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Immunoelectron microscopic localization of ubiquitin in hepatoma cells

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Ubiquitin, a 76 amino acid protein, is covalently attached to abnormal and short-lived proteins, thus marking them for ATP-dependent proteolysis in eukaryotic cells. Free (unconjugated) ubiquitin was localized in hepatoma cells using affinity purified anti-ubiquitin antibodies and colloidal gold immunoelectron microscopy. The anti-ubiquitin antibodies recognize only unconjugated ubiquitin. Ubiquitin is found within the cytoplasm, nucleus, the microvilli, autophagic vacuoles and lysosomes.

Key words: ubiquitin/immunoelectron microscopy/hepatoma

Introduction

Cellular proteins are in a state of constant turnover. The process is extensive and highly selective; specific proteins are degraded within cells at widely different rates. Several distinct mechanisms are responsible for intracellular protein degradation including the lysosomal processes and the soluble ATP-dependent proteolytic pathways. Prominent among these is the ubiquitin-mediated proteolytic system, (recently reviewed by Hershko and Ciechanover, 1986; Ciechanover, 1987; Rechsteiner, 1987). Ubiquitin is a 76 amino acid protein which is highly conserved from yeast to man (Schlesinger and Bond, 1988). In addition to serving as a co-factor in protein degradation for abnormal and short-lived proteins, ubiquitin appears to be involved in several other key cell biological processes such as regulation of the cell cycle. Following its original isolation by Goldstein in 1975 (Goldstein *et al.*, 1975), ubiquitin has been found covalently bound to histone molecules in the nucleus and to cell surface molecules including the platelet derived growth factor receptor (Yarden *et al.*, 1986), the growth hormone receptor (Leung *et al.*, 1987) and the lymphocyte homing receptor (Siegelman *et al.*, 1986).

Despite intensive investigation during the past few years the structural features of the proteolytic substrates which renders them susceptible to ubiquitin-dependent degradation only recently have begun to emerge (Bachmair *et al.*, 1986; Ferber and Ciechanover, 1987; Reiss *et al.*, 1988; Roger *et al.*, 1986). Since ubiquitin conjugates are found in different subcellular organelles, many of which are generated by distinct mechanisms, it is important to define the cellular organization of ubiquitin, ubiquitin-protein conjugates and the processing enzymes. Towards this end, we have localized free (unconjugated) ubiquitin in the eukaryotic cell using monospecific antibodies and colloidal gold immunoelectron microscopy. Our results demonstrate that ubiquitin is found in the cytoplasm, nucleus, the microvilli, the autophagic vacuoles and lysosomes.

Results

In order to enrich specifically the subpopulation of antibody which recognizes ubiquitin and to reduce non-specific contaminating proteins which interfere with the sensitivity of the immunolocalization, we purified anti-ubiquitin antibodies from whole antiserum by sequential affinity chromatography on protein A-Sepharose followed by ubiquitin-Sepharose. The affinity purified antibody is indeed of markedly greater titer as demonstrated by solid-phase radioimmunoadsorbent assay (Figure 1A). Under the conditions of this assay (see Materials and methods), the affinity purified antibody is at least 300-fold more sensitive (Figure 1A). Specificity of the affinity purified antibodies was examined by Western blot analysis. The original description of the antibody to free (unconjugated) ubiquitin showed that the antibody recognized only free ubiquitin and not ubiquitin-protein conjugates (Hershko *et al.*, 1982). However that study was carried out with ^{125}I-labelled ubiquitin conjugates generated *in vitro* and analysed by immunoprecipitation in soluble phase. Therefore, it was necessary to establish the specificity of these antibodies in solid phase analyses. In order to address this problem, Western blot analysis of free ubiquitin and mixures of ubiquitin-protein conjugates was performed. As seen in Figure 2, affinity purified anti-ubiquitin recognizes only free ubiquitin. The presence of multiple high mol. wt ubiquitin-protein complexes in the fraction II + ATP samples is demonstrated by their reactivity to another antibody which recognizes these conjugates as well as free ubiquitin (Figure 2, panel C). Furthermore, extracts of Hep G2 cells do not interfere with detection of ubiquitin by the antibody (Figure 2, panels A, B, lanes e). It should be noted that the sensitivity of this assay was insufficient to detect endogenous free ubiquitin in the Hep G2 because ubiquitin does not quantitatively transfer and bind to nitrocellulose (Swerdlow *et al.*, 1986; and data not shown).

Quantitation of free ubiquitin in Hep G2 was performed by solid phase radioimmunosorbent assay using boiled cell extracts (Figure 1B). As ubiquitin is a heat-stable protein extracts were prepared by boiling cells in PBS prior to spotting onto nitrocellulose (see Materials and methods). In addition, boiling rapidly inactivates isopeptidases which could potentially release ubiquitin from ubiquitin-protein conjugates (Matsui *et al.*, 1982). Direct comparison of

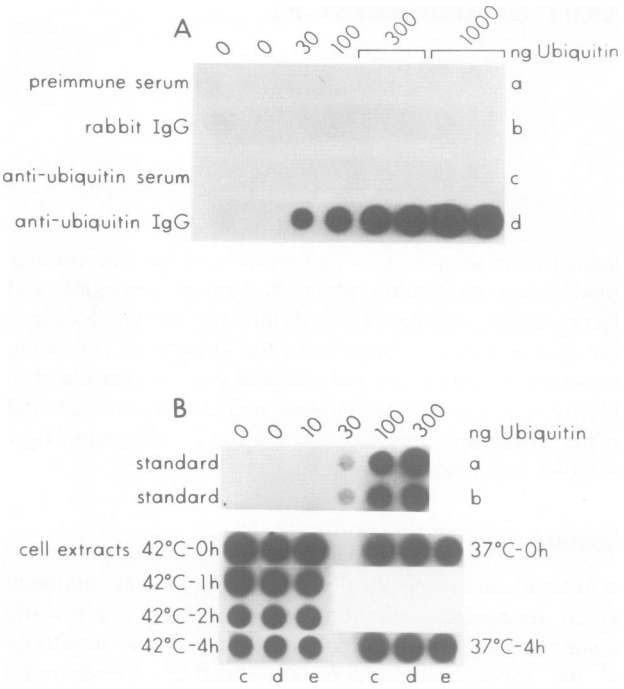

Fig. 1. Solid phase radioimmunosorbent assay for anti-ubiquitin antibodies and ubiquitin quantification. **Panel A**: standards of 0–1000 ng of ubiquitin were applied to nitrocellulose strips, which were blocked with blotto and incubated with (a) rabbit preimmune serum, (b) normal rabbit IgG, (c) rabbit anti-ubiquitin serum or (d) affinity purified rabbit anti-ubiquitin IgG, each at 5 μg/ml. The washed strips were probed with [^{125}I]protein A and exposed to film as described in the text. **Panel B**: standards of 0–300 ng ubiquitin (lanes a and b) or Hep G2 cell extracts (50 μl; 160 μg protein) from triplicate dishes (lanes c–e) of cells incubated at 37°C for 0–4 h or 42°C for 0–4 h were applied to nitrocellulose and probed with affinity purified anti-ubiquitin antibody and [^{125}I]protein A as described above.

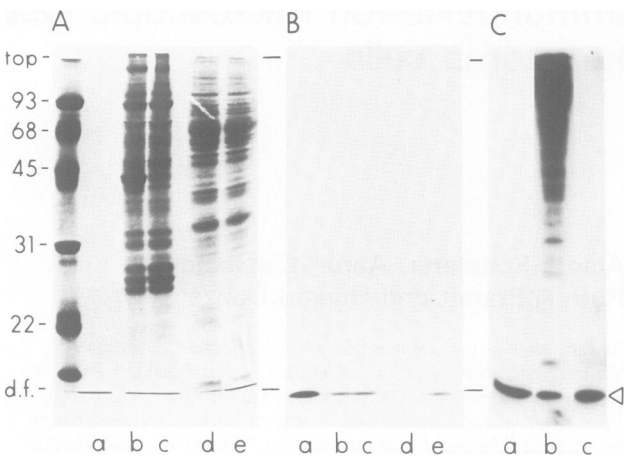

Fig. 2. Specificity of anti-ubiquitin antibodies evaluated by Western blot analysis. Samples of (a) ubiquitin (10 μg), (b) preincubated fraction II (100 μg) + ATP + ubiquitin (10 μg), (c) preincubated fraction II–ATP + ubiquitin, (d) Hep G2 extract (100 μg), (e) Hep G2 extract + ubiquitin (10 μg) were separated by SDS–PAGE prior to Western blot analysis. **Panel A**: Coomassie stain of samples (a)–(e), mol. wt markers are indicated on the left in kilodaltons. The prominent band in lane b is CPK of the ATP regenerating system (40 kd). **Panel B**: Western blot analysis following incubation with affinity purified anti-ubiquitin Ig and [^{125}I]protein A of samples (a)–(e) (unconjugated ubiquitin, arrow). **Panel C**: Western analysis of samples (a)–(c) following incubation with an antibody which recognizes ubiquitin–protein conjugates.

Table I. Specificity of anti-ubiquitin antibodies following cross-linking

	Well coating	Ubiquitin (μg)	Fixation	Anti-ubiquitin IgG A_{410}	Control IgG A_{410}	A_{410}
1.	none	0	none	0	0	0
2.	none	1	none	460	0	460
3.	HSA	0	none	20	0	20
4.	HSA	1	none	10	0	10
5.	HSA	1	A/B	150	110	40
6.	HSA	1	A	80	0	80
7.	HSA	1	B	720	180	540
8.	HSA	1	C	300	0	300

PVC wells were coated with human serum albumin. Thereafter, ubiquitin was added to some wells. As described in the text, fixation was performed with 4% paraformaldehyde (A), 0.5% glutaraldehyde (B), 1% acrolein (C) or a combination. Wells were then probed with either control IgG or affinity purified anti-ubiquitin IgG followed by alkalkine phosphatase-conjugated goat anti-rabbit Ig. Alkaline phosphatase activity was quantitated by *p*-nitrophenol release, determined at 410 nm. Control samples with first or second antibody omitted demonstrated no activity.

several extraction procedures (e.g. Haas and Bright, 1985) including homogenization, sonication, lysis in Triton X-100, SDS or NaOH revealed that each of these procedures markedly interfered with the radioimmunosorbent assay. Quantitation of free ubiquitin in extracts of growing Hep G2 revealed 86 pmol free ubiquitin/10^6 cells in agreement with findings in other cells (Haas and Bright, 1985). In addition, free ubiquitin was quantitated in extracts of Hep G2 cells following heat shock/stress treatment (42°C, 1–4 h or 45°C 0.5–2 h). In four separate experiments, there was a consistent reduction in cellular free ubiquitin. The decrease varied among experiments from 17% to that seen in Figure 1B (50%).

Table I demonstrates the specificity of the affinity-purified anti-ubiquitin antibody following various cross-linking procedures. Less antigenicity was detected following cross-linking with 4% paraformaldehyde or 4% paraformaldehyde/0.5% glutaraldehyde. Cross-linking with either 0.5% glutaraldehyde alone or 1% acrolein was highly efficient. We selected 1% acrolein for cell fixation because of very low non-specific background.

Ubiquitin was localized in cryosections of Hep G2 cells via affinity purified anti-ubiquitin antibodies and colloidal gold–protein A. The electron micrographs in Figures 3–7 show ultrathin cryosections of Hep G2 cells, indirectly immunolabelled with 8nm gold particles for the demonstration of ubiquitin. As seen in Figures 3 and 4, ubiquitin was abundantly localized in the nuclei and cytoplasmic matrix. The localization is specific since (i) preincubation of antibody with 20-fold molar excess of free ubiquitin completely quenched the immunoreaction, (ii) there was no immunoreaction in the absence of anti-ubiquitin antibody, (iii) there was no immunoreaction with rabbit anti-rat pancreatic amylase, and (iv) reaction was confined to the cells (i.e. not present in the embedding matrix) and was localized only to specific compartments. There was no ubiquitin localized to the biosynthetic pathway of secretory and membrane proteins (including the RER, Golgi complex and secretory vesicles). In addition, mitochondria were negative (Figure 4). Ubiquitin

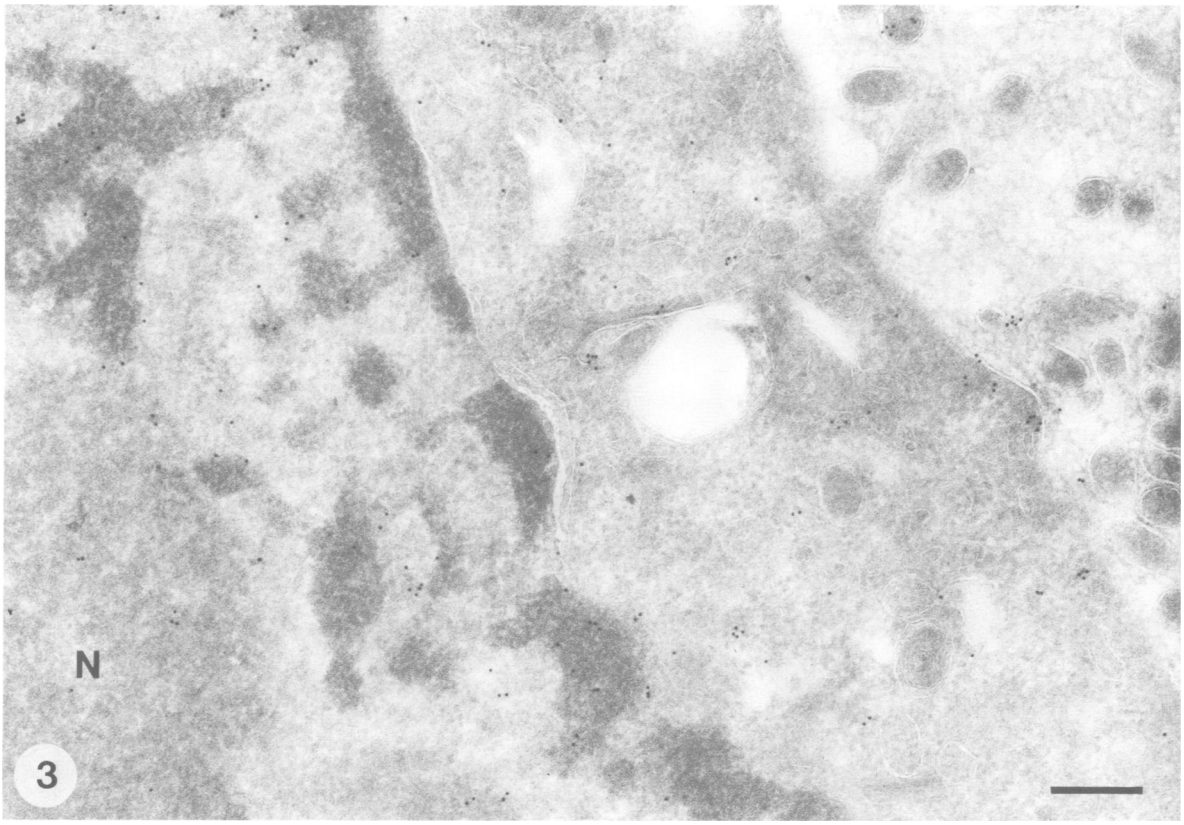

Fig. 3. Ubiquitin labelling is present in the nucleus (left part of the figure) and in the cytoplasm matrix, but not in the gelatin outside the cell. In the nucleus, the gold particles are associated with the electron-dense heterochromatin as well as euchromatin and with the nucleolus (N). Labelling efficiency was increased by an intermediate swine anti-rabbit step. × 39 600. Bar, 0.3 μm.

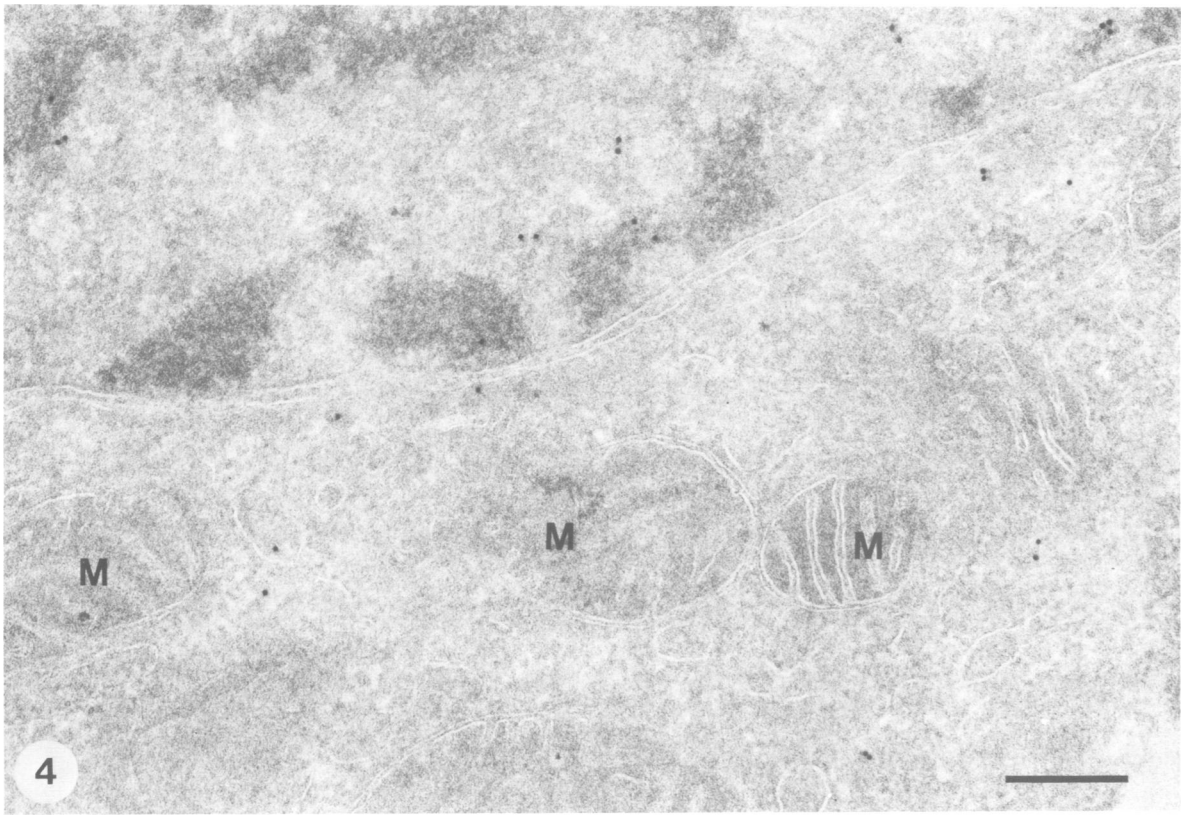

Fig. 4. Portions of a nucleus (above) and cytoplasm, both showing ubiquitin. The mitocondria are negative. The yield of gold particles is lower than in Figure 3 because no enhancing intermediate antibody was used. × 52 200. Bar, 0.3 μm.

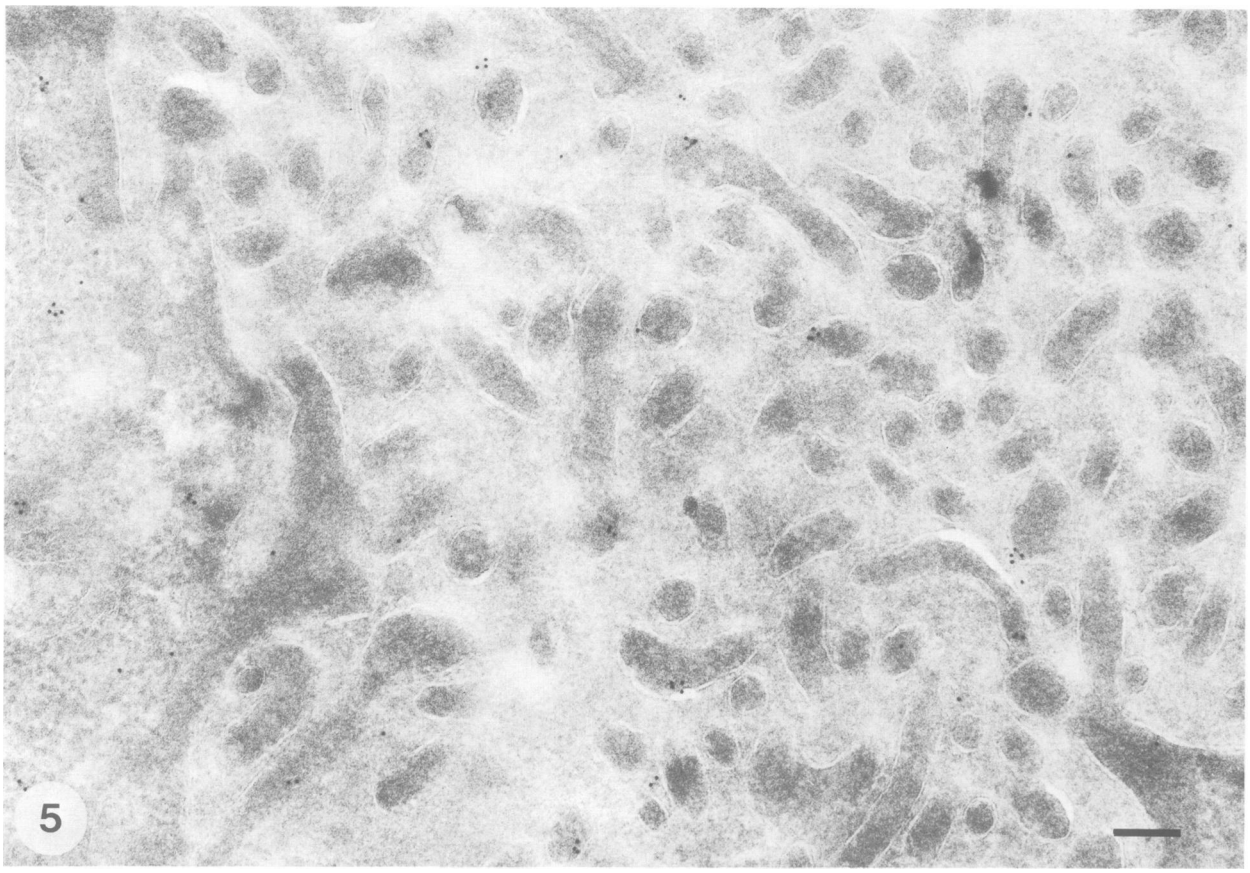

Fig. 5. Bile canalicular-space between Hep G2 cells, with many microvilli which demonstrate ubiquitin labelling. Labelling sequence as in Figure 3. × 44 620. Bar, 0.2 μm.

was present in significant amounts in both autophagic vacuoles as well as lysosome-like structures (Figures 6 and 7). In the nuclei labelling was high over euchromatin as well as heterochromatin (Figure 3). Furthermore, label was present in the nucleoli (Figure 3). Cytoplasmic localization of ubiquitin is also demonstrated in the microvilli (Figure 5).

Since different subcellular organelles within cryosections are variably penetrable by immunoreagents, this may occasionally result in the false impression that one compartment is relatively more heavily labelled than another. To evaluate this possibility, we embedded fixed cells in polyacrylamide prior to cryosectioning and immunolabelling. Under these conditions there is no penetration of the immunoreagents into the sections. Following polyacrylamide embedding, the distribution of the label was maintained (data not shown).

Quantitation of free ubiquitin label by gold particle counting of cryosections over a random intersecting matrix revealed the vast majority of label over cytoplasm and nuclei. Relative densities over cytoplasm, chromatin and nucleolus were 0.8, 1.3 and 1.4, respectively. Ubiquitin label within lysosomes/autophagic vacuoles was a few percent of the specific total label. Similar analysis of ubiquitin label in heat stressed cells revealed relative densities of 0.5 over cytoplasm and 1.7 over the nucleus.

Discussion

Immunoelectron microscopic localization of free ubiquitin in hepatoma cells reveals that the molecule is specifically confined to the cytoplasm, nucleus, microvilli, autophagic vacuoles and lysosomes. It is not surprising that ubiquitin is highly abundant in the cytoplasm since it was originally isolated from high-speed cell supernatants (Goldstein *et al.*, 1979; Ciechanover *et al.*, 1978). Also, degradation of short lived and abnormal proteins seems to occur in the cytosol (for review, see Rechsteiner, 1987). In the nucleus, free ubiquitin may represent the cycling pool responsible for histone targetting (Wu *et al.*, 1981). An interesting finding is the relative abundance of ubiquitin label associated with the euchromatin. Varshavsky and colleagues reported a striking increase in the content of ubiquitin modified histones in nucleosomes of actively transcribed genes compared to nucleosomes confined to inactive chromatin (Levinger and Varshavsky, 1982; Barsoum and Varshavsky, 1985). The finding of ubiquitin label in autophagic vacuoles as well as in the lysosomes is rather surprising. It is possible that ubiquitin and ubiquitin-conjugated proteins are autophagocytosed non-specifically with bulk cytoplasm, or that ubiquitin conjugates are specifically taken up from the cytoplasm or from compartments of the endocytic pathway. In the latter case, ubiquitin must be first released by proteases prior to its recognition by the antibody. The fact that the antibody recognizes at least one intact epitope of ubiquitin in a protease rich environment probably reflects the relative stability of ubiquitin to proteolytic digestion (Goldstein *et al.*, 1985). The precise localization of ubiquitin in the microvillar area is not known. This uncertainty stems from

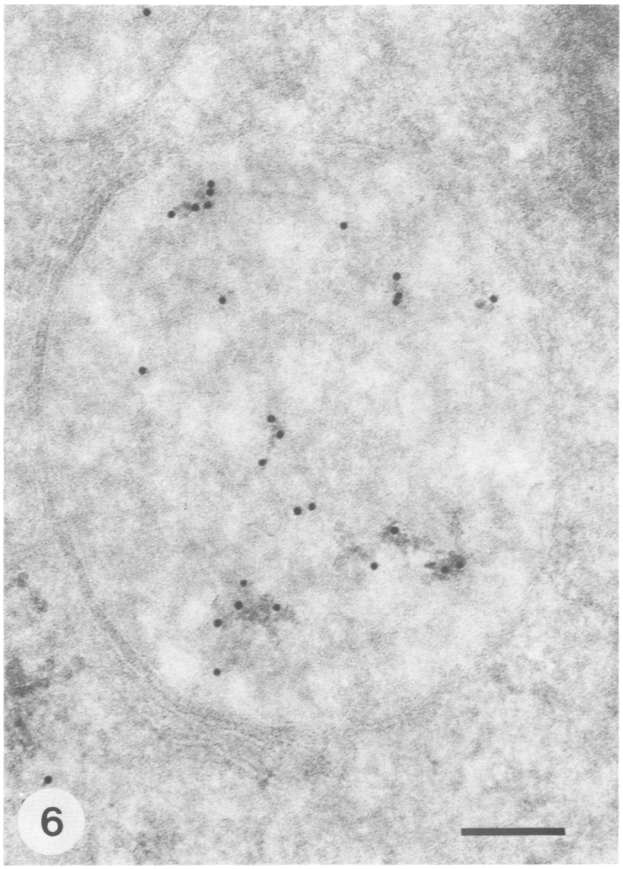

Fig. 6. Vacuole of the endosome/lysosme compartment containing relatively dense labelling of ubiquitin. Labelling as in Figure 3. × 67 450. Bar, 0.2 μm.

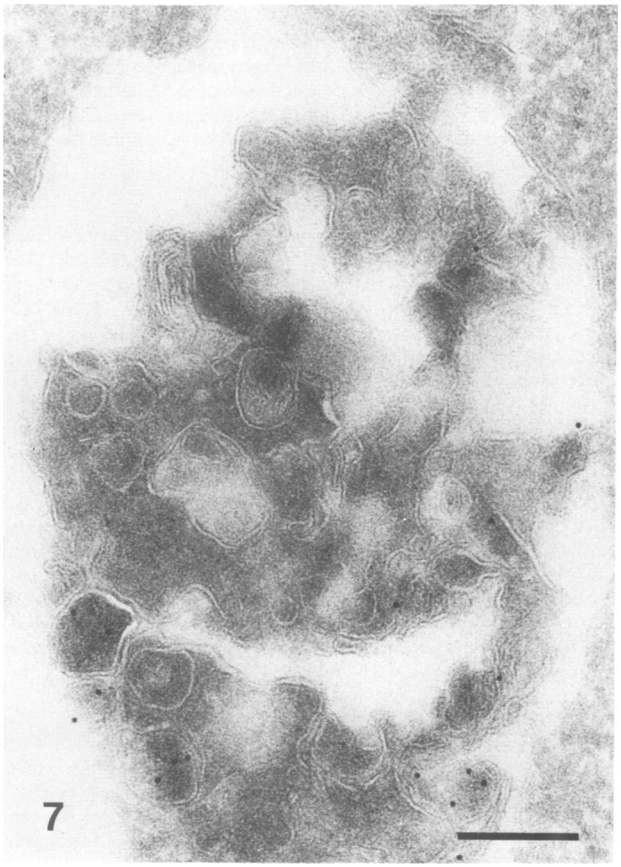

Fig. 7. Ubiquitin labelling in an autophagic vacuole. Labelling as in Figure 4; × 78 850. Bar, 0.2 μm.

the fact that the diameter of the colloidal gold particle and the bridging molecule (protein A, first antibody, second antibody) is larger than the thickness of the plasma membrane. Thus, the possibility still exists that free ubiquitin resides in both the most peripheral cytoplasm as well as at the cell surface. This is of interest because ubiquitin has been found conjugated to both the cytoplasmic and extracellular domains of cell surface proteins (the PDGF receptor and the lymphocyte homing receptor, respectively; Yarden *et al.*, 1986; Siegelman *et al.*, 1986). The cytoplasm associated with the microvilli is a highly ordered array of specific structural cytoskeletal proteins including actin. Recently, Ball *et al.* (1987) reported that flight muscle actin from *Drosophila melanogaster* is modified by ubiquitin. The presence of ubiquitin label in all these locales may reflect a ubiquitin pool in equilibrium with ubiquitin–protein conjugates.

The lack of ubiquitin label within the secretory pathway is of no less interest. Siegelman *et al.* (1986) reported the existence of ubiquitin moieties on the extracytoplasmic domain of the lymphocyte homing receptor. This finding presents a problem of where the ubiquitination occurs. The protein can be ubiquitinated in the cytoplasm cotranslationally or within the lumen of the ER or Golgi. The lack of ubiquitin label in the lumina of the biosynthetic pathway renders the second possibility less likely.

Quantification of ubiquitin during heat stress revealed a consistent and significant decrease similar to that reported in fibroblasts as described and discussed by Bond *et al.* (1988). Immunoelectron microscopic analysis revealed a similar decrease in free ubiquitin label in the cytoplasm of heat-stressed cells. It is possible that heat stress stimulates the formation of proteolysis-sensitive substrates which are tagged by ubiquitin. This may result in a decrease in free ubiquitin concomitant with an increase in conjugated ubiquitin.

This study was limited to analysis of free ubiquitin. Analysis of ubiquitin conjugates was impeded by the fact that different preparations of antibodies which recognize ubiquitin conjugates also recognize immobilized free ubiquitin (see Figure 1C and data not shown). The original experiments with these antibodies were performed in soluble phase and did not demonstrate any reactivity to free ubiquitin (Hershko *et al.*, 1982). Efforts to resolve this problem are currently under way. Future studies will be directed to elucidate the subcellular localization of other components of the ubiquitin system under various physiological conditions, using specific antibodies.

Materials and methods

Materials

Ubiquitin was purified to homogeneity from human erythrocytes as described in detail previously (Ciechanover *et al.*, 1980). [^{125}I]Protein A was prepared from protein A (Pharmacia) by iodination with chloramine T as described earlier (Schwartz *et al.*, 1986). Specific radioactivity was ~2 × 10^4 c.p.m./ng. Nitrocellulose (0.45 micron) was purchased from Schleicher and Schuell. PVC microtiter wells were from Dynatech. Alkaline phosphatase conjugated goat anti-rabbit Ig was obtained from Zymed.

Cells and cell fractions

The well-differentiated human hepatoma Hep G2 al6 cells were grown as

described previously (Schwartz and Rup, 1983). Reticulocyte fraction II was prepared as described previously (Ciechanover *et al.*, 1980). Generation of ATP-dependent ubiquitin–protein conjugates was performed in reticulocyte fraction II in the presence of ubiquitin, ATP and an ATP regenerating system as described previously (Ciechanover *et al.*, 1980). Hep G2 cell extracts for Western blots were prepared as described (Schwartz *et al.*, 1986).

Antibodies

Rabbit polyclonal antibodies to free (i.e. unconjugated) ubiquitin were raised as described previously (Hershko *et al.*, 1982). Similarly, rabbit polyclonal antibodies which recognize ubiquitin–protein conjugates were raised as described previously (Hershko *et al.*, 1982). It should be noted that these antibodies also recognize free ubiquitin (see below). Antibodies to free ubiquitin were affinity purified by a two step procedure, first via protein A–Sepharose (Pharmacia) followed by ubiquitin–Sepharose (5 mg ubiquitin per ml gel). Elution with 0.1 M glycine, pH 2.8 was followed by neutralization, dialysis and concentration. The preparation was homogeneous as determined by SDS–PAGE. Affinity of purified anti-ubiquitin antibody was evaluated by dot-blot radioimmunosorbent assay as follows. Standards of ubiquitin were applied in 50 μl PBS to nitrocellulose using a mini-fold apparatus (Schleicher and Schuell). Strips of nitrocellulose were blocked by incubation for 18 h at 4°C in 'blotto' (as described previously, Schwartz *et al.*, 1986) and thereafter incubated in antiserum, pre-immune serum, affinity purified anti-ubiquitin Ig or control Ig. After extensive washing, the strips were incubated with [^{125}I]protein A (10^6 c.p.m./ml), washed, dried and exposed to Kodak XAR film with Dupont enhancing screens at −70°C.

Quantitation of ubiquitin in cells

Extracts of Hep G2 cells were prepared as follows. Growing cells were rinsed in PBS at 4°C, scraped from the monolayer and boiled within 1 min. Ubiquitin, a heat stable protein (Goldstein *et al.*, 1975; Ciechanover *et al.*, 1978), was quantitated by spotting aliquots of the 13 000 *g* supernatant onto nitrocellulose alongside ubiquitin standards. The nitrocellulose sheets were boiled in water (Swerdlow *et al.*, 1986), incubated in blotto, anti-ubiquitin Ig and [^{125}I]protein A as described above. Quantitation of the dots was performed by counting the individual dots in a Packard gamma counter.

Western blots. Blots for anti-ubiquitin antibody specificity were performed on free ubiquitin and ubiquitin–protein conjugates mixtures (generated by incubation of fraction II, ubiquitin and ATP) following SDS–PAGE and transfer to nitrocellulose. Blots were probed with affinity purified anti-ubiquitin or the antibody which recognizes ubiquitin–protein conjugates followed by [^{125}I]protein A (Schwartz *et al.*, 1986).

Quantitation of ubiquitin fixation. In order to determine that ubiquitin could be quantitatively fixed and recognized by the anti-ubiquitin antibody we used a similar approach to that described for other small NH_2 containing molecules (e.g. Schwartz *et al.*, 1985). Briefly, some PVC microtiter wells were coated with human serum albumin. Wells were then rinsed with PBS. Coated and uncoated wells were incubated with or without 1 μg ubiquitin in 50 μl PBS. Wells were then incubated with 4% paraformaldehyde, 0.5% glutaraldehyde, both or 1% acrolein, followed by 20 mM glycine/1 M NaCl. Thereafter some wells received first antibody at 0.1 μg/ml (control Ig or anti-ubiquitin Ig) followed by second antibody (alkaline phosphatase conjugated goat anti-rabbit Ig) at 1:500. After rinsing, *p*-nitrophenylphosphate was added as described (Schwartz *et al.*, 1985) and the reaction monitored at 410 nm in a Dynatech microplate reader.

Immunolocalization of ubiquitin. This was performed on acrolein-fixed Hep G2 cells essentially as described earlier (Geuze *et al.*, 1981, 1985; Schwartz *et al.*, 1985). Briefly, cryosectioning of the fixed cells, uranyl staining, and methyl cellulose embedding were performed as described previously (Geuze *et al.*, 1981). For indirect immunolabelling, ultrathin cryosections were first incubated with affinity purified rabbit anti-ubiquitin Ig or control rabbit Ig, rinsed, incubated with swine anti-rabbit serum (Nordic Immunology, Tilburg, The Netherlands) 1:1000 diluted with 0.1% BSA, rinsed and finally labelled with 8 nm protein A–colloidal gold particles prepared according to the tannic acid–citrate method (Slot and Geuze, 1985). The swine anti-rabbit step increases the sensitivity of the method 6- to 8-fold, as compared with direct protein A–gold labelling of the first antibody-binding site (Geuze *et al.*, 1987). The increased sensitivity allowed a more precise evaluation of label distribution.

Controls included (i) preincubating antibody with 20-fold molar excess of ubiquitin prior to incubation with the sections, (ii) omitting the first antibody, and (iii) using irrelevant first antibody (i.e. anti-rat pancreatic amylase) (Geuze *et al.*, 1981). Some blocks were embedded in polyacrylamide prior to cryosectioning and immunolabelling (Slot and Geuze, 1982).

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