Related organelles of the endosome–lysosome system contain a different repertoire of ubiquitinated proteins in Sf9 insect cells

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Received 10 May 1995

Abstract Two components of the endosomal/lysosomal compartment of Sf9 cells, multivesicular bodies (MVB) and light vacuoles with membrane complexes (LVMC) have been isolated and probed for ubiquitin protein conjugates with a specific antibody. Immunogold electron microscopy indicates that whereas ubiquitin–protein conjugates are localised to electron dense areas of MVB they are associated with the membranes of LVMC. Five ubiquitinated polypeptides are revealed in MVB by immunoblotting while numerous ubiquitinated species forming a smear following electrophoresis are present in LVMC. We suggest two possible routes for entry of ubiquitin-protein conjugates into these organelles, via the cell surface and via primary lysosomes.

Key words: Ubiquitin; Endosome; Lysosome; Subcellular fractions; Immunogold electron microscopy

1. Introduction

Ubiquitin is a small protein which mediates the non-lysosomal ATP-dependent degradation of short-lived or abnormal proteins. Ubiquitin is conjugated to target proteins by formation of an isopeptide bond between the C-terminal carboxyl group of ubiquitin and an ε-amino group of lysine residues in the target protein. Formation of multiubiquitin chains on the target protein is a signal for degradation by the soluble 26S proteasome [1]. The ubiquitin-mediated proteolytic pathway appeared to be distinct from lysosomal protein degradation; however, recent observations suggest a link exists between protein ubiquitination and the lysosomal degradation system.

We have previously shown in a variety of cell types in normal and diseased tissues that ubiquitin–protein conjugates are enriched in lysosome-related organelles [2–7]. These observations and the results of others [8] suggest that protein ubiquitination may be involved in lysosome biogenesis and function.

The Sf9 insect cell culture and *Autographa californica* baculovirus vector expression system has recently received much attention due to the ease of cell propagation, ability to express eukaryotic proteins of therapeutic and agricultural importance and capacity for post-translational modifications that maintain biological activity and antigenicity [9]. Authors are interested only in the production of the recombinant protein, which sometimes can be very low. There are some proteins which cannot be expressed in this system, or at least the recombinant protein could not be isolated from the medium [10]. In the latter cases the recombinant protein might be expressed but rapidly destroyed by the intracellular protein degradation systems. Studies of the protein degradation systems of Sf9 cells have therefore a great significance to the production of recombinant proteins by the Sf9-*Autographa californica* expression system.

In Sf9 cells clusters of multivesicular bodies (MVB) and light vacuoles with membrane complexes (LVMC) occur normally in the cytoplasm. Sf9 cells are therefore a good system for the study of these organelles as they appear to be far more abundant than in other cell types. These vacuoles are contain varying amounts of the lysosomal enzyme acid phosphatase. Both of them show intense labelling with antibodies against ubiquitin–protein conjugates but in different distribution: in MVB gold particles corresponding to ubiquitin–protein conjugates are mainly concentrated in discrete spherical densities while in LVMC gold-particles are evenly distributed within the vacuoles [7].

Our purpose was to separate these organelles and investigate the ubiquitinated protein content of these structures. Here we report that the two morphologically different lysosome-related organelles have been isolated and characterised. The distribution of ubiquitin–protein conjugates between these two related compartments and between the soluble and sedimentable subfractions of each has been examined and the spectrum of ubiquitinated proteins in each compartment compared.

2. Materials and methods

2.1. Cell culture

Sf9 insect cells were grown as monolayers in antibiotic-free Grace's medium, supplemented with 10% v/v fetal calf serum (Serva, Heidelberg, Germany), 0.33% w/v yeast extract and 0.33% w/v lactalbumin hydrolysate (Sigma, St. Louis, MO, USA) at 26°C [11]. The cells were subcultured three times a week. Cells were depleted of ATP by incubating in 0.2 mM 2,4-dinitrophenol and 20 mM 2-deoxy-D-glucose (Sigma, St. Louis, MO, USA) in PBS for 3 h [12].

2.2. Cell fractionation

All procedures were carried out at 0°C on ice. Cell monolayers were harvested with a rubber policeman from six 75 cm^2 tissue culture flasks. Detached cells were pelleted at 230 × *g* for 10 min in a Spinchron R (Beckman, Palo Alto, CA, USA) centrifuge. Cell pellets were collected into a homogenising tube and homogenising medium (1.5 ml of 0.3 M sucrose and 0.5 ml of 2 M sucrose, pH 7.4/1 ml cell pellet) was added. Homogenisation and fractionation was performed using a modified version of method previously described [13]. Homogenisation was carried out with 100 strokes in Potter-Elvehjem type glass–Teflon homogeniser rotating at 300 rpm. The homogenate was centrifuged at 600 × *g* for 10 min to sediment the nuclei and undisrupted cells (nuclear fraction). The supernatant was then centrifuged at 5,500 × *g* for 10 min. The second supernatant was saved (microsomes/cytosol fraction). The 5,500 × *g* sediment was gently resuspended in 3 ml of 0.3 M sucrose and 1 ml aliquots of this were layered over 4 ml of 50% v/v Percoll (Pharmacia, Uppsala, Sweden) containing 0.3 M sucrose. A self-generating gradient was formed by centrifugation for 15 min at 92,000 × *g* in a 3 × 5 ml swing-out-rotor of a Janetzki VAC 602 (MLW, Engelsdorf, Germany) ultracentrifuge. The resulting two membrane-rich bands that

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SSDI 0014-5793(95)00624-9

were obtained (lower MVB/M fraction and upper LVMC fraction) were diluted with 0.3 M sucrose solution and centrifuged at 5,500 × *g* for 10 min. Some pellets were processed for electron microscopy, others were resuspended in a small volume of 0.3 M sucrose and analysed biochemically. Water soluble and sedimentable components of subcellular fractions were separated by centrifugation at 100,000 × *g* in an Optima TL (Beckman, Palo Alto, CA, USA) ultracentrifuge following freeze-thawing.

2.3. Protein determination

Protein was measured by the method of Lowry et al. [14] scaled down for 96-well microplates using bovine serum albumin (BSA) as standard.

2.4. Marker enzyme assays

Assays were carried out according to the methods described below all scaled down for 96-well microplates. Acid phosphatase (EC 3.1.3.2) marker enzyme for lysosomes and alkaline phosphatase (EC 3.1.3.1) for apical plasma membrane were assayed by the methods of Engström [15] using *p*-nitrophenol phosphate as substrate. Aryl esterase (EC 3.1.1.2) activity, marker for microsomes [16], was determined by the assay of Shephard and Hubscher [17] using indoxyl acetate as substrate. Succinate dehydrogenase (EC 1.3.99.1) for mitochondria was measured according to Pennington [18] using succinate as substrate and *p*-iodonitrotetrazolium violet (INT) as electron acceptor. Optical densities (OD) were measured using a Microplate Reader Model Σ960 (Metertech Inc., Taiwan). Specific activities (SA) of marker enzymes in samples were calculated as (OD of sample − OD of control (no substrate))/mg protein content of sample/incubation time in minutes. Relative specific activity (RSA) of an enzyme was defined as SA of fraction/SA of total homogenate.

2.5. Electron microscopy

For transmission electron microscopy (TEM) pelleted subcellular fractions were fixed in 1.5% (w/v) paraformaldehyde/1% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 1% (w/v) sucrose and 2 mM $CaCl_2$ for 2 h at 20°C. The samples were washed in 0.1 M cacodylate buffer (pH 7.4) and postfixed in 0.5% (w/v) OsO_4. After en block staining with 2% (w/v) uranyl acetate, samples were embedded in Durcupan (Fluka, Buchs, Switzerland). Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined in a JEM-100CX II (Jeol, Tokyo, Japan) electron microscope operating at 60 kV.

Immunogold labelling was carried out by a post-embedding biotin-antibiotin bridge method [3]. Rabbit polyclonal antibody against conjugated ubiquitin was prepared according to the method described by Haas and Bright [12] using denatured ubiquitin cross-linked with keyhole limpet hemocyanin. Rabbit anti-ubiquitin–protein conjugate antibody was used at a dilution of 1:100 followed by biotinylated goat antibody to rabbit IgG (Vector Labs., Peterborough, UK; 1:200 dilution) and anti-biotin gold (20 nm, BioCell, Cardiff, UK; 1:100 dilution). Immunostained sections were counterstained with uranyl acetate and lead citrate and examined as above.

2.6. Polyacrylamide gel electrophoresis and immunoblotting

Samples of whole cell homogenate and isolated subcellular fractions were dissolved in Laemmli buffer [19] and boiled for 5 min. Polypeptides were separated on 5% or 7.5% SDS-polyacrylamide gels as described by Laemmli [19] using a Mini-PROTEAN II apparatus (Bio-Rad, Richmond, CA, USA). After electrophoresis polypeptides were transferred electrophoretically onto Hybond-C super nitrocellulose membranes (0.45 μm, Amersham, Little Chalfont, UK) and developed according to Towbin et al. [20].

The immunodetection of ubiquitin–protein conjugates was carried out using the same affinity-purified rabbit polyclonal antibody against conjugated ubiquitin [12] as for immunogold electron microscopy (dilution of 1:1,000). Primary antibodies were visualised with secondary antibodies labelled with alkaline phosphatase (Sigma, St. Louis, MO, USA; dilution of 1:1,000) for development with Nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indoxyl phosphate (BCIP) method or peroxidase (Sigma, St. Louis, MO, USA; dilution 1:5,000) for ECL (Amersham, Little Chalfont, UK) detection. Control blots were incubated omitting primary antibody for both methods. Before ECL detection polypeptides on blots were stained with copper(II) phtalocyanine 3,4′,4″,4‴-tetrasulfonic acid tetrasodium salt (CPTS; Aldrich, Deisenhofen, Germany) as described by Bickar and Reid [21] followed by destaining and immunodetection.

3. Results

3.1. Isolation and characterisation of subcellular fractions

In normal Sf9 cells two groups of endosome/lysosome-related organelles can be observed which are easily distinguishable morphologically: multivesicular bodies (MVB) and light vacuoles with membrane complexes (LVMC). These two distinct population of vacuoles were separated for further studies.

Four subcellular fractions were obtained following differential centrifugation of Sf9 homogenates. Following homogenisation of cells cell nuclei and undisrupted cells (fraction 1) were isolated from supernatant containing all the other cell organelles and cytosol. Following centrifugation of the postnuclear supernatant the small membrane particles (microsomes) and the cytosol (fraction 4) remained in the supernatant while larger organelles were concentrated in sediment. From this sediment two fractions were separated on a self-generating Percoll gradient (fractions 2 and 3). After pelleting, fixation and embedding these fractions were examined in TEM. In the lower fraction there were two main components: dense multivesicular bodies and mitochondria (MVB/M; Fig. 1A). The bulk of the upper fraction contained light vacuoles with membrane complexes (LVMC; Fig. 1B). The isolated organelles look substantially the same as they did in the whole cells therefore they are not artefacts of homogenisation.

Marker enzyme assays on the four fractions confirmed that fractionation has been achieved (Table 1). The highest acid phosphatase activity, a marker for lysosomes, is observed in LVMC fraction (RSA 2.06) indicating an enrichment of lysosomal/endosomal organelles in this fraction. The MVB/M fraction is less enriched for acid phosphatase (RSA 1.03). The nuclear fraction shows a relatively high acid phosphatase activity due to presence of more or less intact cells in this fraction. Microsomal marker (aryl esterase) activity was found the most enriched in the microsomes/cytosol fraction but relatively high activity is detectable in other fractions as well. Succinate dehydrogenase, a marker for mitochondria is most enriched in the MVB/M fraction as expected because of the high number of mitochondria seen in this fraction (Fig. 1A). Alkaline phosphatase, a plasma membrane marker was very highly enriched in the MVB/M fraction (RSA 5.98) indicating that MVB are endosome related organelles with intravacuolar membrane structures originating partially from the plasma membrane. Electron micrographs of the MVB/M fraction show only small amounts of material suggestive of low levels of free plasma membrane while MVB themselves contain numerous small vesicles some of which could be derived from the plasma membrane (Fig. 1A,C). Alkaline phosphatase activity is lower in all other fractions.

3.2. Immunochemical detection of ubiquitin–protein conjugates

The presence and distribution of ubiquitin–protein conjugates has been studied in the two fractions containing lysosome-related organelles. The spatial distribution of ubiquitinated proteins was detected by immunogold methods on sections of embedded fractions using a ubiquitin–protein conjugate specific antibody. The labelling of isolated organelles (Fig. 1C,D) is the same as in intact cells suggesting that no substan-

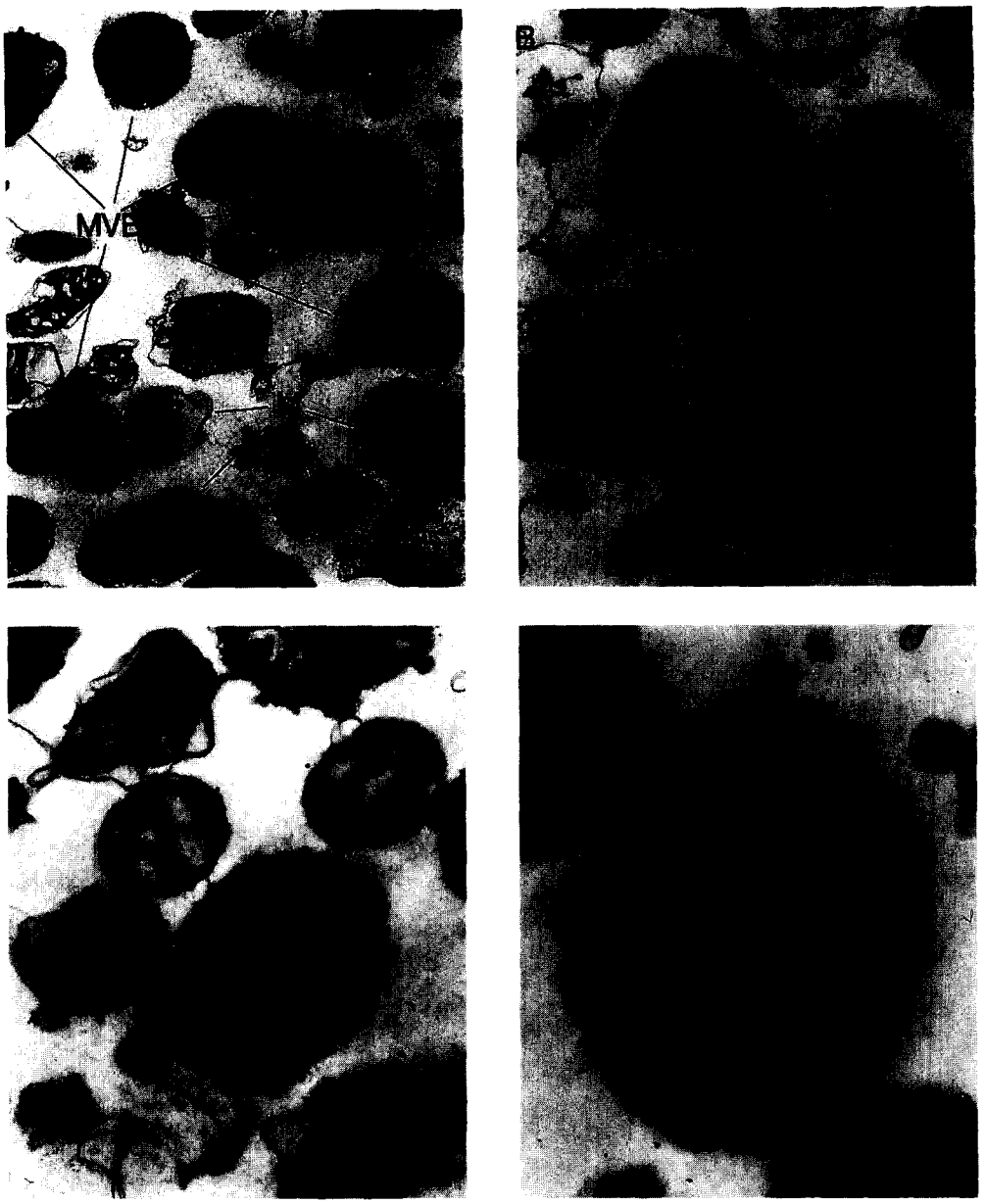

Fig. 1. Electron micrographs of two subcellular fractions of Sf9 cells. (A) Fraction containing dense multivesicular bodies (MVB) and mitochondria (M). (B) Fraction containing light vacuoles with membrane complexes (LVMC). Bars = 1 μm. After labelling with ubiquitin–protein conjugate specific antibody (C) electron-dense area of MVB are decorated by discrete clusters of gold particles and there is no significant labelling above the mitochondria. (D) Gold particles are evenly distributed in LVMC and are located mainly over membrane complexes. Bars = 0.5 μm.

tial changes in the complement of ubiquitin–protein conjugates occurs during fractionation. Electron dense areas of MVB are decorated by discrete clusters of gold particles corresponding to ubiquitin-protein conjugates (Fig. 1C). There is no significant labelling above the mitochondria. Gold particles are evenly distributed on LVMC but mainly on membrane complexes (Fig. 1D).

Further experiments were carried out to investigate the qualitative distribution of ubiquitin–protein conjugates. Polypeptides of total homogenate and subcellular fractions of Sf9 cells were separated by SDS PAGE. Patterns of total polypeptides are very different in the different fractions (Fig. 2A) confirming the effectiveness of fractionation. Major polypeptides can be identified both in the total homogenate and in certain fractions and there are at least 9 polypeptides of similar size in the MVB/M and LVMC fractions (Fig. 2A, lines). On Western blots of the fractions developed by ubiquitin conjugate specific antibody there are notable differences in immunostaining (Fig. 2B). In the MVB/M fraction 5 distinct immunoreactive polypeptides are observable (at about 50, 60, 70, 110 and 130 kDa; Fig. 2B, arrowheads) among which the 110 kDa one gives a very strong signal, while an intense smear appears in the high molecular weight region of LVMC fraction (Fig. 2B). Others have found anti-ubiquitin positive polypeptides with similar molecular weights in a different system, *Mamestra brassicae* (70, 110 and 130 kDa; Sass unpublished observations) using the

Table 1
Relative specific activities of enzyme markers of Sf9 subcellular fractions

	Nuclear fraction	MVB/M fraction	LVMC fraction	Microsome/cytoplasm fraction
Acid phosphatase	0.96 ± 0.08	1.03 ± 0.17	2.06 ± 0.17	0.64 ± 0.12
Aryl esterase	1.34 ± 0.11	1.60 ± 0.52	1.50 ± 0.26	1.66 ± 0.45
Succinate dehydrogenase	2.17 ± 0.16	5.98 ± 1.20	2.57 ± 0.34	0
Alkaline phosphatase	1.63 ± 0.08	5.98 ± 0.61	1.54 ± 0.18	2.30 ± 0.20

Values were calculated as an average of 2 parallel measurements of 3 different samples (±S.D.). Note the high acid phosphatase activity, a marker for lysosomes, in LVMC fraction showing the enrichment of degradative organelles in that fraction. Alkaline phosphatase, a plasma membrane marker, activity is the highest in MVB/M fraction.

same type of antibody. Control blots developed without primary antibody remained completely clear (not shown).

Depleting cell of ATP has been shown to result in disassembly of ubiquitin–protein conjugates [12]. Sf9 cells depleted of ATP by incubation with 2,4-dinitrophenyl hydrazine and 2-deoxy-glucose remained viable (as determined by trypan blue exclusion), but the anti-ubiquitin positive 110 kDa band disappears from MVB/M (Fig. 3A) and the smear of staining in LVMC is greatly reduced (Fig. 3B). This supports the conclusion that these immunopositive polypeptides represent ubiquitin–protein conjugates.

After freezing and thawing of the fractions the anti-ubiquitin immunoblots of the resulting sedimentable components containing membranes are very similar to the immunoblots of the whole fraction while the non-sedimentable components of the fractions show no detectable ubiquitin–protein conjugate immunoreactivity (Fig. 4). At the same time both the soluble and sedimentable components contain numerous polypeptides as revealed by staining with Coomassie blue (not shown).

4. Discussion

Ubiquitin–protein conjugates can be detected not only in the cytosol but also in the sub-compartments of endosomal/lysosomal system in Sf9 cells [7] as we previously showed in a variety of cell types [2–4]. These observations are interesting given that the ubiquitin-mediated proteolytic pathway is cytosolic and appears to be distinct from the lysosomal system. Therefore our goal in this investigation was to separate vacuoles of the endosomal/lysosomal compartment for more detailed examination.

We succeeded in separation of components of the endosomal/lysosomal system of Sf9 cells into two fractions. The more dense fraction (MVB/M) consists of dense, compact multivesicular bodies and mitochondria (Fig. 1A). Multivesicular bodies are thought to be mainly of endosomal origin and therefore will contain plasma membrane derived material, which is supported by the high alkaline phosphatase activity of this fraction (Table 1). Alkaline phosphatase has been localised to

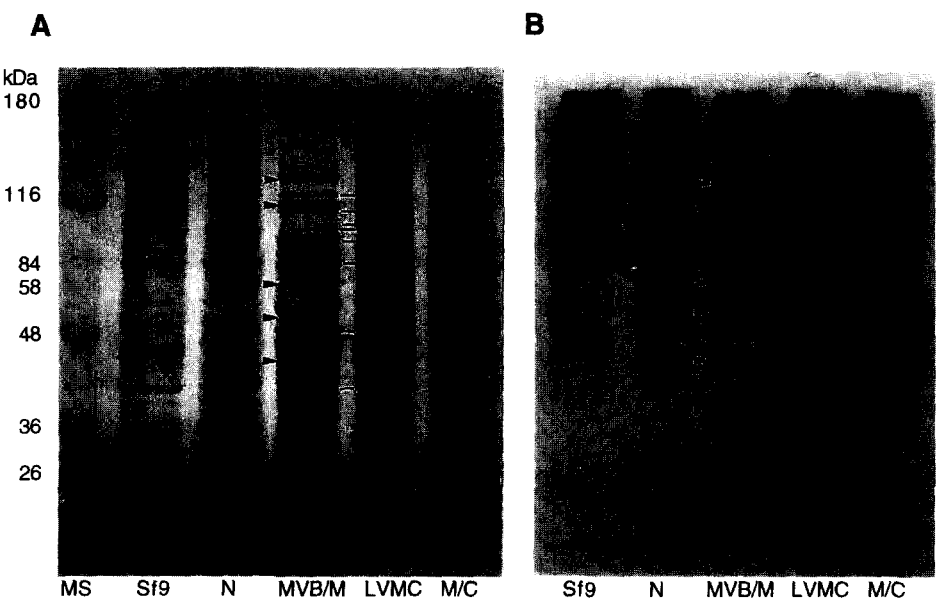

Fig. 2. Anti-ubiquitin antibody detects polypeptides present in the endosomal/lysosomal compartment of Sf9 cells. Polypeptides from subcellular fractions were separated on a 7.5% SDS polyacrylamide gel and blotted onto a nitrocellulose membrane prior to staining for total protein (panel A) or immunostaining with anti-ubiquitin (panel B). Four fractions were obtained from Sf9 homogenate (Sf9): nuclear fraction (N), a fraction containing dense multivesicular bodies and mitochondria (MVB/M), a fraction containing light vacuoles with membrane complexes (LVMC) and a microsomes/cytosol fraction (M/C). Patterns of total polypeptides on nitrocellulose were revealed with CPTS stain (panel A). Major peptides can be identified both in the total homogenate and in samples of certain fractions and there are 9 identical bands between MVB/M and LVMC fractions (lines). Arrowheads on the lane containing MVB/M fraction point to five polypeptides (panel A) which are detected by anti-ubiquitin antibody and ECL (panel B). Molecular mass standards (MS) are indicated on the left. In contrast smears of anti-ubiquitin positive polypeptides are detected in LVMC (panel B).

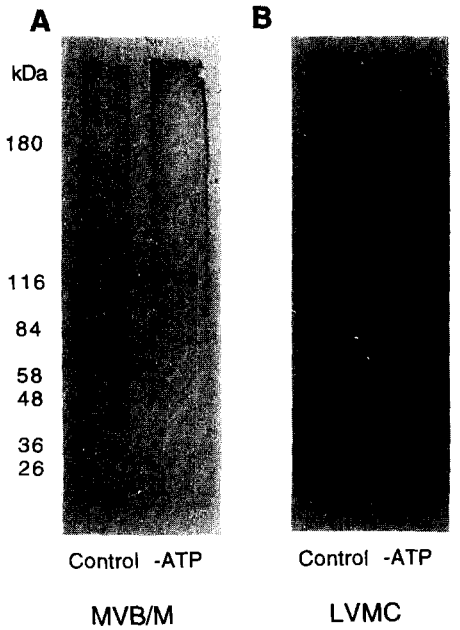

Fig. 3. ATP-depletion of Sf9 cells results in disappearance of anti-ubiquitin antibody immunoreactive polypeptides. Cells were depleted of ATP as described in section 2, followed by electrophoresis, transfer to nitrocellulose and probing with anti-ubiquitin antibody. Anti-ubiquitin immunoreactive polypeptide bands in fractions from control cells (control) are identical to those in Fig. 3B. In fractions obtained from ATP depleted cells these bands disappear (− ATP).

the plasma membrane in a number of insect cell types [22,23]. MVB contain numerous electron dense areas some of which appear to be membrane bordered (Fig. 1A,C and [7]). This may represent the capture by MVB of plasma membrane derived vesicles with their membrane intact. Acid phosphatase is less enriched in the MVB/M fraction compared to the LVMC fraction perhaps because of the diluting effect of the presence of mitochondrial proteins or genuinely lower levels of active acid phosphatase. Histochemical staining suggests that MVB may take up acid phosphatase containing organelles (e.g. primary lysosomes [7]). The action of lysosomal enzymes may lead to breakdown of internal MVB membranes as part of the maturation into LVMC. Indeed, the less dense LVMC contain wide spread acid phosphatase activity [7] and partially degraded membrane structures (Fig. 1B,D). In addition, we have observed that cationized ferritin is taken up by Sf9 cells initially into small endosomal vesicles and subsequently appears in MVB and then later in LVMC [24] supporting the proposed relationship between these compartments with MVB maturing into LVMC. Alternatively, material is transferred from one pre-existing organelle to another [25]. Others have provided evidence for the appearance of endocytosed ligands in an early endosome compartment which matures into dense endosomes and finally very dense endosomes which fuse with lysosomes [26,27]. We suggest that the dense MVB observed here may be analogous to these very dense endosomes and that the less dense LVMC represent the products of MVB and lysosome fusion followed by degradation of MVB contents leading to a lower density organelle.

There are clusters of ubiquitin–protein conjugate specific gold particles over some dense vesicles within MVB (Fig. 1A,C) and on the corresponding immunoblots several discrete ubiquitin containing polypeptides are visible (Fig. 2B, lane MVB/M) which may imply that these ubiquitinated species are selectively present in a sub-population of endosomes. However, gold particles corresponding to ubiquitin–protein conjugates show an even distribution in LVMC (Fig. 1B,D) and on the corresponding immunoblots a large number of ubiquitin containing polypeptides are observed including conjugates of high molecular weight leading to a smear of antibody staining (Fig. 2B, lane LVMC) typical of the presence of numerous ubiquitin-protein conjugates as seen in other cell types [2,12].

The discrete polypeptides present in the MVB/M appear to be absent from the LVMC, although we cannot exclude the possiblity that any of these polypeptides remaining are hidden by the numerous anti-ubiquitin positive polypeptides in the LVMC. The strikingly different pattern of ubiquitin-protein conjugates in the two related compartments is somewhat difficult to explain if LVMC are formed by maturation of MVB rather than vesicular transfer of material (e.g. ferritin [24]) between the two compartments. The discrete bands visible in the MVB/M fraction may represent the uptake of cell surface derived ubiquitinated proteins, for instance receptors, many of which are now known to be ubiquitinated and internalised in response to ligand binding [28–33]. The ABC-transporter Ste6 accumulates at the plasma membrane in a ubiquitinated form in yeast endocytosis mutants, and in the vacuole in mutants deficient in vacuolar proteases [34]. These data provide a mechanism for cell surface proteins, which are ubiquitinated on their cytoplasmic tails to enter the endosomal/lysosomal compart-

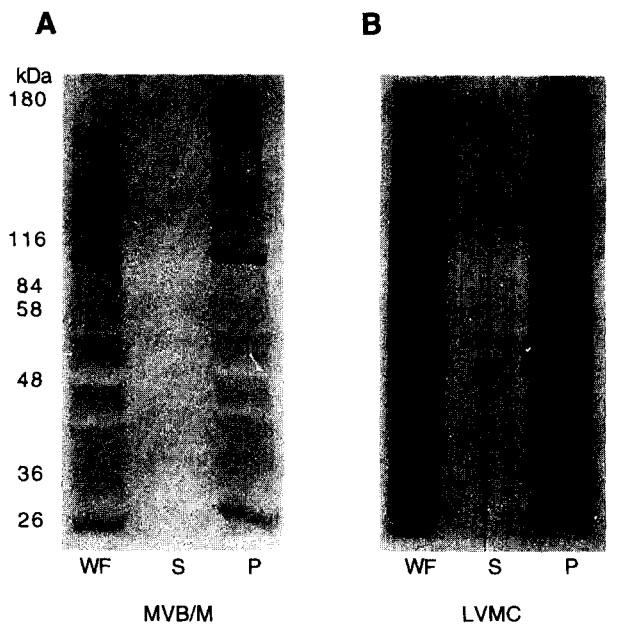

Fig. 4. Ubiquitin containing polypeptides from MVB/M and LVMC are sedimentable following freeze-thawing. After freezing and thawing to induce membrane lysis and release of vacuolar contents subcellular fractions were centrifuged at 100,000 × g and supernatants (S) and pellets (P) were analyzed further. Polypeptides were separated by electrophoresis on a 7.5% SDS polyacrylamide gel. Pellets (P) containing membranes, membrane associated and insoluble proteins contained anti-ubiquitin immunoreactive polypeptides similar to the whole fractions (panel A: whole fraction (WF), S and P from MVB/M; panel B: WF, S and P from LVMC), while ubiquitin containing polypeptides are absent from the supernatants (S).

ment. Rabbit prolactin receptor expressed in Sf9 cells is found in a ubiquitinated form [33] demonstrating that Sf9 cells are capable of ubiquitinating cell surface proteins. These observations together with the high alkaline phosphatase activity of the MVB are suggestive of a possible cell surface origin for the discrete ubiquitinated polypeptides seen in the MVB/M fraction (Fig. 2B, lane MVB/M). Endocytosis per se, or non-receptor-mediated endocytosis do not appear to involve protein ubiquitination as we did not observe any anti-ubiquitin immunogold labelling of primary endosomes containing cationized ferritin while at the same time MVB (and LVMC) were labelled [24]. Interestingly, baculovirus infected Sf9 cells synthesize a ubiquitin, or ubiquitin-like protein, coupled to phosphatidate [35]. It is not yet known if non-infected cells produce this novel form of lipid-modified ubiquitin, but such a modification may serve to anchor ubiquitin to intracellular membranes via the carboxy-terminal glycine of ubiquitin where it may be readily available for conjugation to proteins anchoring them to membranes.

The smear of ubiquitin protein conjugates detected in the LVMC fraction could in part result from the degradation of the ubiquitin–protein conjugates present in the MVB/M fraction. However, the presence of higher molecular weight ubiquitin protein conjugates in the LVMC suggests that additional ubiquitinated polypeptides enter during maturation of the MVB. Possibly, these additional conjugates may be delivered following uptake of primary lysosomes and/or autophagic vacuoles. We have previously detected ubiquitin–protein conjugates in primary lysosomes (transport vesicles [36]) of Sf9 cells by immunogold electron microscopy and have observed the uptake of primary lysosomes by MVB [7].

Our observations support those of Lenk et al. [8] who found that in cells with a thermolabile ubiquitin activating enzyme (E1) early autophagic vacuoles fail to mature into late autolysosomes at the non-permissive temperature. Ubiquitination is not necessary for autophagic segregation [8] and early autophagic vacuoles do not contain ubiquitinated proteins (László, unpublished observations). Mature secondary lysosomes are full of ubiquitinated proteins [2–4,6,7] suggesting that protein ubiquitination is necessary for the maturation of autolysosomes in some, as yet unknown, way. We have been unable to identify Golgi-derived vesicles that may contribute to MVB and LVMC as Sf9 cells do not express mannose-6 phosphate signals on newly synthesized lysosomal proteins [37]. Interestingly, a ubiquitin-activating enzyme is located in the endoplasmic reticulum of yeast [38] and over-expression of ubiquitin rescues clathrin-deficient yeast [39] which may imply a role for protein ubiquitination in the functioning of the endoplasmic reticulum–Golgi pathway.

The ubiquitin–protein conjugates detected in MVB and LVMC are sedimentable following freeze-thawing of these fractions (Fig. 4A, lane P and Fig. 4B, lane P) which may suggest that these ubiquitin–protein conjugates are membrane bound or membrane associated structures. This is consistent with the suggestion that MVB may contain ubiquitinated receptors. However, we cannot exclude the possibility that some at least of the ubiquitin protein conjugates in the endosomal/lysosomal compartment are sedimentable due to extensive aggregation. Indeed, smears of detergent insoluble ubiquitin–protein conjugates have previously been detected in cells with compromised lysosomal proteolysis [2,40].

The observations described here support and extend our previous reports on the concentration of ubiquitinated proteins in the endosomal/lysosomal system. The apparent temporal relationship between MVB and LVMC and the very different repertoire of ubiquitinated proteins in these two fractions suggest that ubiquitinated proteins may enter the lysosomal system via a number of different routes. We would like to suggest that the cell surface may be a source of ubiquitinated proteins destined for degradation, at least partly, in the lysosomal system. In addition, some ubiquitinated protein may be present in primary lysosomes [7]. The role of ubiquitination in the primary lysosome is unknown but it is interesting to note that the biogenesis of at least one other organelle, the peroxisome, appears to involve a ubiquitin-conjugating enzyme [41,42]. Further experiments are in progress to isolate and characterise the major ubiquitinated species in the MVB/M fraction in an attempt to identify them and to isolate primary lysosomes from Sf9 to analyse for ubiquitin–protein conjugates.

Ubiquitin is implicated in programmed cell death (PCD) in insects. Schwartz and co-workers have shown that an early event in the death of the intersegmental muscle (ISM) in hawkmoth *Manduca sexta* is the increased transcription of a polyubiquitin gene [43]. Developmentally programmed cell death can occur by one of three different mechanisms: apoptotic, autophagic and non-lysosomal vesicular degeneration [44]. The features currently held to be characteristic of apoptosis, such as membrane blebbing, chromatin condensation, and DNA fragmentation are not observable during PCD of ISM [45] and motoneurons that innervate them [46]. In contrast, intense autolysosomal activity is detectable in ISM of moths (*Manduca sexta* [45], *Antheraea polyphemus* and *Philosamia cynthia* [47]) and in abdominal motor neurons of *Manduca sexta* [46], so these tissues undergo a special developmentally-regulated cell death involving autophagy. Recent evidence also demonstrates an increase in the activity of the cytosolic protease involved in non-lysosomal degradation of ubiquitinated proteins, the 26S proteasome during ISM death [48]. Therefore the increase in polyubiquitin gene expression in ISM before PCD in muscles may be to fuel both ATP-dependent protein ubiquitination and degradation by the 26S proteasome and protein ubiquitination to enhance the auto-lysosomal destruction of muscle proteins. Although, Sf9 cells, an ovarian cell line from *Spodoptera frugiperda* [49] are not highly specialised like muscle cells or motoneurons they show similar high lysosomal activity and the MVB and LVMC compartments increase on treatment with 20-hydroxyecdysone [7].

Acknowledgements: This work was partly financed by a Copernicus grant from the EU (CIPACT – 930210). The authors would like to thank G. Réz for valuable criticisms of the manuscript and the 'Foundation for the Hungarian Science' for the support of P.L.

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