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Ubiquitinated protein conjugates are specifically enriched in the lysosomal system of fibroblasts

Lajos Laszlo, Fergus J. Doherty+, Natasha U. Osborn+ and R. John Mayer+

Department of General Zoology, ELTE University, Puskin U.3, H-1088 Budapest, Hungary and *+Department of Biochemistry, University of Nottingham Medical School, Queens Medical Centre, Clifton Boulevard, Nottingham, NG 7 2UH, England*

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Ubiquitin-protein conjugates are found by immunogold electron microscopy to be enriched (12-fold) in the lysosomal compartment of 3T3-L1 fibroblasts. Treatment of fibroblasts with the cysteine protease inhibitor E-64 leads to an expansion of the lysosomal compartment and as a result an increase in the cellular content of ubiquitin-protein conjugates. There is no change in the specific enrichment of ubiquitin-protein conjugates in the lysosomal compartment following E-64 treatment. The results suggest that some ubiquitin-protein conjugates may normally be degraded lysosomally following sequestration by microautophagy and imply that protein ubiquitination may be one of the signals for protein uptake into lysosomes.

Ubiquitin; Lysosome; Epoxysuccinyl-leucylamido-(4-guanadino)butane; Electron microscopy; (3T3-L1 fibroblasts)

1. INTRODUCTION

The metabolism of ubiquitin has been extensively studied in reticulocyte lysates where it has been shown to be a cofactor in the rapid extralysosomal ATP-dependent degradation of proteins (reviewed in [1]). However, protein ubiquitination is not only a prerequisite for rapid elimination since in nucleated cells ubiquitinated proteins are found in the nucleus [2] and plasma membrane [3–6]. We have recently shown by biochemical methods that ubiquitinated protein conjugates accumulate in the lysosome compartment in fibroblasts where lysosomal thiol cathepsins have been specifically inhibited [7–10]. At that time immunoblotting and immunogold microscopical examination of untreated cells suggested that ubiquitin-protein conjugates might be normally concentrated in the lysosomal compartment. We have now been able to demonstrate by quantitative immunogold electron microscopy that ubiquitin protein conjugates are indeed selectively enriched in the lysosomal compartment (12-fold) in normal fibroblasts relative to all other organelles. The data indicate a new function for ubiquitin in nucleated cells, namely that protein ubiquitination may serve as a signal for protein uptake into the lysosomal system.

Correspondence address: L. Laszlo, Department of Biochemistry, University Medical School, Queen's Medical Centre, Nottingham NG7 2UH, England

Abbreviations: E-64, epoxysuccinyl-leucylamido-(4-guanidino)butane; LDH, lactate dehydrogenase

2. MATERIALS AND METHODS

3T3-L1 fibroblasts were cultured and inhibitors administered as described previously [10]. Cells were harvested by trypsinization, washed with phosphate-buffered saline then fixed in 2% v/v paraformaldehyde/0.5% glutaraldehyde containing 1% w/v sucrose and 2 mM $CaCl_2$. After post-fixation and block-staining with 1% w/v OsO_4 and 2.5% w/v uranyl acetate the specimens were dehydrated in graded ethanol and embedded in araldite. Cells for immunogold electron microscopy were submitted to the same procedure except for the omission of osmification. Immunogold labelling was carried out by a post-embedding biotin-streptavidin bridge method [11] using affinity-purified rabbit antibody to ubiquitin-protein conjugates [12] (1:20 dilution), affinity-purified rabbit antibody to porcine heart lactate dehydrogenase (1:5 dilution) and biotinylated goat antibody to rabbit IgG (Vector Labs, Peterborough, England; 1:100 dilution) and streptavidin-gold (10 nm, Sigma, Poole, England, 1:20 dilution). Immunostained sections were post-fixed with OsO_4 and counterstained with uranyl acetate and lead citrate.

The total cytoplasmic and lysosomal areas and gold particle densities were estimated [13] using at least 30 different randomly selected electron micrographs of ultrathin sections (covering at least 1000 μm^2). Gold particle numbers from sections treated with non-immune rabbit serum were subtracted from numbers obtained from sections treated with the indicated rabbit primary antibodies (a total of 2829 for anti-ubiquitin protein and 10013 for anti-LDH in untreated cells and a total of 7270 anti-ubiquitin protein conjugate specific gold particles and 8836 anti-LDH specific gold particles in E-64-treated cells) and used to calculate the densities shown in tables 1 and 2.

3. RESULTS AND DISCUSSION

Knowledge of the distribution of ubiquitin-protein conjugates throughout the topologically defined compartments of nucleated mammalian cells is of obvious importance for our understanding of the role of protein ubiquitination. Rabbit antiserum which recognised

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Table 1

Immunogold quantitation of ubiquitin-protein conjugates and lactate dehydrogenase in 3T3-L1 cells

Primary antibody	Immunospecific gold particle density (gold particles/μm^2)			
	Total cytoplasm (Cyt)	Extra-lysosomal cytoplasm (Cyt minus Lys)	Lysosomal compartment (Lys)	Relative density (Lys/Cyt)
Anti-ubiquitin protein conjugates	2.7–0.9	2.2–0.9	33.3–7.0	12.3
Anti-lactate dehydrogenase	9.6–1.3	9.6–1.4	8.3–3.3	0.9

Table 2

Immunogold quantitation of ubiquitin-protein conjugates and lactate dehydrogenase in 3T3-L1 cells treated for 48 h with 0.5 mM E-64

Primary antibody	Immunospecific gold particle density (gold particles/μm^2)			
	Total cytoplasm (Cyt)	Extra-lysosomal cytoplasm (Cyt minus Lys)	Lysosomal compartment (Lys)	Relative density (Lys/Cyt)
Anti-ubiquitin protein conjugates	7.2–2.1	3.9–1.2	32.7–10.5	4.5
Anti-lactate dehydrogenase	8.5–1.8	8.7–2.0	7.5–2.3	0.9

Measurements of total cytoplasmic and lysosomal areas and immunospecific gold particles were as described in section 2

ubiquitin-protein conjugates was raised as described previously [12,14]. Ubiquitin-protein conjugates were readily detectable and free ubiquitin only poorly recognised on immunoblots by affinity-purified antibody [10]. Numerous immunogold particles were observed over sections of freshly prepared rabbit reticulocytes probed with this antibody (not shown). Disassembly of ubiquitin-protein conjugates by depletion of reticulocyte ATP following incubation with dinitrophenol and deoxyglucose resulted in very few immunogold particles over the resulting sections (not shown) suggesting that in contrast to conjugated ubiquitin, free ubiquitin is poorly detected, if at all, in ultrathin sections by this antibody. Examination of sections of 3T3-L1 mouse fibroblast cultures probed with rabbit antibody to ubiquitin-protein conjugates reveals the presence of ubiquitin-protein conjugates throughout the cytoplasm and nucleus of cultured fibroblasts (fig.1a). Structures identified as components of the lysosomal system morphologically as well as biochemically [10,15] were found to be rich in gold particles, indicating the presence of ubiquitin-protein conjugates in this cell compartment (fig.1b). The presence of ubiquitin-protein conjugates in the lumenal space of the lysosomal compartment is surprising given the apparent cytosolic localisation of the enzymes of ubiquitination and proposed role of ubiquitination in targetting conjugated proteins for degradation by a cytosolic large molecular weight protease [16,17]. Gold particles indicating ubiquitin-protein conjugates were also detected in the nucleus and occasionally the Golgi apparatus of 3T3-L1 cells, but at much lower densities than in the lysosomal apparatus (not shown).

Morphometric quantitation of ubiquitin-protein conjugate distribution revealed that the ubiquitin-protein conjugates are normally selectively enriched 12-fold in the lysosomal compartment compared to the total cytoplasm (table 1). Lactate dehydrogenase (LDH) was used both as a marker for the cytosol and to estimate the uptake of cytosolic constituents into the lysosomal compartment by a non-selective mechanism. Immunogold detection demonstrated that LDH is evenly distributed throughout the cytoplasm, with the concentration in the lysosomal compartment being similar to that in the extra-lysosomal cytoplasm (table 1) which would be expected if this protein is lysosomally sequestered by a non-selective pathway. In contrast the high specific enrichment of ubiquitin-protein conjugates in the lysosomal compartment (table 1) suggests that ubiquitin-protein conjugates are sequestered in a selective fashion and implies that ubiquitination normally promotes lysosomal sequestration of proteins.

Treatment of cultured fibroblasts for 48 h with the membrane-impermeant cysteine protease inhibitor E-64, which enters cells by pinocytosis [18], leads to inhibition of lysosomal protein degradation [10] and a large expansion in the cytoplasmic volume fraction occupied by the lysosomal compartment (11.8% compared to 1.7% in untreated cells, i.e. 7-fold, $P < 0.001$), mostly in the form of multi-vesicular dense bodies (MVDB; fig.1d and [19]). The expanded lysosomal compartment contains ubiquitin-protein conjugates as detected by immunoblotting of electrophoretograms [10] and immunogold electron microscopy (fig.1c) largely accounting (i.e. 75%) for the overall increase in the total cytoplasmic content of ubiquitin-protein conjugates (2.7 vs 7.2 gold particles/μm^2, $P < 0.001$; tables 1 and 2). The specific enrichment of ubiquitin-protein conjugates in the lysosome-related compartment remained unchanged following E-64 treatment (approximately 33 gold particles/μm^2, tables 1 and 2) indicating that E-64 treatment does not promote ubiquitin-protein uptake by the lysosomal system.

We have also observed a substantial increase in polyubiquitin mRNA following E-64 treatment (not shown). This may indicate increased cytosolic protein ubiquitination in response to E-64 treatment. There is an increase in the extra-lysosomal density of anti-ubiquitin protein conjugate immunospecific gold par-

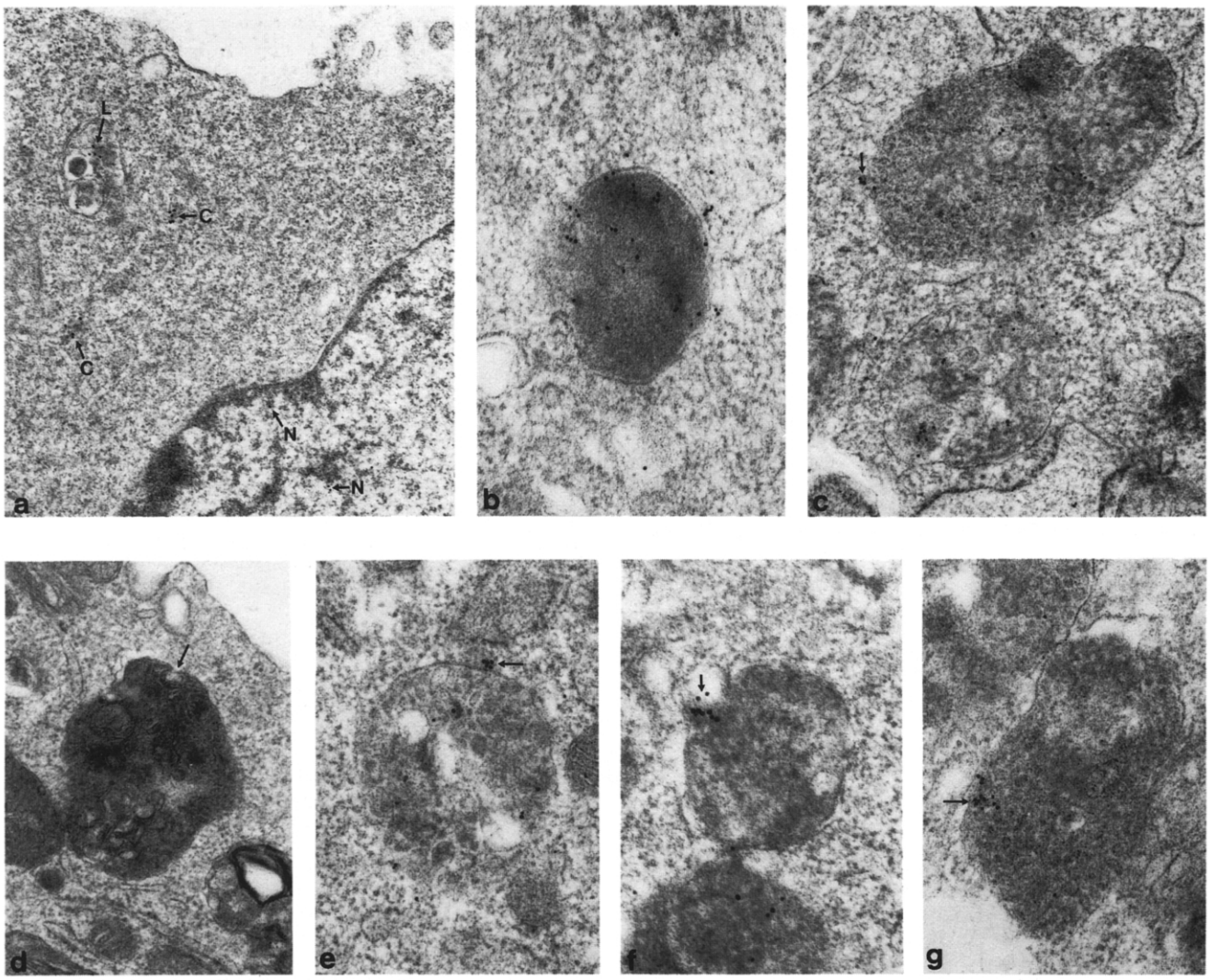

Fig.1. Ubiquitin-protein conjugate immunogold electron microscopy of 3T3-L1 fibroblasts. Gold particles, indicating the presence of ubiquitin-protein conjugates, were detected in the extra-lysosomal cytoplasm (C), nucleus (N) and in multi-vesicular lysosomal bodies (L) of untreated cells (a, arrows). Electron-dense lysosomal bodies in untreated cells are also enriched in ubiquitin-protein conjugates (b). Treatment of cells for 48 h with 0.5 mM E-64 leads to the appearance of numerous electron-dense multivesicular bodies (MVDB; c, d, e, f, g) which are enriched in ubiquitin-protein conjugates (c, e, f, g). Gold particles can be seen close to the cytoplasmic surface of MVDB (c, e), in areas of MVDB surface invagination (f) and close to the lumenal surface of the MVDB limiting membrane (g). The multivesicular structure and surface invaginations present in MVDBs can clearly be seen in samples processed for normal electron microscopy (d). (Magnification: a: × 31200; b: × 67200; c: × 52200; d: × 40800; e: × 63800; f: × 78300; g: × 56000).

ticles following E-64 treatment (3.9 vs 2.2 particles/μm^2, $P < 0.001$; table 2 vs table 1). The increase in extra-lysosomal ubiquitin-protein conjugates could be due to slowed sequestration into the functionally compromised lysosomal system. The increase in polyubiquitin gene expression could result from the cells attempt to maintain the relative concentrations of cytosolic free ubiquitin and ubiquitin-protein conjugates [20]. This would imply that E-64 leads to the trapping in conjugated form in the lysosome of ubiquitin which under normal conditions can recycle out to the cytosol.

Examination of the electron micrographs (fig.1c,e; arrows) shows that groups of gold particles can be found in the cytosolic space close to the surface of the limiting membrane of MVDB. The limiting membrane of the MVDB can be seen invaginating in many cases (fig.1d,f). These observations indicate that the uptake of ubiquitin-protein conjugates, perhaps as aggregates, is occurring by selective microautophagy [21]. Alternative mechanisms for the sequestration of ubiquitin-protein conjugates into the lysosome related system includes by endocytosis of ubiquitinated cell surface proteins [3–6] or fusion of ubiquitin-protein conjugate containing secretory vesicles with components of the lysosomal system. However, the presence of cytosolic ubiquitin-protein conjugates close to the limiting membrane of MVDB (fig.1e,f) indicates that some con-

jugates are sequestered into the lysosomal compartment from the cytosolic space. Whatever the mechanism whereby ubiquitinated proteins enter the lysosomal system, the observations described here suggest that yet another role may be added to ubiquitin's growing repertoire of intracellular functions. Ubiquitin may have a pivotal role in the two major systems of protein catabolism in nucleated cells. Such a role would explain why ubiquitin is only found in eukaryotic cells and why the protein is found in cytosolic and vesicular bodies in a variety of human neurodegenerative diseases where impairments of protein catabolism may occur [9,22–27].

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