Volume 316, number 2, 152–156 FEBS 12039 January 1993

Immunogold localisation of ubiquitin–protein conjugates in Sf9 insect cells

Implications for the biogenesis of lysosome-related organelles

Péter Löw[a], Fergus J. Doherty[b], Miklós Sass[a], János Kovács[a], R. John Mayer[b] and Lajos László[a]

[a]*Department of General Zoology, Eötvös University, Puskin u. 3, Budapest, H-1088, Hungary* and [b]*Department of Biochemistry, University of Nottingham Medical School, Queen's Medical Centre, Nottingham, NG7 2UH, UK*

Received 25 November 1992; revised version received 11 December 1992

Immunogold electron microscopy with antibodies which primarily detect ubiquitin–protein conjugates shows conjugate-specific gold particles enriched severalfold in acid phosphatase-positive lysosomes and multivesicular bodies in insect Sf9 cells. The observations demonstrate that ubiquitinated proteins are associated with small acid phosphatase-containing primary lysosomes (transport vesicles) and indicate a pathway in which primary lysosomes fuse with multivesicular bodies to generate mature lysosome-related structures.

Sf9 insect cell; 20-Hydroxyecdysone; Lysosome, acid phosphatase; Ubiquitin

1. INTRODUCTION

Increases in 20-hydroxyecdysone preceding moulting are closely followed by enhanced autophagic/lysosomal activity in insect tissues [1,2]. Elevated ecdysone affects virtually all tissues during metamorphosis as the entire animal is reorganized; most larval tissues and organs break down and are replaced by adult structures which develop from discrete clusters of imaginal progenitor cells.

We have previously shown in a variety of cell types in normal and diseased tissues in animals and man that ubiquitin–protein conjugates are enriched in lysosome-related organelles [3–5]. The availability of insect cell lines which respond to 20-hydroxyecdysone led us to explore the effect of this hormone on the lysosomal and ubiquitin systems in Sf9 cells, a clonal isolate derived from the immature ovaries of the pupae of the Fall armyworm *Spodoptera frugiperda* [6]. We show here that ubiquitin–protein conjugates are enriched in lysosomes and multivesicular bodies.

The observations suggest a sequence of events by which lysosome-related organelles may mature in animal cells and support the notion that protein ubiquitination may be involved in lysosome biogenesis and function.

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

2. MATERIALS AND METHODS

Cells, subcultured three times weekly, were maintained at 26°C in antibiotic free Grace's medium supplemented with 10% fetal calf serum. Hormone-treated cells were grown for 72 h in the presence of 10^{-6} M 20-hydroxyecdysone (20-HE). For transmission electron microscopy (TEM) cells were fixed for 1 h in 1.5% (v/v) paraformaldehyde/1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 1% (w/v) sucrose and 2 mM $CaCl_2$. The samples were postfixed in 1% (w/v) OsO_4 for 1 h, then dehydrated in acetone and embedded in Araldite (Durcupan ACM, Fluka, Buchs, Switzerland). Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined in a Philips 410 TEM operating at 80 kV.

For ultracytochemical localisation of acid phosphatase, cells were incubated at 37°C for 3 h in medium containing 0.1 M acetate buffer (pH 5.0), 1 mM β-glycerophosphate (Sigma, Poole, Dorset, UK), 2 mM $CeCl_3$ (Sigma), and 5% (w/v) sucrose [7]. Immunogold labelling was carried out by a post-embedding biotin–antibiotin bridge method [3,4] using an affinity-purified sheep antibody, which recognises ubiquitin conjugated to other proteins [5,8], at a dilution of 1:100 (non-immune sheep IgG was applied for the controls at the same concentration) followed by biotinylated goat antibody to sheep IgG (Vector Labs., Peterborough, UK; 1:200 dilution) and antibiotin-gold (10 nm, Bio-Rad, Hemel Hempstead, UK; 1:200 dilution). Immunostained sections were counterstained with uranyl acetate and lead citrate and examined as above.

For morphometric evaluation 24 micrographs were taken randomly from the ultrathin sections of each sample (final magnification 30,000×). Morphometric measurements were made by the point-counting method [9] with the help of a double-square lattice (5 × 5 and 25 × 25 mm). The cytoplasmic volume fraction of the components of the lysosomal system (expressed in %) and the density of gold particles above the cytoplasm and lysosome-related organelles (number of gold particles/μm^2) were computed from each micrograph. The resulting data were subjected to statistical evaluation, including computation of mean ± S.E. and application of F and t-tests. Differences were considered significant at $P < 0.001$.

Published by Elsevier Science Publishers B V

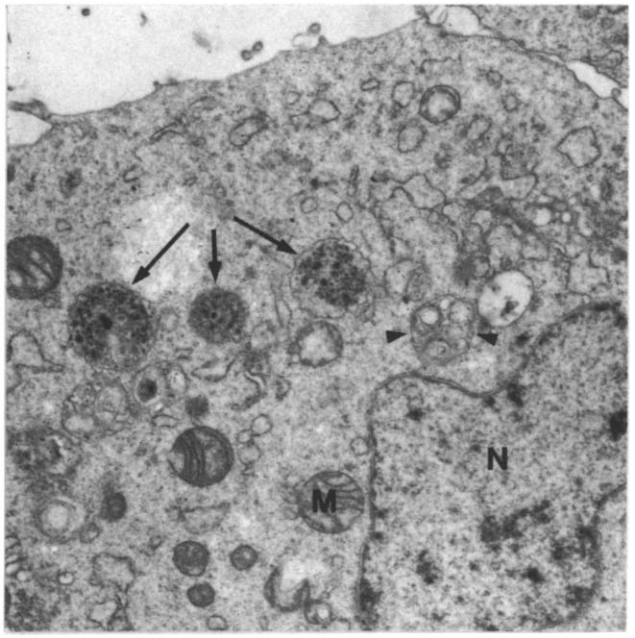

Fig. 1. Light vacuole with membrane complexes (LVMC, arrowheads) and multivesicular bodies (MVB, arrows) in Sf9 cells treated with 20-HE (10^{-6} M); mitochondrion, M; nucleus, N; magnification 15,000×.

3. RESULTS AND DISCUSSION

Clusters of multivesicular bodies (MVB) and light vacuoles with membrane complexes (LVMC) of different sizes can be easily seen in Sf9 cells treated with 20-hydroxyecdysone (Fig. 1) when the cytoplasmic volume fraction of MVB and LVMC is expanded approximately fourfold by treatment of the cells with the moulting hormone (16.32 ± 2.55% compared to 4.26 ± 1.12% in untreated cells, $P < 0.001$).

The electron microscopical analysis of the cells immunolabelled for ubiquitin–protein conjugates and stained for a lysosomal marker enzyme, acid phosphatase, suggest that MVB and LVMC may represent two families of endosome/lysosome-related organelles (or two different stages of biogenesis and maturation).

In MVB gold particles corresponding to ubiquitin–protein conjugates are mainly concentrated in discrete peripheral spherical densities (Fig. 2C,D,E). The acid phosphatase staining is similar to the distribution of ubiquitin–protein conjugates in these organelles (not shown). In LVMC the ubiquitin-specific gold particles are evenly distributed (Fig. 3A) and these electron-lucent vacuoles show intense reaction product of acid phosphatase also distributed uniformly (Fig. 3B). The presence and distribution of acid phosphatase indicates that both families of organelles (MVB and LVMC) are part of the lysosome related system and the LVMC may represent late mature (secondary) lysosomes in these cells.

MVB-containing discrete areas of acid phosphatase and ubiquitin–protein conjugates may arise in different ways. One of the possibilities is that MVB with discrete peripheral densities containing ubiquitin-protein conjugates (Fig. 2C,D,E) are the result of fusion of MVB with primary lysosomes (defined elsewhere as transport vesicles containing acid hydrolases [10,11]). Small primary lysosomes containing acid phosphatase (Fig. 2A) and ubiquitin–protein conjugates (Fig. 2B) can be seen in the cytoplasm. A primary lysosome fusing with an MVB may be seen in Fig. 2C (arrow). The origin of the MVB is unknown but they resemble components of the multivesicular-endosomal compartment [10,11]. If this is the case, then a sequence of events can be envisioned in which early endosomes derived from the plasma membrane mature to MVB (defined elsewhere as endosome carrier vesicles [11]). Following acidification, the MVB acquire lysosomal enzymes by fusion with primary lysosomes and complete their maturation into LVMC (defined as endolysosomes since they are derived from endosomes) with uniform distribution of ubiquitin–protein conjugates and acid phosphatase (Fig. 3A,B) through the digestive activities of the hydrolytic enzymes. The acquisition of ubiquitin–protein conjugates by MVB from primary lysosomes (Fig. 2) suggests that protein ubiquitination may not be directly involved in the initial events of endocytosis. This notion is supported by the observation that early tubulovesicular endosomes do not contain acid phosphatase activity or ubiquitin–protein conjugates (Fig. 2E, asterisk).

Large complex vacuoles with dense membrane limited structures are also seen; they contain ubiquitin–protein conjugates (Fig. 4A) and acid phosphatase (Fig. 4B). These lysosome-related organelles may develop through fusion of double-membraned autophagic vacuoles with other vacuolar organelles (e.g. MVB). Digestion of materials by hydrolytic enzymes within such structures will then also generate large LVMC with uniformly distributed acid phosphatase and ubiquitin–protein conjugates similar to those seen in Fig. 3A,B.

Quantitation of gold particle densities in lysosome-

Table I

Enrichment of ubiquitin–protein conjugates in the lysosome system of Sf9 cells

	Treatment	Gold particle density (particles/μm^2 ± SE)
	Control	
Cytoplasm		4.13 ± 0.24
LVMC + MVB		24.52 ± 2.88
		($P < 0.001$)
	Ecdysone	
Cytoplasm		4.64 ± 0.49
LVMC + MVB		26.87 ± 3.45
		($P < 0.001$)

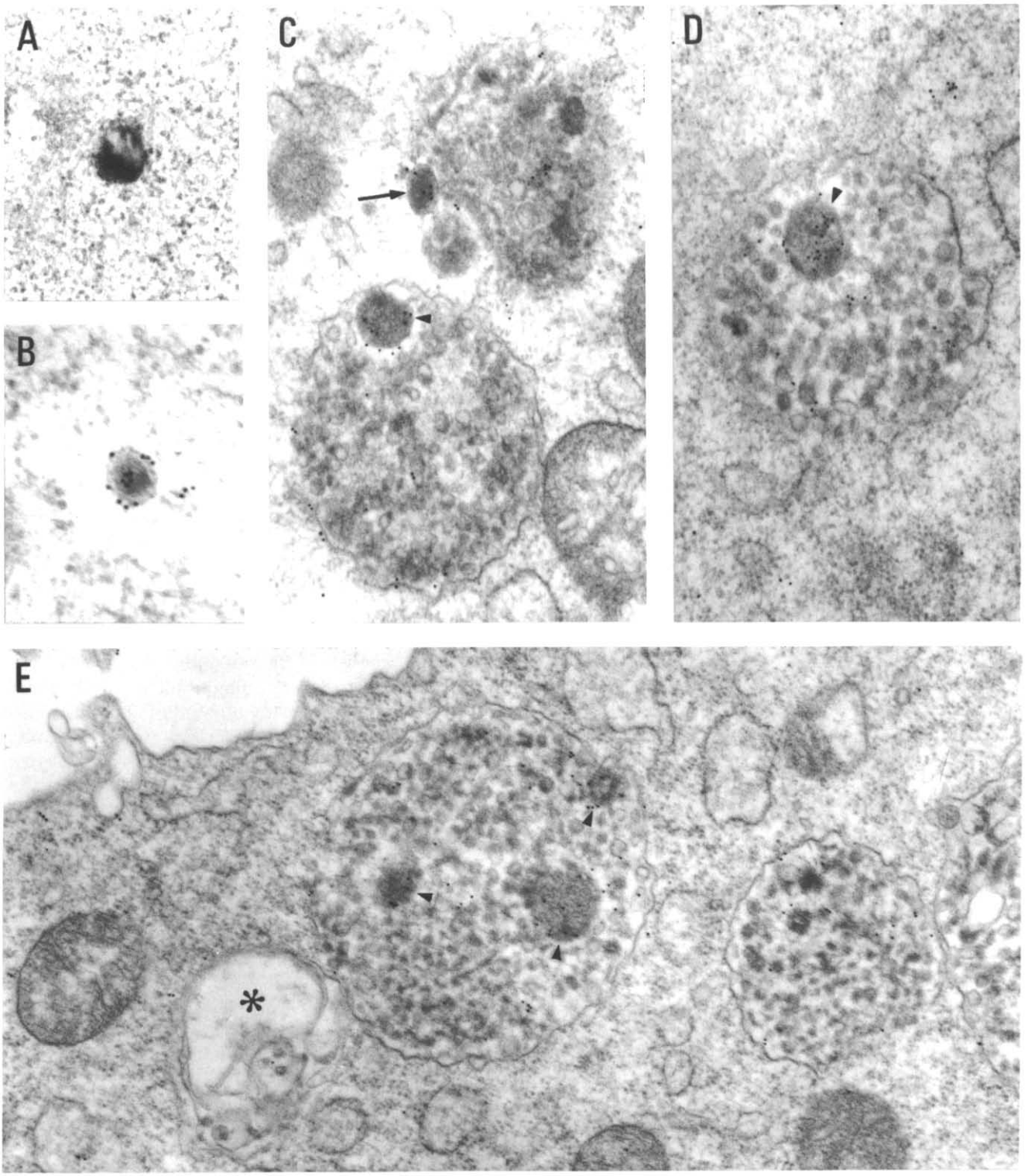

Fig. 2. (A) Acid phosphatase reaction product in cytoplasmic primary lysosome; magnification 81,200×. (B) Ubiquitin–protein conjugates associated with cytoplasmic primary lysosome; magnification 84,000×. (C) Ubiquitin–protein conjugate positive primary lysosome at the surface (arrow) and in the periphery (arrowhead) of MVB; magnification 64,000×. (D) Ubiquitin–protein conjugates in large spherical density (arrowhead) in MVB; magnification 59,000×. (E) Several heterogeneous densities containing ubiquitin–protein conjugates specific gold particles (arrowheads) in a large MVB; early tubulovesicular endosome (asterisk) demonstrating no gold particles corresponding to ubiquitinated proteins; magnification 56,100×.

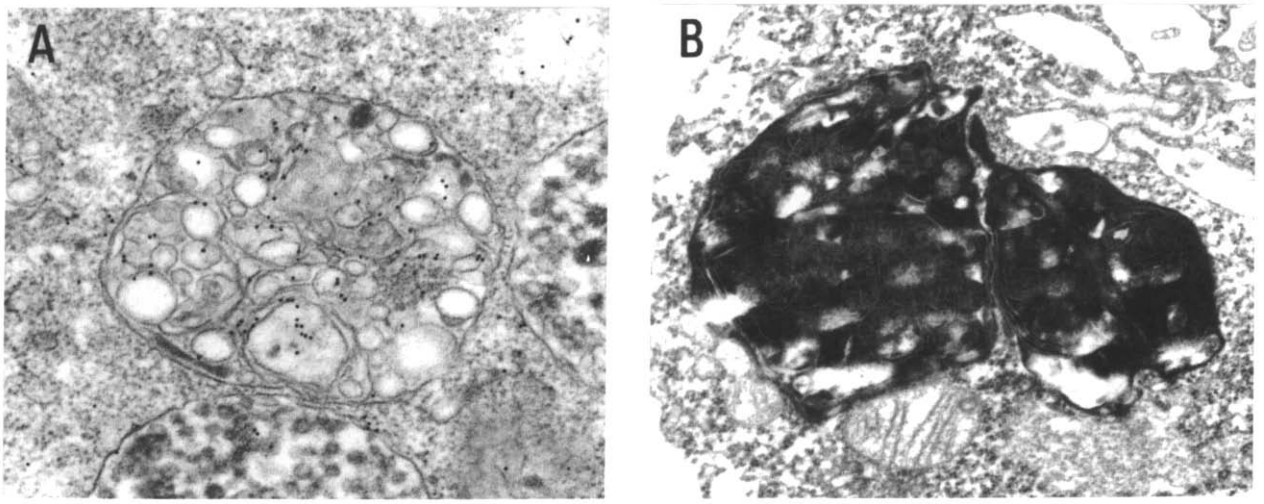

Fig. 3. (A) Gold particles corresponding to ubiquitin–protein conjugates uniformly distributed in an LVMC; magnification 59,000×. (B) Uniformly distributed acid phosphatase reaction product in two adjacent LVMC; magnification 36,000×.

related organelles (LVMC and MVB) relative to cytoplasm shows a comparable enrichment in 20-hydroxyecdysone treated cells and in control cells (Table I). These results are similar to our previous findings in fibroblasts [3,5], and indicate that the large volume expansion of the lysosome-related compartment by steroid action (approx. 4-fold) is accompanied by a similar increase in the concentration of ubiquitin–protein conjugates in the organelles. This implies a critical role of protein ubiquitination in the lysosomal system which is supported by the immunogold electron microscopical observations. The involvement of protein ubiquitination in the biogenesis and maturation of lysosomes by the pathway suggested here is compatible with recent data on cells with a temperature sensitive defect in the activity of the ubiquitin activating enzyme, E1 [12]. At the non-permissive temperature these cells acquire acidic autophagic vacuoles which fail to mature into late autolysosomes (residual bodies): the defect could be in the ubiquitin-dependent formation or transport of pri-

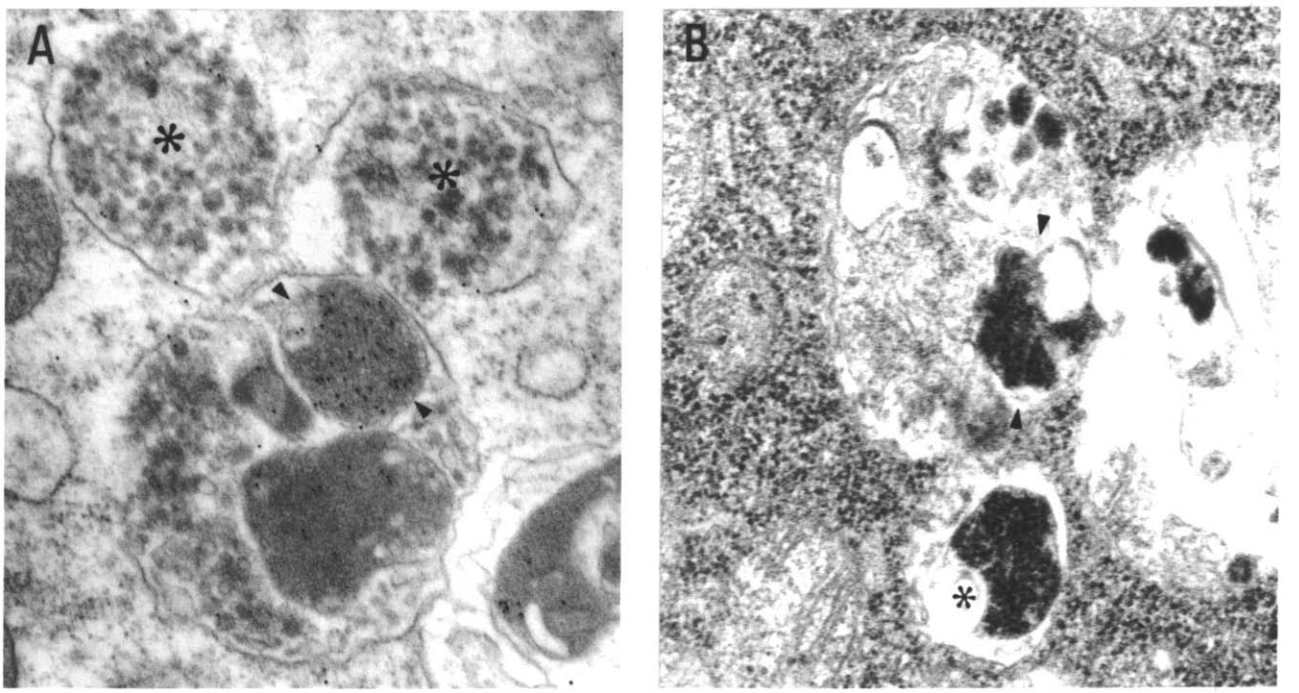

Fig. 4. Large complex vacuoles with small vesicles and dense membrane-bordered structures of different size. (A) Gold particles corresponding to ubiquitin–protein conjugates enriched in large membrane-limited densities (arrowheads); two MVB (asterisk) fusing with a complex vacuole; magnification 51,000×. (B) Intense reaction product of acid phosphatase in discrete membrane-bound area of a complex vacuole (arrowheads) and in a double membrane-bordered autophagic vacuole (asterisk) adjacent to a complex vacuole; magnification 72,000×.

mary lysosomes which therefore cannot introduce hydrolytic enzymes efficiently into the autophagosomes.

The enrichment of ubiquitin–protein conjugates in lysosome-related organelles in insect cells supports and extends our previous findings in several types of unrelated mammalian cells [3–5] and the work of others indicating the involvement of the ubiquitin system in lysosomal protein degradation during stress [13–15]. In conclusion, Sf9 cells may provide a convenient model system for studying regulation of the endosomal-lysosomal system in insect cells and may shed further light on the relationship between protein ubiquitination and lysosomal function.

Acknowledgements: We would like to thank the Wellcome Trust for the support of L.L., the TEMPUS initiative of the EC for support of P.L. and Dr. Michael Landon for critically reading the manuscript.

REFERENCES

[1] Kömüves, L.G., Sass, M. and Kovács, J. (1985) Cell Tissue Res. 240, 215–221.
[2] Sass, M., Kömüves, L.G., Csikós, G. and Kovács, J. (1988) Comp. Biochem. Physiol. 92A, 285–291.
[3] László, L., Doherty, F.J., Osborn, N.U. and Mayer, R.J. (1990) FEBS Lett. 261, 365–368.
[4] László, L., Doherty, F.J., Watson, A., Self, T., Landon, M., Lowe, J. and Mayer, R.J. (1991) FEBS Lett. 279, 175–178.
[5] Doherty, F.J., Osborn, N.U., Wassell, J.A., Heggie, P.E., László, L. and Mayer, R.J. (1989) Biochem. J. 263, 47–55.
[6] Vaughan, J.L., Goodwin, R.H., Tompkins, G.J. and McCawley, P. (1977) In Vitro 13, 213–217.
[7] Zhang, S.X., Kobayashi, T., Okada, T., Gracia del Saz, E. and Seguchi, H. (1991) J. Submicrosc. Cytol. Pathol. 23, 431–437.
[8] Haas, A.L. and Bright, P.M. (1985) J. Biol. Chem. 260, 12464–12473.
[9] Weibel, E.R., in: International Review of Cytology (G.H. Bourne and J.F. Danielli, Eds.) Academic Press, New York, 1969, pp. 235–302.
[10] Ludwig, T., Griffiths, G. and Hoflack, B. (1991) J. Cell Biol. 115, 1561–1572.
[11] Griffiths, G., Hoflack, B., Simons, K., Mellman, I. and Kornfeld, S. (1988) Cell 52, 329–341.
[12] Lenk, S.E., Dunn, W.A., Trausch, J.S., Ciechanover, A. and Schwartz, A.L. (1992) J. Cell Biol. 118, 301–308.
[13] Gropper, R., Brandt, R.A., Elias, S., Bearer, C.F., Mayer, A., Schwartz, A.L. and Ciechanover, A. (1991) J. Biol. Chem. 266, 3602–3610.
[14] Schwartz, L.M., Myer, A., Kosz, L., Engelstein, M. and Maier, C. (1990) Neuron 5, 411–419.
[15] Schwartz, A.L., Brandt, R.A., Geuze, H. and Ciechanover, A. (1992) Am. J. Physiol. 262, C1031–C1038.