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Immunoelectron microscopic localization of the ubiquitin-activating enzyme E1 in HepG2 cells

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ABSTRACT **As the first enzyme in the ubiquitin system the ubiquitin-activating enzyme E1 plays a pivotal role in all pathways of protein ubiquitination. In an effort to learn more about the cell biology of this pathway, we have purified the 110-kDa enzyme to homogeneity and generated a panel of distinct monoclonal antibodies to it. Using quantitative electron microscopic immunolocalization with these anti-E1 monoclonal antibodies, we find that E1 is abundant both within the cytoplasm and nucleus. Within the cytoplasm, E1 was found throughout the cytoplasmic volume as well as enriched along the cytoplasmic face of the rough endoplasmic reticulum and associated with the dense material along the desmosomal junctions. E1 was also found associated with the cytoplasmic surface of endosomal/lysosomal vacuoles. Interestingly, E1 was also found within the mitochondria. The lumen of rough endoplasmic reticulum, Golgi complex, endosomes, and lysosomes were negative. The specific localization of E1 to distinct subcellular organelles suggests that E1 may play multiple physiological roles within the cell.**

Ubiquitination is a covalent modification of various cellular proteins. This posttranslational modification is involved in various physiological processes. The most thoroughly characterized conjugation event occurs in the ubiquitin-dependent degradative pathway (1). In this case, multiply ubiquitinated proteins are targeted for degradation. In addition, stable mono-ubiquitin adducts are found intracellularly—for example, those involving nuclear histones and cytoplasmic arthrin (2, 3).

The first reaction in ubiquitin conjugation is the activation of ubiquitin to a high-energy intermediate. This reaction is catalyzed by E1, the ubiquitin-activating enzyme (4). The ubiquitin is then transferred to one of the ubiquitin carrier proteins (E2s) (5). At this point, ubiquitin can either be linked directly to a target protein or conjugated to proteins destined for degradation via the ubiquitin–protein ligases (E3s) (4). Thus, E1 provides the initial activated form of ubiquitin necessary for any conjugation reaction to occur. In this capacity, E1 plays a key regulatory role in any process affected by ubiquitin modification.

Recently, we (6) and others (7–9) have cloned and sequenced the cDNA for E1 from human, yeast, and wheat. A single ubiquitous E1 mRNA of 3.5 kilobases (kb) predicts a mature protein of ≈110 kDa. Despite intensive investigation during the past few years, the structural features of the proteolytic substrates that render them susceptible to ubiquitin-dependent degradation only recently have begun to emerge (for review, see ref. 10). We have recently defined the subcellular localization of ubiquitin in HepG2 cells (11). Ubiquitin was localized to the cytoplasm, nucleus, microvilli, autophagic vacuoles, and lysosomes. In addition, Laszlo *et al.* (12) have similarly immunolocalized ubiquitin–protein conjugates within the cytoplasm as well as within lysosomes (12). Because ubiquitin–protein conjugates are found in different subcellular organelles, it is important to define the cellular organization of ubiquitin, ubiquitin–protein conjugates, and the processing enzymes.

In a recent and surprising series of genetic experiments Mitchell *et al.* (13) and Kay *et al.* (14) have cloned the mouse *Spy* gene, which controls spermatogenesis and lies within the sex-determining locus. The mouse *Spy* gene is highly homologous to the human ubiquitin-activating enzyme E1 gene.

In the present study we have developed monoclonal antibodies (mAbs) to human E1 and used these probes for colloidal gold immunoelectron microscopic localization of E1 in HepG2 cells. We demonstrate E1 within the nucleus and cytoplasm, including the demosomal junctions, rough endoplasmic reticulum (RER), ablumenal membranes of endosomes and lysosomes, and within the mitochondria.

MATERIALS AND METHODS

Materials. Ubiquitin was obtained from Sigma and covalently coupled to Sepharose. Nonspecific mouse IgM, rabbit anti-mouse IgM, rabbit anti-mouse IgM agarose, alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin, and mAb isotyping kit were from Zymed Laboratories. Rabbit anti-mouse IgM was iodinated with ^{125}I and chloramine T or Iodo-Gen as described (15). Nitrocellulose (0.45 μm) was from Schleicher & Schuell. Poly(vinyl chloride) microtiter wells were from Dynatech. α-Chymotrypsin was from Worthington. Ribi adjuvant was obtained from Ribi Immunochem. All other reagents were of analytical grade.

Purification of Human E1. E1 was purified from human erythrocytes via covalent affinity chromatography on immobilized ubiquitin as described (6, 16). Routinely the enzyme was >95% pure as judged by SDS/PAGE and Coomassie blue stain and silver stain (ref. 6; Fig. 1). The purified enzyme was assayed for ubiquitin-dependent pyrophosphate–ATP exchange (17).

Anti-E1 mAbs. To obtain mAbs to human E1, Swiss Webster mice were repeatedly injected with 25 μg of purified E1 in Ribi adjuvant. After development of a high-titer anti-E1 antiserum, spleens were removed and fused to P3X63Ag8.653 myeloma cells. Initial screening of 800 wells from two fusions via ELISA with immobilized E1 yielded three mAb-producing lines (1C5, 2D4, and 2C1). Subsequent screening was via immunoblot analysis (see below), and positive clones were subcloned in soft agar as stable lines. The immunoglobulin species were isotyped and found to be IgM, κ. The mAbs were raised in ascites of pristane-primed mice and purified to homogeniety via gel-filtration chromatography and affinity chromatography on goat anti-mouse IgM.

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Abbreviations: mAb, monoclonal antibody; RER, rough endoplasmic reticulum.

Epitope Mapping of Anti-E1 mAbs. To determine whether 1C5, 2D4, and 2C1 recognize distinct epitopes on the E1 molecule we performed immunoblots on partial proteolytic digests of E1. E1 (600 ng) was incubated at 37°C with α-chymotrypsin (20 ng) for 0–120 min. After incubation, the samples were boiled in Laemmli sample buffer, and replicate SDS/PAGE and immunoblots were probed for total protein via silver stain and reactivity to individual mAbs.

Quantitation of E1 in Cells. *Immunoblots.* Blots for anti-E1 antibody specificity were done on purified E1, erythrocyte lysates, and HepG2 cell lysates (11). After SDS/PAGE and transfer to nitrocellulose, blots were probed with anti-E1 mAbs (generally at 1 μg/ml) in Blotto (15) followed by ^{125}I-labeled rabbit-anti-mouse IgM (specific radioactivity, ≈2 × 10^4 cpm per ng) (15). After exposure to Kodak XAR film and DuPont enhancing screens at −70°C, autoradiograms of immunoblots were quantitated via densitometry.

Immunolocalization of E1. HepG2 cells were fixed in 4% paraformaldehyde/0.5% glutaraldehyde as described earlier (11). Cryosectioning of the fixed cells, uranyl staining, and methylcellulose embedding were done as described (18, 19). For indirect immunolabeling, ultrathin cryosections were first incubated with one of the anti-E1 monoclonal IgMs (1C5, 2D4, or 2C1) or nonspecific mouse IgM (Zymed Laboratories). Thereafter sections were incubated with rabbit anti-mouse IgM (Zymed Laboratories) followed by protein A–colloidal gold particles prepared according to the tannic acid-citrate method (19).

Controls included (*i*) use of nonspecific mouse IgM as the primary antibody, (*ii*) omission of the first antibody, and (*iii*) preincubation of anti-E1 mAb 1C5 with 20-fold molar excess of purified E1.

RESULTS

To define precisely the subcellular localization of the ubiquitin-activating enzyme E1, we generated several mAbs to human E1. As seen in Fig. 1, E1 purified from human erythrocytes is a 110-kDa protein on SDS/PAGE. Often E1 migrates as a tight doublet, which may represent a posttranslational modification (see below; ref. 6). Mouse mAbs to human E1 (1C5, 2D4, and 2C1) recognize only the 110-kDa E1 species and no additional proteins in whole-cell lysates (Figs. 1–3). Using these mAbs and quantitative immunoblots of whole HepG2 cell lysates, we have determined that E1 is a rather abundant protein representing ≈0.1% of total cellular protein or ≈10^7 molecules per HepG2

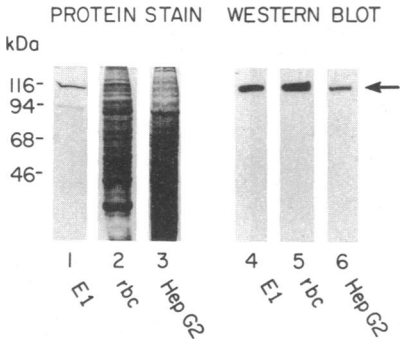

FIG. 1. Specificity of anti-E1 mAb (1C5) evaluated by immunoblot analysis. Samples of E1 (100 ng of protein) (lanes 1 and 4), red blood cell (rbc) lysate (10 μg of protein) (lanes 2 and 5), or HepG2 lysate (10 μg of protein) (lanes 3 and 6) were separated by SDS/PAGE before immunoblot analysis. (*Left*) Silver stain of samples 1–3, molecular size markers are at left in kDa. (*Right*) Immunoblot (Western) analysis of samples 4–6 after incubation with anti-E1 mAb 1C5 and ^{125}I-labeled rabbit anti-mouse IgM. E1 is denoted by an arrow. The minor band at ≈90 kDa in lane 1 may be a degradation product of E1 detected in this particular preparation and not recognized by mAb 1C5.

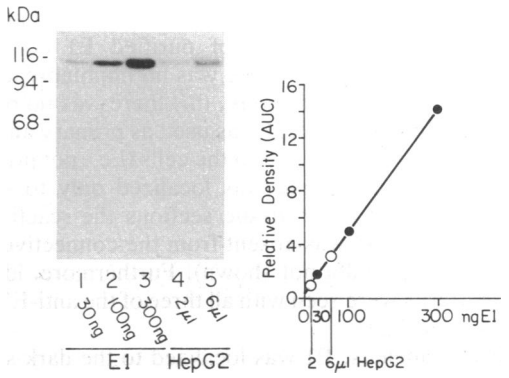

FIG. 2. Quantitation of E1 in HepG2 cells via immunoblot. HepG2 cells were solubilized directly in boiling Laemmli sample buffer. Aliquots were analyzed via SDS/PAGE and immunoblot analysis with mAb 1C5. (*Left*) Immunoblot of E1 standards (30, 100, 300 ng; lanes 1–3) and HepG2 lysate (10 μg protein per μl; 2 μl and 6 μl, lanes 4 and 5). Molecular mass standards are at left in kDa. (*Right*) Plot of relative density of E1 peak (area under curve, AUC) versus sample volume aliquot. ○, HepG2; ●, E1.

cell (Fig. 2). Each of these mAbs (1C5, 2D4, and 2C1) recognizes distinct epitopes as shown in Fig. 3. In this experiment immunoblot analyses with each mAb were done on partial-proteolytic digests of E1. Each mAb recognizes a distinct subset of the proteolytic fragments.

E1 was localized in ultrathin cryosections of HepG2 cells via affinity-purified mouse anti-E1 mAbs by using rabbit anti-mouse secondary antibodies and colloidal gold–protein A (Figs. 4–6). As seen in Fig. 4, E1 was abundantly localized in the nucleus, mitochondria, and cytoplasmic matrix. The

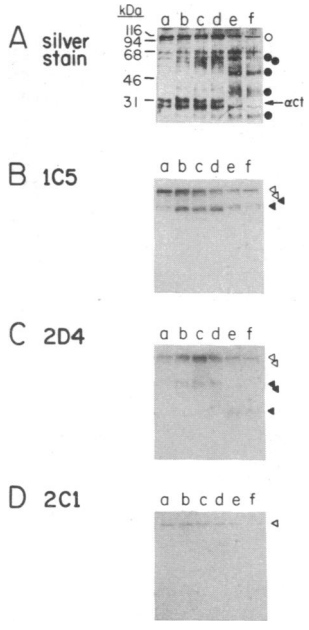

FIG. 3. Epitope map of anti-E1 mAb reactivity. E1 (600 ng) was incubated at 37°C with α-chymotrypsin (20 ng) for 0 (lanes a), 0 (lanes b), 2 (lanes c), 10 (lanes d), 60 (lanes e), or 120 (lanes f) min. In sample a the E1 was boiled in Laemmli sample buffer before adding α-chymotrypsin. After incubation, the samples were boiled in Laemmli sample buffer, and replicate SDS/PAGE and immunoblots were probed for total protein via silver stain (*A*), 1C5 (*B*), 2D4 (*C*), and 2C1 (*D*). In *A* molecular size markers are at left in kDa. ○, Position of intact E1; ●, position of proteolytic fragments. Position of α-chymotrypsin (αct) doublet is noted at right. There is a minor band at ≈60 kDa, which either represents a proteolytic fragment of E1 or contaminating protein. (*B–D*) △, Intact E1; ▲, proteolytic fragments recognized by respective mAbs.

localization is specific because (*i*) preincubation of antibody with a 20-fold molar excess of purified E1 completely quenched the reaction, (*ii*) there was no immunoreaction in the absence of primary antibody, (*iii*) there was no reaction when nonspecific mouse IgM was used as primary antibody, (*iv*) the reaction was confined to the cells (i.e., not present in the embedding matrix) and was localized only to specific compartments, and (*v*) in tissue sections the reaction was confined to cells and was absent from the connective tissue ground substance (data not shown). Furthermore, identical labeling patterns were seen with all three of the anti-E1 mAbs (1C5, 2D4, and 2C1).

Within the nucleus, E1 was localized to the dark-staining heterochromatin (Fig. 4). Little E1 was found within euchromatin. Quantitative assessment of E1 localization revealed 37% of the total E1 was contained within the nucleus (Table 1).

Cytoplasmic staining for E1 was abundant (Figs. 4–6) and represented 56% of total E1 (Table 1). E1 was not associated with the plasma membrane, except at desmosomal junctions where intense staining was seen (Figs. 4 and 5). However, this desmosomal localization only represented 0.4% of total E1 (Table 1). E1 staining was minimal at the apical plasma membrane (Fig. 5). E1 was absent from the lumen of the biosynthetic and endocytotic pathway organelles (endoplasmic reticulum, Golgi complex, endosomes, lysosomes) (Figs. 4 and 6). On occasion some E1 reactivity was found within lysosomes (data not shown). However, E1 clearly was found associated with the cytoplasmic surface of endosomes and lysosomes (Fig. 4) and represented 5% of total E1 (Table 1). The cytoplasmic surface of the RER also was found enriched

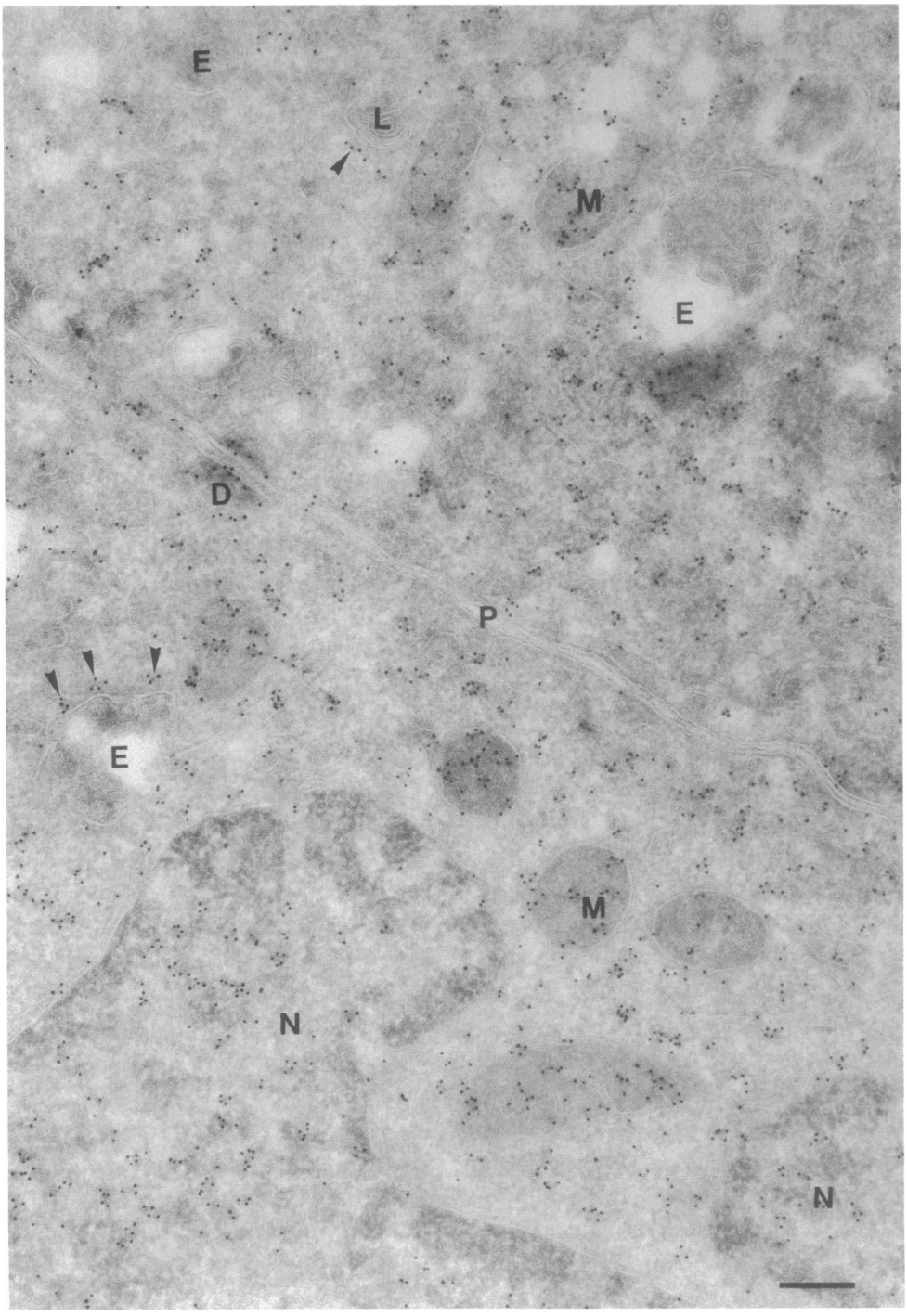

FIG. 4. Low-power immunoelectron micrograph of HepG2 cells labeled with anti-E1. E1 labeling is seen in the nucleus (N), especially at the margins of the heterochromatin fields, in mitochondria (M), and in the cytoplasm. Label is also associated with the electron-dense fibrillar material of desmosomes (D). Note that the contents of multivesicular endosomes (E) and lysosomes (L) are negative, but that E1 labeling is found at their cytoplasmic faces (arrowheads). P, plasma membrane. (×52,800; bar = 0.2 μm.)

Table 1. Distribution of E1 gold labeling in HepG2 cells

Area	Gold labeling, %
Nucleus	**37**
Cytosol	**56**
Endosome/lysosome-associated	5
Desmosomal junction-associated	0.4
Ribosomal face of endoplasmic reticulum	1.6
Mitochondria	**7**

Cryosections were indirectly immunolabeled with mAb 1C5 and an 8:1 mixture of protein A-gold^{10nm} and protein A-gold^{20nm} to allow for precise quantitation. Only 20-nm gold particles were counted. Particles within a distance of 40 nm (i.e., two times the gold size) from the endosomal/lysosomal membrane were assigned as endosome/lysosome-associated. Total gold particles = 9812; total cell profiles = 24. Boldface numbers total 100%.

in E1 labeling (Fig. 6). The 1.6% of E1 found associated with the RER is clearly an underestimate because the endoplasmic reticulum cisternae are often tangentially sectioned. Thus, label associated with such profiles without apparent ribosomes were attributed to cytosol (Table 1). No E1 was identified within the RER (data not shown). Mitochondria were enriched for E1 (Figs. 4 and 6) and represented 7% of total E1 (Table 1).

DISCUSSION

Using mAbs and colloidal gold immunoelectron microscopy we demonstrate the subcellular localization and abundance of the ubiquitin-activating enzyme E1 in human hepatoma HepG2 cells. As the initial step in ubiquitin conjugation, E1 provides a key regulatory role in any process affected by ubiquitin modification, including ATP-dependent degradation and generation of stable ubiquitin–protein adducts. Quantitative immunoblot analysis reveals $\approx 10^7$ E1 molecules per HepG2 cell. Of note is that the abundance of free ubiquitin in these cells is approximately the same (5×10^7 molecules per HepG2 cell; ref. 11). This result is somewhat unexpected because multiple ubiquitin residues are required to target a specific substrate molecule for degradation, and, thus, one might expect a substantially greater amount of substrate (i.e., ubiquitin) than enzyme (i.e., E1) (20, 21).

The presence of ubiquitin–protein conjugates within the cytoplasm as well as nucleus has been well established (2, 22). Furthermore, ubiquitin–protein conjugates are found within the endosomes/lysosomes of the vacuolar apparatus (12). Thus, either the requisite enzymes are available at these varied subcellular sites or ubiquitin–protein conjugates generated within the cytoplasm are subsequently targeted to their ultimate site or both. Interestingly, native E1 is $\approx$220 kDa (subunit equals 110 kDa) and contains a highly charged putative nuclear localization sequence near its amino terminus (6, 7, 9). Eukaryotic cells harboring temperature-sensitive mutations in E1 display a cell-cycle arrest phenotype, suggesting putative roles for E1 within the nucleus (see refs. 7 and 23). Indeed, deletion of E1 in yeast is lethal (9).

The recent demonstration of mouse E1 as the spermatogenesis controlling gene *Spy* is also important in this context, because deletion of *Spy* from Y chromosome (e.g., XO mouse) results in arrest of A spermatogonia, azoospermia, and sterility. However, whether these effects are attributable to intranuclear effects of E1 is not yet known.

Within the cytoplasm, E1 is found in several discrete compartments. Both free ubiquitin (11) as well as ubiquitin–protein conjugates (12) have been found within the endosomal–lysosomal vacuolar compartment. The mechanism(s) underlying their formation and targeting has not been resolved. Three possibilities exist—namely, (*i*) uptake of E1, E2, and ubiquitin followed by intravacuolar conjugation, (*ii*) directed uptake of the preformed ubiquitin–protein conjugates, similar to that described by Dice and colleagues (30), or (*iii*) autophagy (24). The first possibility is unlikely because E1 is apparently not found within endosomes/lysosomes (Figs. 4 and 6). Alternatively, E1 may have been rapidly proteolyzed by the acid proteases within the endosomes/lysosomes. The enrichment of E1 along the cytoplasmic membrane of endosomes (Fig. 4) suggests a role in either the second or third possibilities. Perhaps this enrichment plays a role in autophagy. Eukaryotic cells harboring a temperature-sensitive mutation in E1 fail to undergo the autophagic response in response to elevated temperatures (25). This observation directly links E1 to autophagy. The sequence of

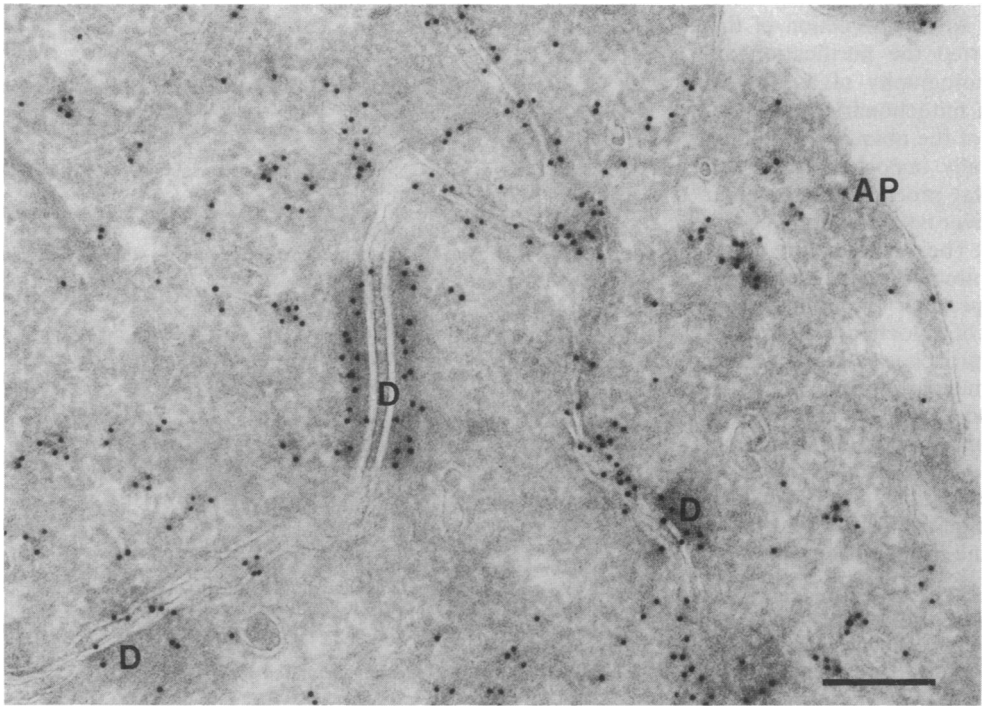

FIG. 5. Immunolabeling of E1 on HepG2 plasma membranes. Partially grazing section through the lateral plasma membranes of HepG2 cells shows E1 labeling associated with the dense material of desmosomes (D). Label is also present in the cytoplasm. AP, apical plasma membrane. (×73,600; bar = 0.2 μm.)

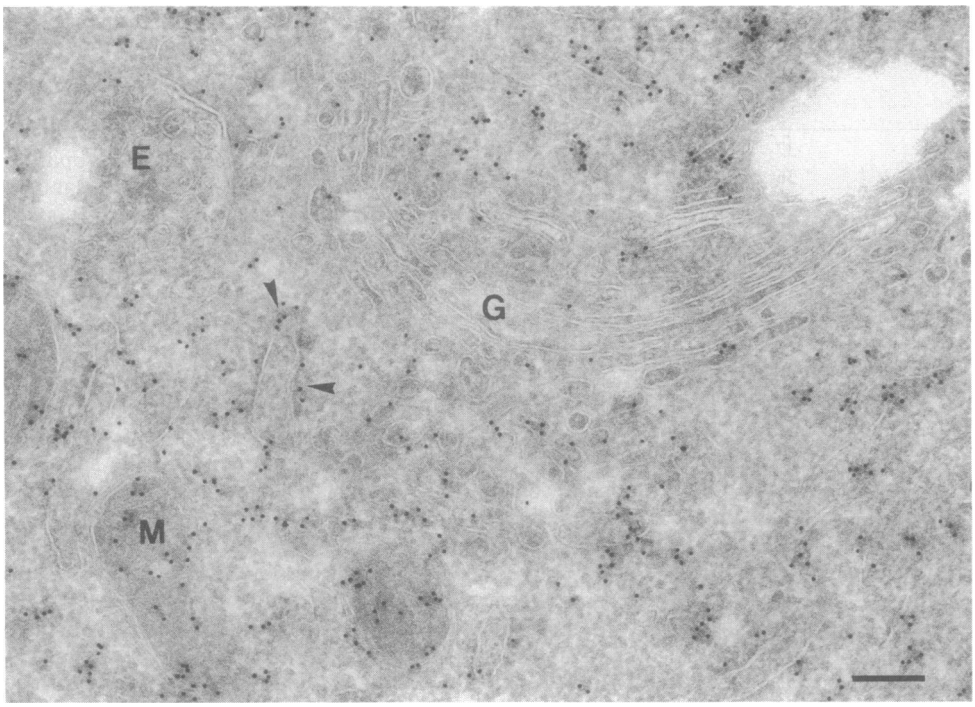

FIG. 6. Immunolabeling of HepG2 for E1. E1 labeling is absent from the Golgi complex (G) and multivesicular endosome (E) and is associated with the ribosomal face of the endoplasmic reticulum (arrowheads) and present in mitochondria (M). (×48,000; bar = 0.2 μm.)

events during autophagy has been recently illuminated by Dunn (26, 27), who demonstrated that autophagic membrane buds from the endoplasmic reticulum, the resultant vacuole acidifies, and the vacuole thereafter fuses with endosomal/lysosomal hydrolase-containing vacuoles. Our demonstration of marked enrichment of E1 along the cytoplasmic RER membrane (Fig. 6) is consistent with the notion that the ubiquitin system is linked to autophagy. In addition, the evolving concept that cytoplasmic proteins are degraded to peptides, transported across the RER membrane, and presented together with major histocompatibility complex class I molecules for antigen recognition may relate to the localization of E1 along the cytoplasmic RER membrane.

A surprising finding in the present study was the localization of E1 within mitochondria. This pattern of E1 localization was also seen in several other cell types, including BHK, CaCo, and JY (data not shown). After completion of this study, Magnani *et al.* (28) reported the purification via ubiquitin covalent affinity chromotography of a 110-kDa protein from purified rabbit brain mitochondria and endoplasmic reticulum. Thus, the role of the ubiquitin system in mitochondria may be physiologically important. It is well known that different mitochondrial proteins turn over at distinct rates. In addition, overall liver mitochondrial protein degradation is ATP-dependent (29). The precise nature of this pathway and its relationship to the ubiquitin-dependent pathway are not fully understood at present. Experiments designed to examine the degradation of mitochondrial proteins in E1 mutant cells will provide approaches to this problem.

Thus, we have defined the immunolocalization of E1 in HepG2 cells, within the nucleus and cytoplasm along desmosomal junctions, endosomes/lysosomes, and RER; in addition, E1 was found within mitochondria. These findings provide a variety of avenues for further investigations into the cellular biology and physiology of the ubiquitin system in protein modification, in protein turnover, and in spermatogenesis.

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