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Ubiquitin metabolism in HeLa cells starved of amino acids

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Radio-iodinated ubiquitin (Ub) was introduced into HeLa cells by red blood cell-mediated microinjection. The half-life and solubility of Ub, as well as the molecular weight distributions of Ub conjugates, were then measured in HeLa cells grown in complete medium or in medium lacking amino acids and fetal calf serum. Ub metabolism was similar in the two sets of cells. Thus, the dramatic changes in Ub metabolism induced by thermal stress are not observed upon amino acid deprivation.

Protein degradation; Ubiquitin; Autophagy; Amino acid starvation; Microinjection

1. INTRODUCTION

Ubiquitin (Ub) is a highly conserved eucaryotic protein capable of being reversibly crosslinked to a variety of intracellular proteins. Although attachment of Ub may serve many functions, the molecule is best characterized as a necessary component for intracellular proteolysis. In this role, Ub is covalently linked to intracellular proteins, thereby targeting them for destruction (see [1–3] for reviews). Conceptually, intracellular proteolysis in eucaryotes has often been divided into two broad categories, lysosomal and cytosolic pathways. Several years ago it would have been reasonable to assume that Ub functions exclusively within the cytosol. However, newer observations suggest that Ub may also participate in lysosomal protein degradation. Immunohistochemical procedures have been used in three studies to demonstrate either that Ub is present within lysosomes [4] or that Ub conjugates accumulate within lysosomes under certain conditions [5,6]. Recently, Gropper et al. [7] observed that the Ub-activating enzyme, E1, is required for stress-induced lysosomal degradation in the mutant mouse cell line, ts85. The latter finding could reflect the targeting of Ub conjugates to autophagosomes following stress. Alternatively, Ub may play a direct role in autophagosome formation and/or function.

If cells have the ability to direct Ub conjugates to autophagosomes, one might expect an accumulation of Ub conjugates upon amino acid deprivation since autophagy increases markedly under such conditions [8]. In addition, if Ub conjugates are degraded within lysosomes, one should observe faster turnover of Ub after transfer of cells to amino acid-deficient medium, and the enhanced degradation should be sensitive to ammonia or chloroquine. In this study, we have tested these hypotheses and have found that although starvation for amino acids and serum does enhance the degradation of Ub, the proportion of Ub degraded in lysosomes does not increase significantly. We also found that starvation for amino acids, unlike other forms of stress, does not alter the molecular weight distribution of Ub conjugates [9–12].

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2. EXPERIMENTAL

2.1. *Radiolabeling of proteins*

Proteins (~150 μg) were labeled with [^{125}I]Bolton-Hunter reagent as described previously [13]. Typical specific activities ranged from ~10^6 cpm/μg to ~5×10^6 cpm/μg.

2.2. *Cell culture*

The HeLa cell line, D98/AH2, was routinely grown in McCoys Medium supplemented with 5% fetal calf serum (FCS), 100 U/ml penicillin and 20 μg/ml streptomycin. However, 48 h prior to an experiment, the cells were cultured in Minimum Essential Medium (MEM) medium (Gibco-BRL). HeLa cells were starved for amino acids by culturing in MEM medium containing 0.5% dialyzed FCS and lacking amino acids.

2.3. *Measurement of endogenous protein degradation*

HeLa cells were trypsinized and plated at about 50% confluency. They were then grown for 24 h in MEM complete medium containing 10 μCi/ml [^{35}S]methionine, washed and chased for an additional 36 h in MEM containing a 10-fold excess of unlabeled methionine. The chase medium was changed every 12 h. [^{35}S]Methionine-labeled cells were harvested by trypsin, fused to mock-loaded red blood cells (RBCs), and plated in 75 cm^2 T-flasks. Proteolysis was measured by acid precipitation of culture medium taken at various times after rinsing the cells. The released [^{35}S]methionine was compared to the total [^{35}S]methionine incorporated. The latter was determined by dissolving the cell monolayer in 1% SDS and counting a portion of this sample in a liquid scintillation spectrometer.

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2.4. *Loading of RBCs and Sendai-mediated fusion*

Radiolabeled proteins were introduced into freshly drawn human RBC by the preswell method, and 3×10^9 loaded RBCs were fused with 3×10^7 HeLa cells as described previously [13]. For experiments in which [^{35}S]methionine-labeled HeLa cells were used, RBCs were simply lysed in 10 mM Tris, pH 7.5, and re-sealed to produce mock-loaded RBCs.

2.5. *Measurement of the degradation of injected proteins*

HeLa cells were fused with loaded red blood cells and plated into 75 cm^2 T-flasks. After thoroughly rinsing the attached HeLa cells to remove unfused RBCs, the flasks were incubated in various media as described in the figure legends. At various times, 2 ml of medium was removed, and 2 ml of prewarmed medium was re-added to each flask such that the volume of medium did not change over the course of the experiment.

Proteolysis was measured by acid precipitation. A 0.9 ml sample of the collected medium was mixed with 0.1 ml of 100% trichloroacetic acid. After 15 min on ice, the precipitated protein was sedimented in an Eppendorf centrifuge, and the rate of proteolysis was determined from the ratio of soluble ^{125}I to total [^{125}I]protein in the cells at 0 time. The latter value was obtained by dissolving the cellular monolayer in 1.0 N NaOH at the end of the experiment and adding the ^{125}I from this sample to all ^{125}I released into the medium. Student's *t*-tests were used to determine whether differences in rates of proteolysis were statistically significant [14].

2.6. *Triton X-100 extraction*

Monolayers of injected cells or cells labeled with [^{35}S]methionine were rinsed twice with PBS and 1.5 ml of 0.5% Triton X-100 in 100 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 200 mM sucrose, 0.15 mg/ml phenylmethylsulfonyl fluoride, 10 mM Tris, pH 7.5, was added to the flask. After 4 min the supernatant was removed, and the Triton X-100-insoluble fraction was dissolved in 1 N NaOH. The distribution of isotope between the two fractions constitutes the Triton X-100 solubility for each radio-iodinated protein or the collection of [^{35}S]methionine labeled cellular proteins.

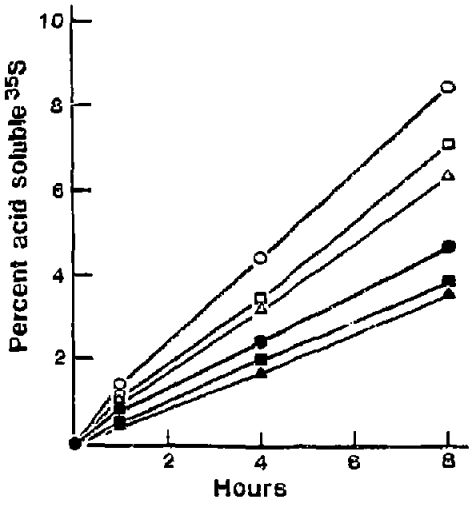

Fig. 1. Effects of lysosomotropic agents on the degradation of endogenous HeLa proteins. HeLa cells were labeled with [^{35}S]methionine and fused to mock-loaded human red blood cells as described in section 2. The fused cells were plated into six 75 cm^2 T-flasks, allowed to attach for 1 h and then rinsed 3 times with MEM containing a 10-fold excess of unlabeled methionine. After 1 h three flasks were rinsed with prewarmed complete MEM. Individual flasks then received 6 ml of complete MEM containing 4 mM NH_4Cl (■), 10 μM chloroquine (▲) or no lysosomotropic agents (●). The other three flasks were rinsed with MEM lacking amino acids (MEM incomplete) and then cultured in 6 ml of incomplete MEM containing 4 mM NH_4Cl (□), 10 μM chloroquine (△) or no lysosomotropic agent (○). Degradation of [^{35}S]methionine-labeled proteins was measured by acid precipitation as described in section 2.

Table I

Stabilities of endogenous and injected proteins in HeLa cells

Culture conditions	Half-lives (h)			
	Ub_{76}	Ub_{74}	BSA	^{35}S-labeled endogenous proteins
MEM complete	18.3 ± 2.6[a] (n = 7)	8.5 ± 1.5 (n = 4)	14 ± 1.6 (n = 4)	107 ± 12[c] (n = 3)
MEM complete + 4 mM NH_4Cl	22.0 ± 5.3 (n = 4)	8.5 ± 0.9 (n = 2)	13	125 ± 20 (n = 3)
MEM complete + 10 μM Chloroquine	22.5 ± 2.8[b] (n = 4)	8.1 ± 2.0 (n = 2)	12	111 ± 29 (n = 3)
MEM lacking amino acids and FCS (= incomplete)	13.3 ± 2.5[a] (n = 7)	6.8 ± 1.4 (n = 4)	11 ± 0.1 (n = 4)	64 ± 8[c,d] (n = 3)
MEM incomplete + 4 mM NH_4Cl	17.4 ± 5.6 (n = 4)	5.9 ± 2.0 (n = 2)	10	86 ± 7 (n = 3)
MEM incomplete + 10 μM Chloroquine	17.9 ± 3.9[b] (n = 4)	6.8 ± 2.2 (n = 2)	10	86 ± 8[d] (n = 3)

[a] The difference in half-life of Ub_{76} in MEM complete vs. MEM incomplete is statistically significant ($P \leq 0.05$).

[b] The half-life of Ub_{75} in medium containing chloroquine is significantly ($P \leq 0.05$) longer than in medium lacking the lysosomotropic agent.

[c] The average half-life of [^{35}S]methionine-labeled HeLa proteins in MEM incomplete is significantly ($P \leq 0.02$) reduced over that in complete medium.

[d] The average half-life of [^{35}S]methionine-labeled HeLa proteins in MEM incomplete medium containing chloroquine is significantly longer ($P \leq 0.03$) than in the absence of the lysosomotropic agent.

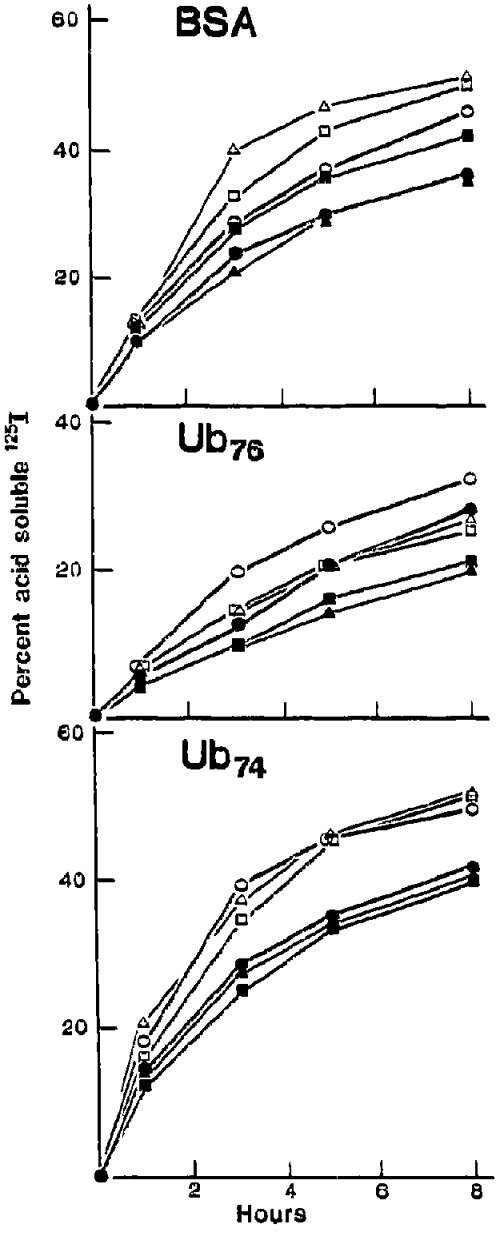

Fig. 2. Effects of lysosomotropic agents on the degradation of microinjected proteins in HeLa cells cultured in complete or amino acid-deficient medium. HeLa cells were fused with red blood cells loaded with the radio-iodinated protein indicated above, and distributed into six 75 cm^2 T-flasks. The cells were then treated as described in the legend to Fig. 1, and proteolysis of the injected proteins was measured by acid precipitation (see section 2). Open symbols denote amino acid-deficient MEM; closed symbols signify complete MEM. Circles represent proteolysis in the absence of lysosomotropic agents, squares indicate proteolysis in medium containing 10 μM chloroquine and triangles signify proteolysis in the presence of 4 mM NH_4Cl.

2.7. *Electrophoretic analysis of ubiquitin conjugates*

Samples of HeLa cells injected with [^{125}I]Ub_{76} were prepared as described [13] and analyzed on 10% SDS-PAGE gels by the method of Laemmli [15]. The molecular weight distribution of radiolabeled conjugates was determined by autoradiography using Kodak XAR film.

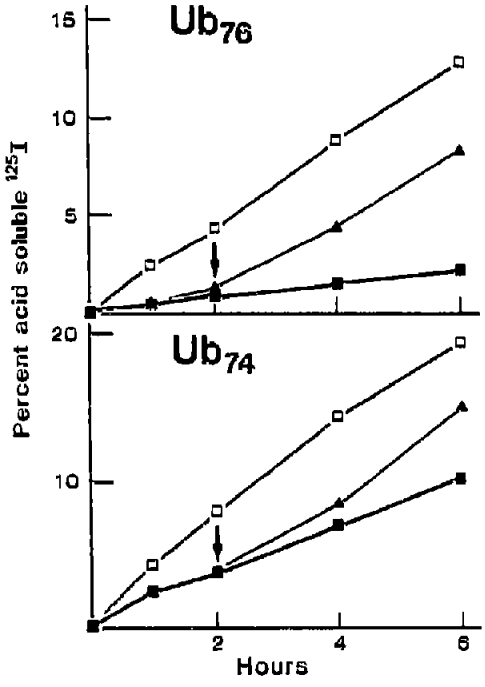

Fig. 3. Effects of ATP depletion on the degradation of [^{125}I]ubiquitin 76 and [^{125}I]ubiquitin 74. Suspensions containing 3 × 10^7 HeLa cells were injected with [^{125}I]Ub_{76} or [^{125}I]Ub_{74} by the RBC-mediated procedure. After washing to remove unfused RBCs, injected cells from each set were plated into three 75 cm^2 T-flasks, washed 1 h later with 6 ml of McCoys medium and incubated for an additional 18 h. A flask of cells from each set was then incubated in MEM complete medium, and proteolysis was measured over a 6 h interval by the rate at which TCA-soluble ^{125}I entered the medium. Cells of the other two flasks from each set were incubated in MEM lacking glucose and amino acids and containing 1 mM 2,4-dinitrophenol plus 10 mM 2-deoxyglucose (solid symbols). Proteolysis was measured in cells cultured in ATP depletion medium for 2 h, and then measured for an additional 4 h in cells left in ATP depletion medium (■) and in cells restored to normal culture conditions (▲).

2.8. *Materials*

[^{35}S]Methionine (spec. act. = 649 Ci/mmol) was obtained from New England Nuclear. The Bolton-Hunter reagent was purchased from Behring Diagnostics. All components of MEM medium were from Gibco; McCoy's medium, Triton X-100, Ubiquitin 76 and bovine serum albumin (BSA) were from Sigma; Ubiquitin 74 was a gift from K. Wilkinson (Emory University).

3. RESULTS

Using the labeling protocol described in section 2 the average half-life of [^{35}S]methionine-labeled proteins was ~110 h in complete MEM. This value decreased to almost half in MEM lacking amino acids (see Fig. 1 and Table I). Chloroquine and NH_4Cl were used to assess the contribution of lysosomes to overall proteolysis under both conditions of culture. Whereas these two lysosomotropic agents had little effect on proteolysis in HeLa cells grown in MEM complete, they inhibited protein degradation by 25% in HeLa cells starved of amino acids and FCS. Thus, the data in the far right-

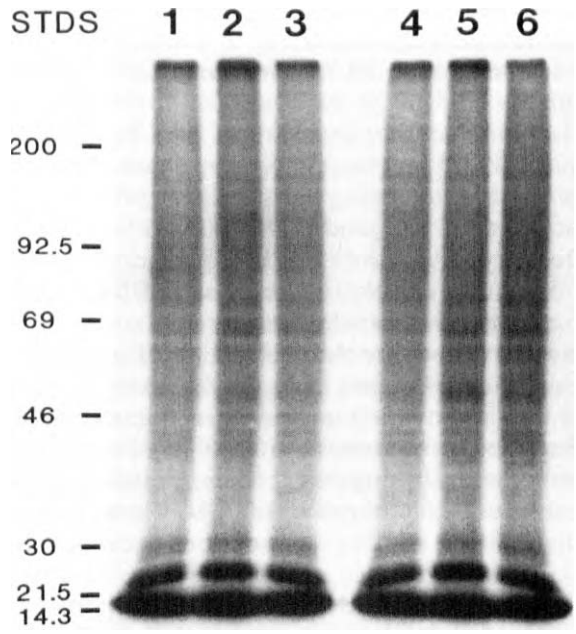

Fig. 4. Molecular weight distributions of Ub conjugates in HeLa cells cultured in complete or amino acid-deficient medium. [^{125}I]Ub was introduced into 6×10^7 HeLa cells by red cell-mediated microinjection. 1 h after plating in six 75 cm^2 T-flasks, the cells were rinsed with complete MEM. Cells of two flasks were trypsinized immediately and dissolved in SDS-PAGE sample buffer. The remaining cells were cultured in complete MEM (2 flasks) or MEM lacking amino acids (2 flasks). After an additional 3 or 8 hs cells were harvested from a flask from each set and dissolved in SDS-PAGE sample buffer. The molecular weight distribution of Ub conjugates was then determined by electrophoresis on 10% acrylamide gels followed by autoradiography. Lanes 1 and 4, 0 h samples; lane 2, 3 h sample from cells in MEM complete; lane 3, 3 h sample from cells in MEM lacking amino acids; lane 5, 8 h sample from cells in MEM complete; lane 6, 8 h sample from cells in MEM lacking amino acids. Each lane contained approximately 5,500 cpm ^{125}I and autoradiographic exposure was for 23 days.

hand column of Table I demonstrate that the degradation of HeLa proteins almost doubles in MEM lacking amino acids and serum; about two-thirds of the increased proteolysis can be attributed to autophagy/lysosome pathways based on inhibition by ammonia or chloroquine.

With a clear indication for enhanced autophagy in nutrient-deprived HeLa cells, we asked whether the degradation of Ub_{76}, Ub_{74} or BSA was altered in incomplete MEM. Ub_{74} and BSA were included in these experiments for the following reasons. Ub_{74} is not conjugated to HeLa proteins [16], and thus serves as a control for the possibility that Ub itself is specifically targeted for destruction under step-down conditions. Similarly, BSA is a rapidly degraded protein whose catabolism is known to be exclusively cytosolic in HeLa cells [17,18].

Each of the three proteins was radio-iodinated and injected into HeLa cells by RBC fusion. The degradation rate of each protein was then determined in complete MEM and in MEM lacking amino acids and FCS. According to the data in Fig. 2 and Table I, all three injected proteins were degraded faster under step-down conditions, although only the degradation of Ub_{76} was increased by a statistically significant amount. In addition, degradation of the injected proteins responded differently to lysosomotropic agents when compared to endogenous HeLa proteins and among themselves. Although the degradation of each injected protein increased by 25–40% in step-down medium (MEM incomplete), sensitivity of their turnover to lysosomotropic agents was unaltered. That is, Ub_{74} and BSA appeared to be degraded exclusively in the cytosol under both methods of culture. By contrast, the degradation of Ub_{76} was inhibited 20–25% by lysosomotropic agents in either medium.

Because the degradation of Ub_{76} and Ub_{74} clearly differed in rate and with regard to sensitivity to lysosomotropic agents, we asked whether both proteins were degraded by ATP-dependent pathways. For these experiments, HeLa cells were injected with either [^{125}I]Ub_{76} or [^{125}I]Ub_{74} and transferred to medium containing 2,4-dinitrophenol and 2-deoxyglucose to deplete intracellular ATP pools. The data in Fig. 3 demonstrate that degradation of Ub_{76} was severely inhibited

Table II

Triton X-100 solubilities of injected or endogenous proteins

Conditions	Protein	Triton X-100 solubility (%) at various hours after medium change		
		0	3	8
MEM complete	Ub_{76}[a]	77	77	75
	Ub_{74}[b]	84	83	79
	[^{35}S]met-labeled[b] endogenous	76	74	74
MEM incomplete	Ub_{76}[a]	77	80	70
	Ub_{74}[b]	84	72	72
	[^{35}S]met-labeled[b] endogenous	76	74	78

[a] The results for Ub_{76} are the averages from two experiments.
[b] Results are from a single experiment.

in ATP- depleted HeLa cells; the degradation of Ub_{74} also slowed appreciably, but less so than that of Ub_{76}. The decreased turnover of both molecules can be attributed to energy depletion rather than cell death since degradation returned to normal upon removing the inhibitors.

The molecular weight distribution of Ub changes markedly when HeLa cells are stressed by heat, ethanol or oxidizing agents [10]. There is a rapid disappearance of uH2A and the free Ub pool. These changes are accompanied by a marked increase in high molecular mass (M_r > 100,000) conjugates. To determine whether nutrient deprivation induced similar changes in Ub pools, we injected HeLa cells with [^{125}I]Ub_{76} and compared the SDS-PAGE profiles of radio-iodinated Ub from cells grown in complete MEM or MEM lacking amino acids. The autoradiogram presented in Fig. 4 clearly shows that amino acid starvation did not induce any major redistribution of Ub among the various size classes of conjugates. Equally important, there was no loss of uH2A. Since the redistribution of Ub from uH2A to high molecular weight conjugates is characteristic of the stress response [9,11], the persistence of uH2A can be taken as evidence that starvation of amino acids does not induce the stress response, at least with regard to shifts in Ub metabolism.

The heat-shock response affects Ub in another way. Following stress there is a marked decrease in the solubility of Ub conjugates in buffers containing Triton X-100 [9]. To determine whether starvation of amino acids affected the solubility of Ub conjugates, HeLa cells were injected with [^{125}I]Ub_{76} and cultured in MEM with or without amino acids. At various times thereafter, cells in each medium were extracted in solutions containing 0.5% Triton X-100. Ub conjugates remained soluble in both sets of cells (see Table II).

4. DISCUSSION

The experiments presented above address two major questions. Does amino acid depletion produce the dramatic changes in Ub pools seen after heat shock or oxidative stress? And, is starvation-induced autophagy accompanied by increased degradation of Ub within lysosomes? Fig. 4 provides a clear answer to the first question. The molecular weight distribution of Ub conjugates does not change perceptibly when HeLa cells are transferred to culture medium lacking amino acids and FCS. Moreover, the solubility of Ub conjugates remains unchanged in step-down medium. We conclude that unlike the effect seen with heat, ethanol and oxidizing agents [10], amino acid depletion does not lead to major perturbations in Ub pools.

The second question has proved more difficult to answer. In an extensive series of experiments published several years ago [19], we showed that lysosomes contributed to the degradation of injected proteins only when the half-life of a specific protein is longer than 40 h. The data in Table I and Fig. 2 demonstrate that Ub is a clear exception to the previous observations. Although its half-life is less than 20 h, lysosomotropic agents inhibited the degradation of Ub_{76} by 20% in complete MEM and by 25% in step-down medium, the levels of inhibition being statistically significant. Degradation rates for Ub_{74} and BSA, on the other hand, were unaffected by ammonia or chloroquine, in agreement with the earlier correlation between half-life and degradation by the lysosomal pathway.

One can rationalize the destruction of some Ub molecules within lysosomes from the fact that Ub is conjugated to a wide variety of specific cellular proteins, including membrane components [20–26]. Presumably, by virtue of being conjugated to these proteins, some Ub molecules are catabolized within lysosomes. Still, a substantial portion of Ub_{76} degradation is resistant to ammonia and chloroquine and can therefore be attributed to a cytosolic degradative pathway(s). Although the responsible pathways remain to be discovered, we have presented correlative evidence that proline endopeptidase is required for Ub destruction [16]. The data in Fig. 3 demonstrate that virtually all Ub_{76} degradation is ATP-dependent, indicating that factors other than or in addition to proline endopeptidase must be involved since the latter enzyme does not require energy for peptide bond hydrolysis.

Whether the conjugation of Ub to specific proteins actually promotes their transfer to autophagic vacuoles cannot be determined from the present studies. Several observations, however, argue against this possibility. First, there are numerous studies showing that, for the most part, cytosolic proteins are randomly transferred to autophagic vacuoles ([27] and refs. therein). Because most evidence indicates that ubiquitin targets specific proteins for destruction, it is not an attractive candidate for enhancing autophagic degradation. Second, calculations based on the data in Table 1 demonstrate that lysosomes degrade a minimum of 6 cellular proteins other than Ub for each ubiquitin destroyed in the organelle. For example, 10^7 HeLa cells contain about 4 mg of protein or about 6×10^9 molecules per cell, assuming a molecular mass of 40,000 for the average HeLa protein. The same HeLa cell contains 2×10^8 Ub molecules [13]. Under step-down conditions endogenous HeLa proteins are degraded at 1.1% per h and Ub_{76} is degraded at 5% per h. Considering the fractional inhibition by chloroquine, one can estimate that lysosomes are responsible for degrading 2.5×10^6 Ub molecules and at least 1.5×10^7 cellular proteins per h. Clearly then, most protein transferred to lysosomes are not conjugated to Ub. Third, the portion of Ub molecules degraded in lysosomes is almost unchanged upon step-down (e.g. 19% in complete MEM vs. 25% in MEM lacking amino acids). These considerations suggest that

the enhanced proteolysis during autophagy does not specifically select for proteins conjugated to Ub.

The enhanced turnover of HeLa proteins under step-down conditions accords well with previous studies using other cell lines or primary cultures [28–31]. However, it was somewhat surprising to find that NH_4Cl and chloroquine were almost completely ineffective inhibitors of proteolysis in HeLa cells grown in complete MEM medium (Table I). The results of Gropper et al. [7] also indicate that lysosomotropic agents had little effect on proteolysis in the Chinese hamster line, E36, or in the mouse line, FM3A, under non-stressful conditions. Since other investigators find at least 30% inhibition of proteolysis after adding lysosomotropic agents to primary cultures [30,31], it appears that in some permanent cell lines lysosomal pathways play a smaller role in overall protein degradation under normal growth conditions.

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