E-64-treated cells

Cells were treated for 48 h with E-64 and after trypsinization the pellets were processed for electron microscopy and embedded in Araldite or Lowicryl (immunogold) before sectioning. (*a*) Numerous multivesicular dense bodies (MVDB) were present in treated cells and almost entirely absent from control cells (not shown). (*b* and *c*) Sections were incubated with antibody specific for ubiquitin–protein conjugate followed by biotinylated anti-(rabbit) IgG, then streptavidin–gold. Gold particles could be seen close to, and over, dense bodies. (*d*) Primary antibody was omitted. (*e*) Gold particles over the nucleus of an E-64-treated cell and (*f*) over the endoplasmic reticulum–Golgi area of an E-64-treated cell. Fixation followed by embedding in epoxy resin and osmification revealed morphological details (*a*) not readily seen in samples processed for immunogold microscopy (*b*, *c* and *d*). Bars, 0.1 μm. The arrow in (*a*) indicates the membrane of a multivesicular body invaginating to enclose a small vesicle. Single arrows in (*b*) and (*c*) indicate gold particles near the bordering membrane of the multivesicular body (arrow with star, gold particles within invaginating membrane). Double arrows (*c*) indicate a group of gold particles in the neighbouring cytosol.

formed in the cytosol (e.g. Fig. 5*c*; double arrow) could be taken up into multivesicular bodies by microautophagy as suggested by the electron micrographs shown here (Fig. 5*a–c*). There appears to be an increase in ubiquitin–protein conjugates in the cytosolic fractions from E-64-treated cells (Fig. 2, fractions 1–8 and Fig. 3, fractions 1–8), which could be due to slowed uptake of cytoplasmic material into the constipated lysosomal system in inhibitor-treated cells.

Free ubiquitin is not thought to be degraded lysosomally (Haas & Bright, 1987); therefore, its apparent occurrence in the lysosomal system (Fig. 3, *a* versus *b*, fractions 15–22) suggests that it may have entered the lysosomal system in conjugated form and been released after degradation of the target protein or hydrolysis of the isopeptide bond. Ubiquitin is remarkably resistant to both low pH (Lenkinski *et al.*, 1977) and proteolytic digestion (Schlesinger *et al.*, 1975) and therefore could accumulate in the lysosome. The increase in ubiquitin–protein conjugates in the lysosomal sysem (Fig. 3) could therefore be an underestimate of the amount of ubiquitin–protein conjugates that have entered the lysosome, owing to lysosomal isopeptidase activity or incomplete inhibition of lysosomal proteolysis. Iodoacetamide and haemin were included in the homogenization medium to inhibit isopeptidase (Vierstra & Sullivan, 1988) and ATP- and ubiquitin-dependent proteinase action (Hough *et al.*, 1987) during the fractionation procedure.

We are not able to say whether uptake of ubiquitin–protein conjugates into the lysosomal system is faster than that of unconjugated proteins, i.e. that ubiquitination leads to enhanced lysosomal sequestration. It has been suggested that the relatively hydrophobic *N*-terminus of ubiquitin may act as a signal for membrane translocation (Wilkinson, 1987). Alternatively, ubiquitination may lead to partial unfolding of target proteins exposing hydrophobic sites which bind to lysosomal membranes and are subsequently taken up by microautophagy. Ubiquitination may act as a signal for membrane invagination as seen during microautophagy in multivesicular bodies (Figs. 5*a* and *b*). Ubiquitin has been found to be secreted from cultured cells (Haas & Bright, 1987; Carlson & Rechsteiner, 1987) and free ubiquitin is found relatively concentrated in lysosomes and autophagic vacuoles of hepatoma cells (Schwartz *et al.*, 1988) compared with the surrounding cytoplasm. Therefore, ubiquitin must be able to find its way into vesicular cell compartments, possibly in conjugated form.

Lysosomal inhibitors have been reported to lead to an increase in expression of some heat shock genes (Hsp 70; Ananthan *et al.*, 1986) and the polyubiquitin gene in chicken and human fibroblasts is also subject to a heat shock promoter (Bond & Schlesinger, 1985; Choi *et al.*, 1988). We have found that ammonium chloride and chloroquine lead to a small increase, and E-64 a large increase, in the transcription of a polyubiquitin gene in 3T3-L1 cells (Doherty *et al.*, 1989). Therefore, lysosomal inhibitors, particularly inhibitors of lysosomal cysteine proteinases, may lead not only to the accumulation of ubiquitin–protein conjugates, but also to an increase in ubiquitin gene expression to provide free ubiquitin for increased ubiquitination of cytoplasmic proteins.

We have recently provided evidence that intermediate filament-containing inclusions in chronic degenerative (Lowe *et al.*, 1988*a–c*; Lennox *et al.*, 1989) and viral diseases (Lowe *et al.*, 1988*d*) are ubiquitinated. In Alzheimer's disease we have shown that in areas of granulovacuolar degeneration in hippocampal neurones (Lowe *et al.*, 1988*a*), the granular material in membrane-limited vacuoles is ubiquitinated, i.e. intravesicular material is ubiquitinated in a chronic neurodegenerative disease. It is possible that the intravesicular accumulation of ubiquitin–protein conjugates in E-64-treated cells provides a model for this phenomenon.

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