

# Ubiquitin-activating Enzyme, E1, Is Associated with Maturation of Autophagic Vacuoles

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**Abstract.** The ubiquitin-activating enzyme, E1, is required for initiating a multi-step pathway for the covalent linkage of ubiquitin to target proteins. A CHO cell line containing a mutant thermolabile E1, ts20, has been shown to be defective in stress-induced degradation of proteins at restrictive temperature (Gropper et al., 1991. *J. Biol. Chem.* 266:3602-3610). Parental E36 cells responded to restrictive temperature by stimulating lysosome-mediated protein degradation twofold. Such a response was not observed in ts20 cells. The absence of accelerated degradation in these cells at 39.5°C was accompanied by an accumulation of autolysosomes. The fractional volume of these deg-

radative autophagic vacuoles was at least sixfold greater than that observed for either E36 cells at 30.5° or 39.5°C, or ts20 cells at 30.5°C. These vacuoles were acidic and contained both acid phosphatase and cathepsin L, but, unlike the autolysosomes observed in E36 cells, ubiquitin-conjugated proteins were conspicuously absent. Combined, our results suggest that in ts20 cells, which are unable to generate ubiquitin-protein conjugates due to heat inactivation of E1, the formation and maturation of autophagosomes into autolysosomes is normal, but the conversion of autolysosomes into residual bodies is disrupted.

**W**ITHIN mammalian cells, proteolysis occurs by both nonlysosomal and lysosomal mechanisms. Protein ubiquitination has been implicated in the selective nonlysosomal degradation of normal as well as abnormal proteins (4, 10, 14, 18). Ubiquitination of proteins involves a series of ordered events, the first being the activation of ubiquitin to a high energy intermediate by ubiquitin-activating enzyme, E1 (4, 10, 14, 24). The activated ubiquitin is then transferred to one of a variety of ubiquitin-carrier proteins, E2, either for conjugation of a single ubiquitin molecule to a protein substrate (i.e., mono-ubiquitin adducts) or for conjugation catalyzed by ubiquitin-protein ligase, E3, resulting in multi-ubiquitin adducts. Once conjugated to ubiquitin, the protein is rapidly degraded by a soluble high molecular weight ATP-dependent protease complex (17, 30). Using cells with a thermolabile E1, we have shown that this enzyme is required for the degradation of short-lived proteins in the cytosol as well as for stress-induced enhanced degradation of cellular proteins within lysosomes (12). Cells exposed to stress (e.g., nutrient deprivation or elevated temperature) enhance the degradation of endogenous proteins by stimulating cellular autophagy (12, 23). Autophagy is a complex cellular process involving four major events: (a) sequestration of cellular proteins and organelles into autophagosomes; (b) acidification of autophagosomes; (c) ac-

quisition of acid hydrolases upon fusion of autophagosomes with lysosomes; and (d) conversion of autolysosomes into residual bodies upon complete hydrolysis of the sequestered contents (8, 9). A major regulatory event of autophagy is the formation of the autophagosome. Nascent or early autophagosomes arise from an invagination of ribosome-free regions of the rough endoplasmic reticulum. In response to a variety of environmental factors, regions of cytoplasm including organelles are sequestered within these enveloping membranes which fuse to form an autophagosome. Early autophagosomes are limited by a double membrane, and neither acidic nor contain lysosomal hydrolases. Once formed, the early autophagosome matures in a step-wise fashion into a residual body. The first step of maturation is the acidification of the early autophagosome presumably due to the acquisition of an ATP-dependent H<sup>+</sup> pump. This intermediate autophagic vacuole or late autophagosome has characteristics of both early autophagosomes (e.g., bound by two limiting membranes) and autolysosomes (e.g., the contents are acidic). Although the late autophagosome does not yet contain lysosomal hydrolases, a lysosomal membrane protein (e.g., LGP120) has been identified at its outer limiting membrane (9). Next, the acidic late autophagosome then acquires acid hydrolases by fusing with late endosomes or lysosomes, thereby becoming an autolysosome. Finally, luminal con-

tents within the autolysosome are completely hydrolyzed and this vacuole is gradually transformed into an electron-dense residual body.

The inability of E1 ts mutant cells to enhance protein degradation at restrictive temperatures suggests that one or more events of autophagy is altered in these cells. We have recently examined the density profile of subcellular vesicles containing lysosomal enzymes in ts mutant cells (28). After incubation of cells at elevated temperature, there was a shift in lysosomal enzymes to a lighter buoyant density, similar to that observed in the parental cells. One possible interpretation of these results is that autophagic vacuole formation occurred in both ts mutant and parental cells. In this study, we sought to define which of the four events of autophagy requires a functional ubiquitin-activating enzyme. We have examined this linkage in parental, E36, and thermolabile E1 mutant, ts20, CHO cells using biochemical, morphologic, and morphometric approaches. In the absence of a functional ubiquitin-activating enzyme, heat-induced enhanced protein degradation is suppressed coincident with an accumulation of acidic autolysosomes containing acid hydrolases. These data thus suggest that a functional E1 is not required for the formation of the autophagosome nor its maturation into an autolysosome, but is required for the final event of autophagy, the conversion of the autolysosome into a residual body.

## Materials and Methods

### Materials

Rabbit polyclonal antibodies were prepared against ubiquitin which had been covalently coupled to bovine  $\gamma$ -globulin (15). These antibodies preferentially recognize ubiquitin-protein conjugates on Western blots (see Fig. 2 C, reference 27). *N*-[3-[(2,4-dinitrophenyl)-amino]propyl]-*N*-(3-aminopropylmethylamine) dihydrochloride (DAMP)<sup>1</sup> was kindly provided by Dr. K. Madsen (University of Florida Health Science Center, Gainesville, FL). Mouse anti-dinitrophenol was kindly supplied by Dr. R. G. W. Anderson (University of Texas Health Sciences Center, Dallas, TX). Anti-cathepsin L was a gift of Dr. C. Gabel (Pfizer Pharmaceuticals, Groton, CT). Immunogold probes, Na<sup>125</sup>I, and L-[4,5-<sup>3</sup>H]-leucine were purchased from Amersham Corp. (Arlington Heights, IL). All other reagents were purchased from Fisher Scientific (Springfield, NJ) or Sigma Chemical Co. (St. Louis, MO).

### Culturing of Cells

E36 and ts20 cell lines were grown at 30.5°C in MEM containing 4.5 g glucose/l and 10% FCS as described previously (12, 19). Cells were cultured for several days attaining 75–90% confluence at the time of study. The media was refreshed 15–24 h before each experiment. For studies at the permissive temperature, cells were incubated in MEM buffered with 20 mM Hepes, pH 7.4, for up to 5 h at 30.5°C. For studies at the restrictive temperature, cells were incubated in Hepes-buffered MEM for 60 min in a water bath at 41–44°C (to inactivate E1 in the ts20 cells) and then switched to 39.5°C for up to 4 h.

### Quantitation of Degradation of Long-lived Proteins

To determine the effect of incubation at elevated temperature on the rate of degradation of long-lived proteins in parental, E36, and ts mutant, ts20, monolayers were evaluated at ~75% confluency, as described previously (12). Briefly, the growth medium was removed and the cells incubated at 30.5°C for ~16 h in fresh medium (MEM with 10% FCS) containing [<sup>3</sup>H]leucine (2  $\mu$ Ci/ml), pulse period. After a 16-h incubation, the medium was removed and cells were washed twice in Hepes-buffered media contain-

ing 2 mM unlabeled L-leucine and thereafter incubated for 1 h in this medium at either 30.5° or 41°C (to inactivate E1 in the ts mutant cells). After replacement of the medium with fresh pre-warmed Hepes-buffered media containing 2 mM unlabeled L-leucine (chase), cell incubations were continued at 30.5° (untreated cells) or at 39.5°C (heat-treated cells). Aliquots of the culture medium were sampled hourly in duplicate and processed for evaluation of TCA-soluble radioactivity following the addition of carrier human serum albumin to each sample. At the end of the experiment, the remaining medium was removed, the cells washed in ice-cold PBS, scraped from the dish, and TCA-precipitable radioactivity in the cell pellet determined. Radioactivity in cellular proteins at the beginning of the experiment (beginning of the chase period) was calculated by addition of the amount of label released during the experiment to that found in the cells at the end of the experiment. Degradation rates are expressed as the acid-soluble radioactivity as percentage of the initial radioactivity in the cells.

### Quantitation of Protein Ubiquitination Activities in Cell Lysates

The ubiquitin conjugation activity in cellular extracts was used as a measure of E1 (ubiquitin-activating enzyme) activity (12, 21). Quantitative determination of conjugation of <sup>125</sup>I-labeled ubiquitin to protein acceptors was carried out using a modification of the resin-binding assay (21). The assay is based on the observation that free ubiquitin has a neutral isoelectric point and thus is not adsorbed to either an anion or cation exchange resin at neutral pH. On the other hand, ubiquitin-protein conjugates are adsorbed to such resins. The reaction is dependent upon the presence of ATP. In the absence of ATP, the resin-bound radioactivity was <8% of that found in the presence of ATP. Briefly, extracts were prepared from parental or ts mutant cells in ice-cold 20 mM Tris-HCl, pH 7.2, 0.25% Triton X-100, and 2 mM DTT (11). After centrifugation at 13,000 g for 15 min, an aliquot of the supernatant was incubated with <sup>125</sup>I-ubiquitin in the presence or absence of ATP and ATP regenerating system. Thereafter, the mixture was incubated at neutral pH with a combination of anionic and cationic resins, washed extensively at pH 7.0, and counted. Alternatively, the <sup>125</sup>I-ubiquitin-protein conjugates were determined after separation of <sup>125</sup>I-ubiquitin-labeled species on SDS-PAGE, autoradiography, and densitometry (12). Results from each experiment were normalized to 100% activity for the parental cells maintained at 30.5°C.

### Morphology and Cytochemistry

**Acid Phosphatase.** Parental and ts mutant cells incubated at either permissive or restrictive temperatures were fixed for 1 h in 1% glutaraldehyde/2% paraformaldehyde/25 mg% CaCl<sub>2</sub>/0.1 M sodium cacodylate, pH 7.4. Acid phosphatase activity was localized using cytidine 5'-monophosphate and cerium chloride as described by Robinson (25). Cells were stained with 2% uranyl acetate, dehydrated, gently scraped from the dish, and the cell pellets embedded in Polybed 812 (Polysciences, Inc., Warrington, PA) (8).

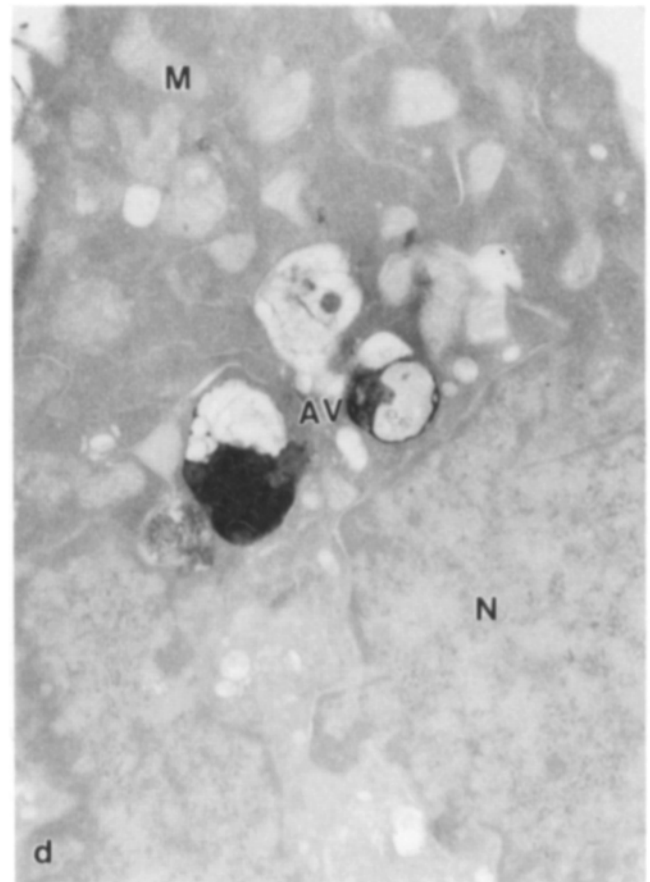
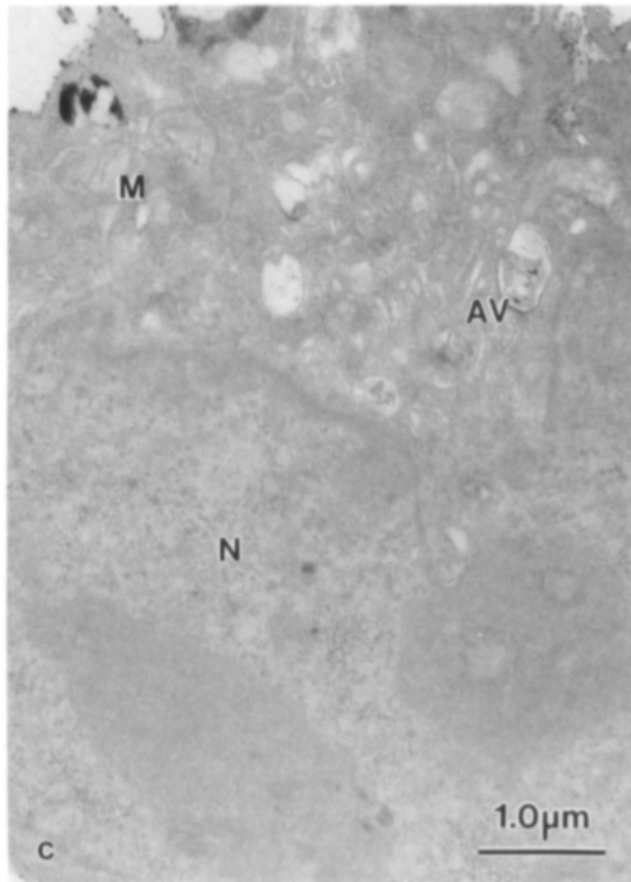
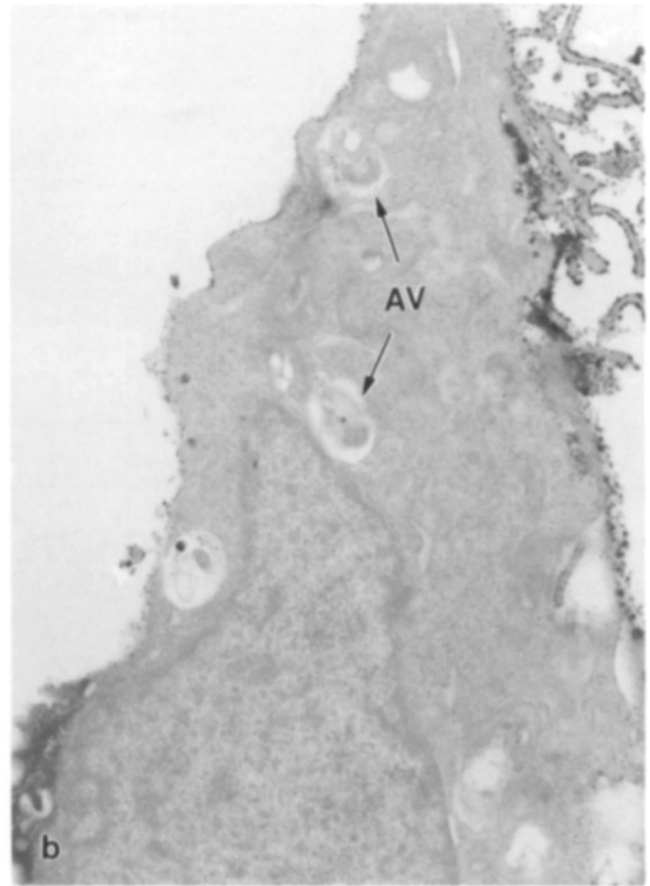
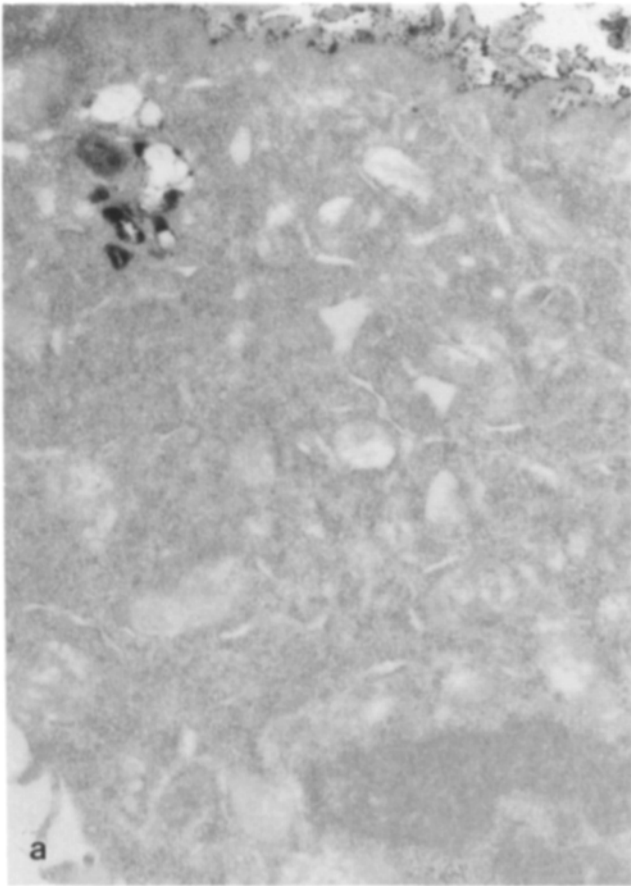
**Immunogold.** Parental and ts mutant cells were incubated at either the permissive or restrictive temperature. 50  $\mu$ M DAMP was added to the medium for the last 30 min of incubation. The cells were then fixed in 1% glutaraldehyde/2% paraformaldehyde/25 mg% CaCl<sub>2</sub>/0.1 M sodium cacodylate, pH 7.4, as described above. The cells were washed in 50 mM NH<sub>4</sub>Cl in PBS, dehydrated in a graded ethanol series, gently scraped from the dishes, and the cell pellets embedded in LR Gold resin (8). The blocks were polymerized at -20°C over an ultraviolet light for 24 h. Ultrathin sections were cut and mounted on 400-mesh nickel grids (Ernest F. Fullum, Inc., Schenectady, NY). Immunogold labeling and staining of the sections was carried out as previously described (8, 9, 29).

### Morphometric Quantitation

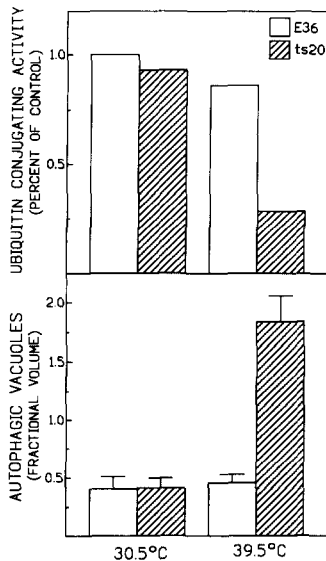
Cells from each cell line, E36 or ts20, were incubated at either permissive or restrictive temperatures and processed for acid phosphatase cytochemistry as described above. The fractional volume of the cell represented by autophagic vacuoles was quantified from 10–20 micrographs (15–40 cell profiles) per experiment using a digitizing tablet interfaced with SigmaScan software (Jandel Scientific, Corte Madera, CA) (8).

The relative concentrations of ubiquitin-conjugated proteins and DAMP in autophagic vacuoles were determined by quantifying the density of gold particles (gold particles/U area) in individual vacuoles (9). These values were compared to the density of gold particles over mitochondria, which are neither acidic nor contain high concentrations of ubiquitinated proteins.

1. *Abbreviation used in this paper:* DAMP, *N*-[3-[(2,4-dinitrophenyl)-amino]propyl]-*N*-(3-aminopropyl-methylamine)dihydrochloride.



**Figure 1.** Morphological characterization of parental E36 and mutant ts20 cells. Parental E36 (*a* and *b*) and mutant ts20 (*c* and *d*) cells were incubated at either 30.5°C (*a* and *c*) or exposed to heat stress of 41°C for 1 h followed by 39.5°C for 4 h (*b* and *d*). Cells were fixed and processed for acid phosphatase cytochemistry as described in Materials and Methods. Exposure to heat stress resulted in the accumulation of acid phosphatase positive autophagic vacuoles (AV) in both E36 and ts20 cells. N, nucleus; M, mitochondrion.



**Figure 2.** Quantitation of heat inactivation of  $^{125}\text{I}$ -ubiquitin conjugation to acceptor proteins and autophagic response in parental E36 and mutant ts20 cells. Parental E36 and mutant ts20 cells incubated at either permissive ( $30.5^\circ\text{C}$  for 5 h) or restrictive ( $41^\circ\text{C}$  for 1 h followed by  $39.5^\circ\text{C}$  for 4 h) temperatures were homogenized to measure ubiquitin conjugating activity (A) or fixed to quantify the fractional volumes of autophagic vacuoles (B). The ability of cell extracts to conjugate  $^{125}\text{I}$ -ubiquitin to cellular proteins was determined as described under Materials and Methods. Results from each of two experiments were normalized to 100% activity for the parental E36 cells maintained at  $30.5^\circ\text{C}$ .

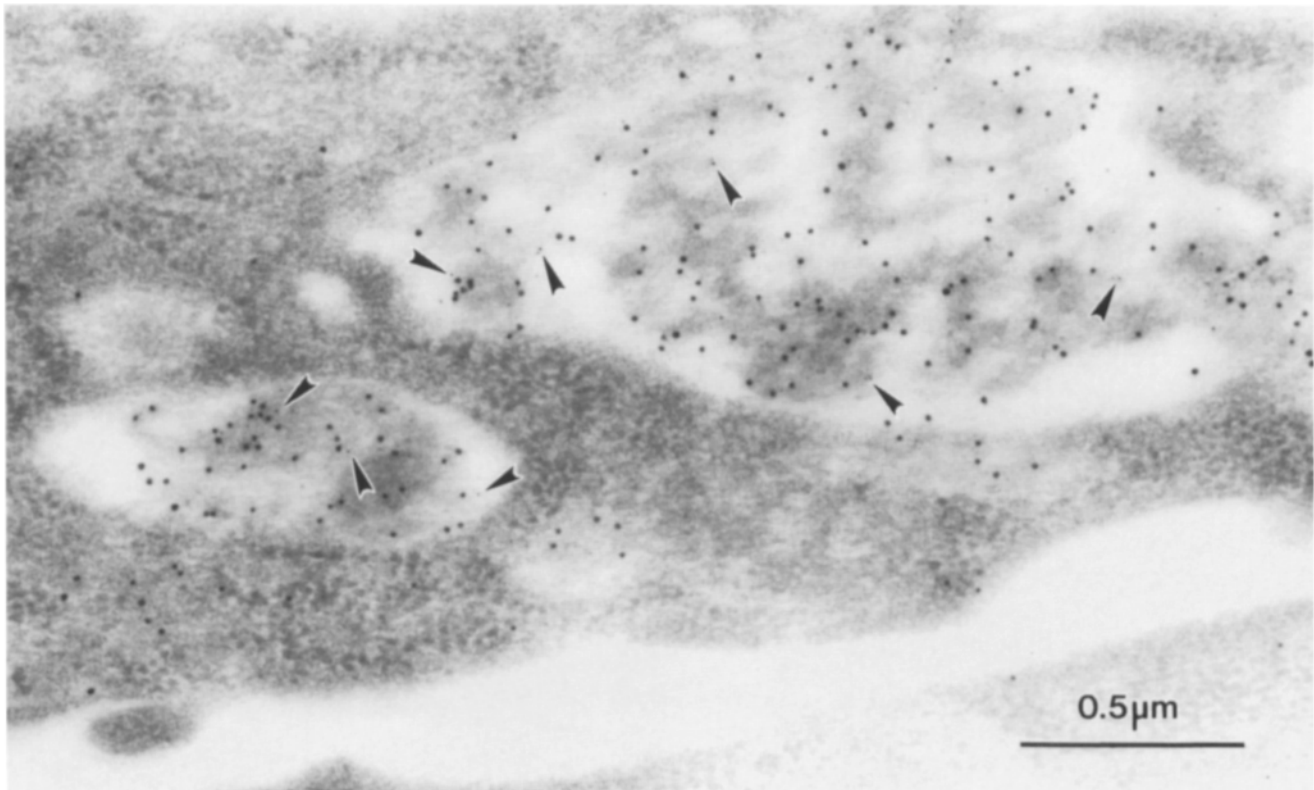
The fractional volumes of autophagic vacuoles in the aldehyde-fixed cells were measured and expressed as a percentage of the total cell volume as described in Materials and Methods. The numbers presented represent the mean  $\pm$  SEM from four separate experiments.

## Results

### *In ts20 Cells at Restrictive Temperatures both Ubiquitin Conjugation and Heat Stress-induced Degradation of Long-lived Proteins Is Deficient*

ts20 cells contain a thermolabile ubiquitin activating enzyme, E1. As seen previously (12) following incubation of ts20 cells at restrictive ( $41^\circ/30.5^\circ\text{C}$ ) temperatures, there was a  $74\% \pm 6\%$  (SEM) ( $n = 7$  experiments) reduction in reduced E1-dependent ubiquitin-conjugating activity in cell lysates compared to cells maintained at the permissive temperature ( $30.5^\circ\text{C}$ ) (data not shown). There was no difference in ubiquitin-conjugating activity in the parental E36 cells at either temperature.

Protein degradation is enhanced in cells exposed to stressful conditions (23). Parental E36 cells respond to heat stress by accelerating the degradation of long-lived proteins approximately twofold (data not shown). As seen earlier, this heat-induced acceleration of degradation is inhibited by  $\text{NH}_4\text{Cl}$  and therefore presumed to be lysosome mediated (12). However, the rate of protein degradation in ts20 mutant cells is unaltered from basal at elevated temperatures. The data suggest a potential linkage between ubiquitin conjugation and lysosomal-mediated protein degradation.



**Figure 3.** Characterization of autophagic vacuoles accumulating during stress in ts20 mutants. Mutant ts20 cells were incubated for 1 h at  $41^\circ\text{C}$  and then 4 h at  $39.5^\circ\text{C}$ , fixed, and processed for immunogold cytochemistry as described in Materials and Methods. Both DAMP (10 nm gold) and cathepsin L (5 nm gold, arrowheads) were located in autolysosomes or degradative autophagic vacuoles in heat-stressed ts20 cells.

**Table I. Quantitation of DAMP in Subpopulations of Autophagic Vacuoles as Determined by the Density of Immunogold Labeling\***

	DAMP accumulation (gold particles/unit area)	
	E36	ts20
Mitochondria	2.01 ± 0.56 (17)	1.92 ± 0.41 (33)
Autophagic vacuoles		
Early autophagosomes	3.95 ± 0.71 (13)	4.15 ± 0.54 (18)
Late autophagosome/ autolysosomes	45.33 ± 2.78 (26)	36.02 ± 2.82 (43)

\* Cells were incubated at 41°C for 1 h and then at 39°C for 4 h. The cells were then fixed, processed for immunogold cytochemistry, and the densities of gold particles quantified as described in Materials and Methods. Vacuoles were categorized into nonacidic (*Early autophagosomes*) and acidic (*Late autophagosomes/autolysosomes*) compartments. The density of gold particles over mitochondria was used as a measure of background labeling. The measurements represent the mean ± SEM. The numbers in parentheses represent the number of organelles counted.

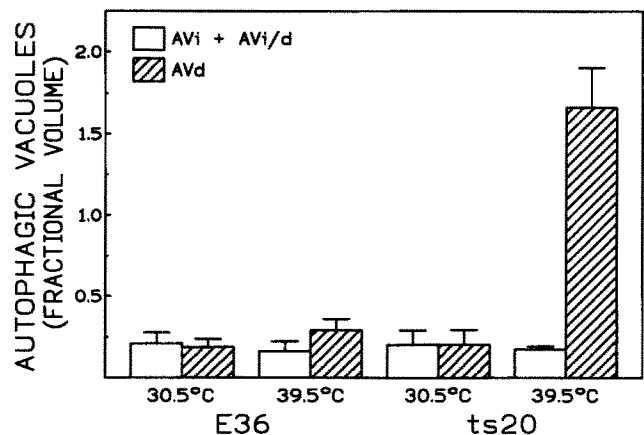
### The Effects of Elevated Temperature on the Appearance of Autophagic Vacuoles in E36 and ts20 Cells

One of the major cellular responses to stressful conditions is an increase in the formation of autophagic vacuoles (23). Therefore, we compared the morphology of E36 parental and ts20 mutant cells under non-stressed and heat-stressed conditions (Fig. 1, *a-d*). The overall ultrastructure was comparable between cells incubated at the permissive and those incubated at the restrictive temperature. Autophagic vacuoles were present in both cell types regardless of the temperature conditions. However, we observed an increase in the number and size of autophagic vacuoles in ts20 cells at the restrictive temperature (Fig. 1 *d*).

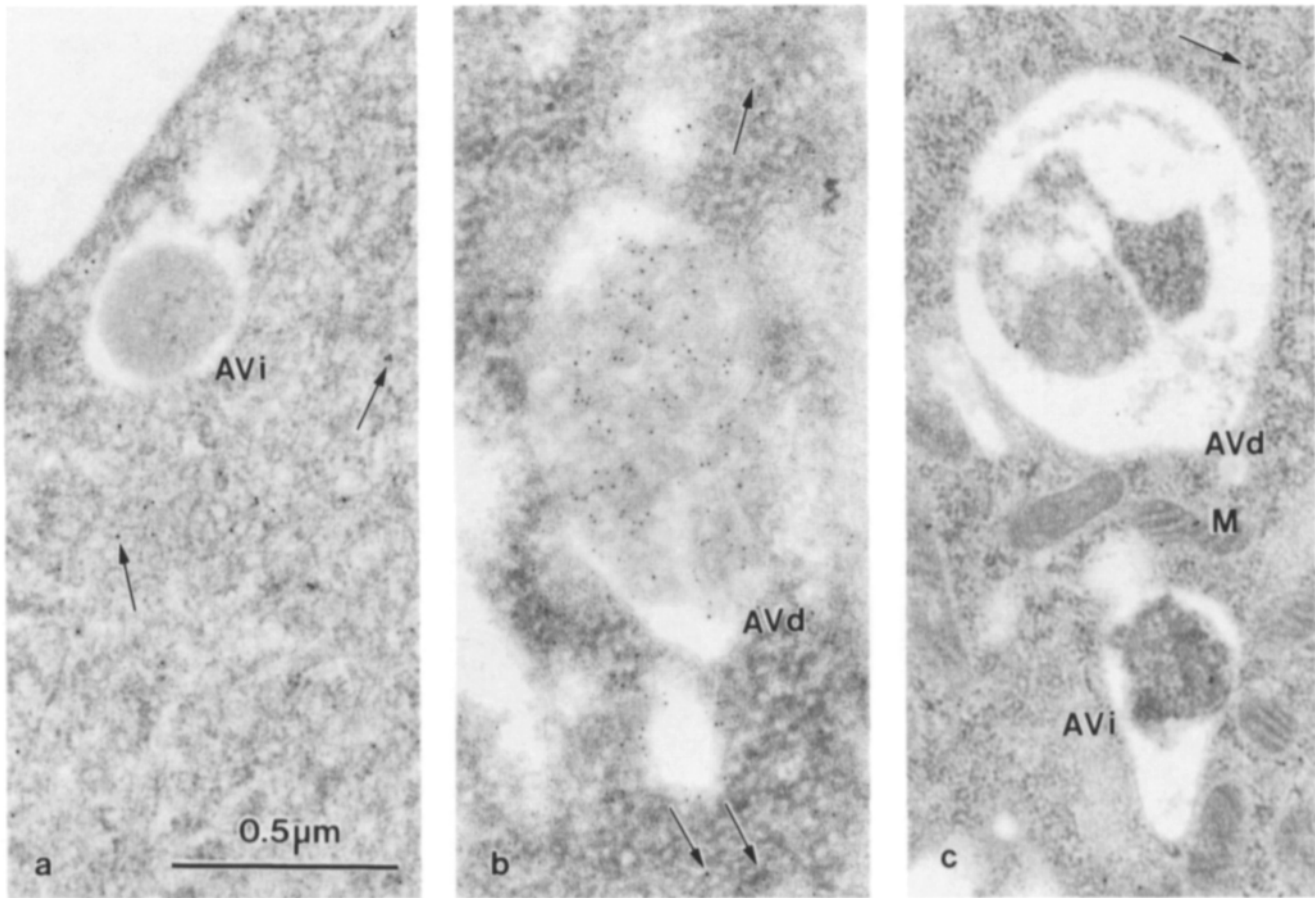
E1-dependent ubiquitin conjugation is inactivated in ts20 cells after incubation at elevated temperature. We have estimated the degree of E1 inactivation by quantifying the ability of cell lysates to conjugate <sup>125</sup>I-ubiquitin to acceptor proteins (Fig. 2 *a*). Cells were incubated at permissive and restrictive temperatures, cell lysates prepared, and ubiquitin-conjugating activity quantified in vitro. The ability of E36 cell lysates to ubiquitinate proteins was suppressed by 10% after incubation of the cells at restrictive temperature. On the contrary, ubiquitin-conjugating activity was reduced by ~70% in lysates of ts20 cells after incubation at restrictive temperatures. At the permissive temperature (30.5°C), the fractional volume represented by autophagic vacuoles was comparable in both cell types (Fig. 2 *b*). In E36 parental cells, the fractional volume of autophagic vacuoles was essentially unaffected by incubation at elevated temperatures. However, in the ts20 mutant cells, incubation at the restrictive temperature resulted in a four- to fivefold increase in the fractional volume of autophagic vacuoles when compared with E36 cells at 30.5° or 39.5°C or ts20 cells at 30.5°C (Fig. 2 *b*). Thus, these data suggest that the accumulation of autophagic vacuoles is coincident with the temperature-dependent inactivation of E1-dependent ubiquitin conjugation.

### Quantitation of Subpopulations of Autophagic Vacuoles in Heat-stressed E36 and ts20 Mutant Cells

We have observed an accumulation of autophagic vacuoles in heat-stressed ts20 mutant cells. Using morphological and immunological criteria described previously, we have classified autophagic vacuoles into three subpopulations: nascent or early autophagosome, intermediate or late autophagosome, and degradative or autolysosome (8, 9). Both early and late autophagosomes are bound by two membranes and contain recognizable cellular components. Late autophagosomes are morphologically similar to early autophagosomes, but differ in that they are acidic. On the other hand, autolysosomes are bound by a single membrane and contain cellular components in various stages of degradation. In addition, these vacuoles are acidic and contain hydrolytic enzymes. Following this criteria, we next characterized which class of vacuoles accumulated in the heat-stressed ts20 cells. This was accomplished by defining the acidic environment of the vacuolar lumen and identifying the presence of acid hydrolases within the vacuoles. Vacuole acidification was examined by incubating cells in the presence of DAMP, which accumulates in acidic compartments (2). DAMP was present in a majority of the autophagic vacuoles of both E36 (not shown) and ts20 (Fig. 3) cells at both permissive and restrictive temperatures. The amount of DAMP which had accumulated within autophagic vacuoles of E36 and ts20 cells incubated at the restrictive temperature revealed a similar degree of acidification in both late autophagosomes and autolysosomes (Table I). Thus, incubation at the restrictive temperature did not appear to alter the vacuole acidification process in either cell type. Acid phosphatase and cathepsin L were also pres-



**Figure 4.** Quantitation of subpopulations of autophagic vacuoles accumulating during stress in parental E36 and ts20 mutant cells. Cells were incubated at either 30.5°C or heat stressed at 41°C for 1 h, then 39.5°C for 4 h, fixed, and processed for acid phosphatase cytochemistry as described in Materials and Methods. Relative volumes of autophagic vacuoles were measured and expressed as a percent of the total cell volume as described in Materials and Methods. Vacuoles were classified as early autophagosomes (*AVi*), late autophagosomes (*AVi/d*), or autolysosomes (*AVd*) according to the descriptions of Dunn (8, 9). The fractional volumes of nascent and intermediate vacuoles were combined and are presented together. The numbers presented represent the mean ± SEM of four separate experiments.



**Figure 5.** Distribution of ubiquitin-protein conjugates within subpopulations of autophagic vacuoles. Parental E36 (*a* and *b*) and mutant ts20 (*c*) cells were incubated at 41°C for 1 h followed by incubation at 39.5°C for 4 h. The cells were fixed and processed for immunogold cytochemistry as described in Materials and Methods. Ubiquitin-protein conjugates (arrows) are found primarily in the cytosol and nucleus of parental E36 (*a* and *b*). Ubiquitin conjugates are present in degradative (*AVd*) but not nascent (*AVi*) autophagic vacuoles of E36 cells. Ubiquitin-protein conjugates are conspicuously absent from both the nascent (*AVi*) and degradative (*AVd*) autophagic vacuoles of mutant ts20 cells. Labeling of the mitochondria (*M*) in both cell lines is minimal.

ent in many of the autophagic vacuoles observed in heat-stressed E36 (not shown) and ts20 (Fig. 1 d and see Fig. 3) cells. Labeling was confined to those vacuoles bound by a single membrane. As expected, these hydrolases were absent from early and late autophagosomes distinguished by the presence of two limiting membranes (not shown).

We next quantified the fractional volumes represented by these distinct vacuolar compartments (Fig. 4). Incubation at either basal or elevated temperature had no significant effect on the fractional volumes of both types of autophagosomes. However, the fractional volume of the autolysosomes was approximately sixfold greater in ts20 cells than E36 cells after incubation at the restrictive temperature. Taken together (Figs. 3 and 4), the results suggest that autolysosomes accumulate within cells in which the activity of the ubiquitin-activating enzyme has been reduced.

#### ***Intracellular Distribution of Ubiquitin Conjugates***

The above results suggest that the maturation of autolysosomes is dependent upon activity of the ubiquitin-activating enzyme. Therefore, we examined the presence of ubiquitin-protein conjugates within autophagic vacuoles using a polyclonal antibody that preferentially recognizes conjugated

ubiquitin. In addition to the labeling of nuclear and cytoplasmic compartments, these antibodies also identified ubiquitin conjugates within many of the autophagic vacuoles present in heat-stressed parental E36 cells (Fig. 5, *a* and *b*). When present, the conjugates were found almost exclusively within the lumen of the autolysosome. These degradative vacuoles contained 14-fold more ubiquitin conjugates than both early and late autophagosomes as estimated by the density of immunogold labeling (Table II). However, ubiquitin conjugates were conspicuously absent from those autolysosomes which had accumulated in heat-stressed mutant ts20 cells (Fig. 5 *c*, Table II). In fact, antibody labeling of these vacuoles was reduced by 80% compared to that observed for similar vacuoles found in E36 cells.

#### ***Discussion***

The degradation of long-lived proteins is enhanced after incubation of cultured cells at elevated temperatures (12). This heat stress-induced accelerated degradation is inhibited by lysosomotropic amines (i.e., NH<sub>4</sub>Cl and chloroquine). Thus, we suggested that the heat stress-induced increase in lysosomal degradation of proteins was mediated by au-

**Table II. Quantitation of Ubiquitin Conjugates in Subpopulations of Autophagic Vacuoles in Heat-stressed E36 and ts20 Cells as Determined by the Density of Immunogold Labeling\***

	Ubiquitin-Protein conjugates (gold particles/unit area)	
	E36	ts20
Mitochondria	0.97 ± 0.33 (26)	1.21 ± 0.18 (117)
Autophagic vacuoles		
Early and late autophagosomes	0.58 ± 0.36 (12)	0.62 ± 0.27 (18)
Autolysosomes	8.23 ± 1.31 (22)	1.52 ± 0.40 (30)

\* Cells were incubated at 41°C for 1 h and then at 39°C for 4 h. The cells were then fixed, processed for immunogold cytochemistry, and the densities of gold particles quantified as described in Materials and Methods. Vacuoles were categorized into two groups (*Early and late autophagosomes* and *Autolysosomes*) based on the presence or absence, respectively, of a double limiting membrane. The density of gold particles over mitochondria was used as a comparison of labeling conditions between the cell types. The measurements represent the mean ± SEM. The numbers in parentheses represent the number of organelles counted.

tophagy. However, in the present study, we do not observe a significant accumulation of autophagic vacuoles coincident with accelerated protein degradation in parental E36 cells incubated at 39.5°C. One possible explanation for these findings is that accelerated protein degradation is not associated with macroautophagy. That is, endogenous proteins are sequestered within lysosomes by either chaperone-mediated transfer (i.e., *hsc73*) or microautophagy upon incubation at elevated temperature. Chaperone-mediated transfer of proteins into lysosomes is enhanced in cells upon exposure to stress. However, this effect is dependent upon the synthesis of *hsp73* which requires a minimum of 2 h under stress conditions (19). The heat stress-induced acceleration in protein degradation seen in the E36 cells is already detected by 1 h (12). Microautophagy has been shown to be suppressed during nutrient deprivation (22). In addition, we have not seen any morphological evidence that this pathway is activated in the heat-stressed cells. A second possibility is that autophagic vacuoles are formed at a faster rate in heat-stressed parental E36 cells but that vacuole maturation is also enhanced at elevated temperatures. Indeed, cellular processes associated with lysosome-mediated degradation (e.g., endocytosis, lysosomal acidification, and lysosomal enzyme activity) are directly related to temperature (1, 7, 16, 26). Therefore, the elevated rates of vacuole formation and maturation which would likely occur at elevated temperatures may not result in a detectable net accumulation of autophagic vacuoles.

At the non-permissive temperature mutant ts20 cells are unable to conjugate ubiquitin to proteins secondary to inactivation of a temperature-sensitive ubiquitin-activating enzyme, E1. We have shown that, unlike parental E36 cells, autolysosomes accumulate in these cells at the restrictive temperature. At least three possible explanations may account for this accumulation: (a) the formation of autolysosomes is accelerated; (b) the maturation of autolysosomes to residual bodies is suppressed; or (c) both. In the first possibility, we would predict that an enhanced sequestration of

cellular proteins into autolysosomes would be associated with accelerated protein degradation in ts20 cells at restrictive temperature. However, heat-induced acceleration of protein degradation occurred only in the parental E36 cells containing functional E1. Mutant ts20 cells, which contain a thermolabile E1, did not exhibit an acceleration of protein degradation at the restrictive temperature. Maturation of autolysosomes requires hydrolysis of its luminal contents. Therefore, in the second possibility, a reduction in the degradation of vacuolar contents would result in the accumulation of autolysosomes which under normal circumstances would mature into residual bodies. Such appears to be the case, as heat stress-induced degradation is suppressed in ts20 cells. The inability of these cells to induce protein degradation in response to stress is not, however, accompanied by an inhibition of autophagic vacuole formation. The third possibility remains open. However, if formation of autophagic vacuoles was accelerated and their maturation suppressed, we might expect to observe an increase in the fractional volumes of both nascent and intermediate autophagic vacuoles in the ts20 mutants at the restrictive temperature. As shown in Fig. 4, such increases were not detected. Therefore, the data support the concept that functional E1 is not required for the formation of autophagic vacuoles but rather for the maturation of autolysosomes to residual bodies and its attendant protein degradation.

Under many circumstances ubiquitin conjugation is linked to protein degradation (5, 11, 12). The cascade of events involved in protein ubiquitination is initiated by the ubiquitin-activating enzyme, E1 (10, 11). Within the cytoplasm, multi-ubiquitination of a protein is associated with an increased susceptibility of the conjugated protein to proteolysis (10). Whether protein ubiquitination serves a similar function within autolysosomes is not known. Using an antibody that preferentially recognizes ubiquitin-protein conjugates, we have found such conjugates localized to both cytoplasmic and nuclear compartments. In addition, we have identified ubiquitinated proteins within autolysosomes, but not early and late autophagosomes. The absence of ubiquitinated proteins from early and late autophagosomes suggests that these conjugates are either formed within or transported into autolysosomes. We have previously reported the presence of free ubiquitin within autophagic vacuoles (27). This free ubiquitin may serve as a substrate for ubiquitination or is the product of proteolysis and isopeptidase activity. However, we have not detected E1 on Western blots of autophagic vacuoles isolated from rat liver (unpublished observations). Although the vacuolar localization of E2, E3, and the multicatalytic proteasome is not known, it is unlikely that the same mechanisms (e.g., neutral pH and ATP availability) which govern their function within the cytoplasm are operative within the acidic autophagic vacuole (10). Therefore, in the absence of protein ubiquitination within the autophagic vacuole, entry of ubiquitinated proteins into autolysosomes may occur by either chaperone-mediated transport (e.g., *hsc73*) or fusion of intermediate autophagic vacuoles with vesicles containing ubiquitin conjugates (i.e., lysosomes) or both. The KFERQ sequence which is recognized by *hsc73* and responsible for targeting cytosolic proteins to lysosomes is not present in ubiquitin (3, 6, 24). Morphological evidence provided by Mayer and co-workers suggests that ubiquitin conjugates may be incorporated into lysosomes by mecha-

nisms similar to microautophagy (20). We have found a substantial reduction in the concentration of ubiquitin conjugates present in autolysosomes that accumulate in ts20 cells at the restrictive temperature. This reduction may be the result of E1 inactivation or reflect volumetric changes in the autolysosome compartment. Kulka et al. have shown that in ts20 cells, the cellular amounts of ubiquitinated proteins are dramatically decreased coincident with heat inactivation of E1 (19). At this time, it is not clear whether this absence of ubiquitin conjugates is a cause of or only an association with the defect in autophagic vacuole maturation. We propose that protein ubiquitination is required for the degradation of proteins to free amino acids within autolysosomes. It is possible that ubiquitin conjugation is either essential for rendering proteins susceptible to hydrolytic enzymes or necessary for activating proteins or enzymes required for proteolysis. That is, unconjugated proteins may be poor substrates for lysosomal enzymes or a regulatory protein must be ubiquitinated for hydrolysis to proceed. At this time, we do not know what event in the hydrolytic scheme is defective. We know that the radioactivity present in the ts20 cells is acid insoluble, but we have yet to determine whether or not the proteins that have accumulated within the autolysosome at the restrictive temperature are intact or high molecular weight degradative fragments.

In the past, nonlysosomal and lysosomal pathways were thought to function independently of one another. Evidence presented here and elsewhere suggests that lysosome-mediated protein degradation requires protein ubiquitination (12). The present study was undertaken to identify which of the four autophagic events is dependent upon the ubiquitin system. Our data demonstrate that autophagic vacuoles form, acidify, and acquire acid hydrolases independent of E1 activity. However, the proteolytic events responsible for the transformation of an autolysosome into a residual body is contingent upon a functional E1.

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