Regulation of lysosomal fusion during deprivation-induced autophagy in perfused rat liver

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Previous studies have demonstrated that autophagic vacuoles induced in hepatocytes of the perfused rat liver can be effectively separated from secondary lysosomes in self-generating gradients of colloidal silica/polyvinylpyrrolidone. Here we show that, as autophagy is increased by graded amino acid deprivation, the lysosomal marker *N*-acetyl- β -D-glucosaminidase shifts progressively from dense to buoyant gradient fractions, an effect attributable to enhanced lysosome-autophagosome fusion and the formation of additional autolysosomes. Both the amount of the shift and the aggregate volume of autolysosomes were directly proportional to the increase in protein degradation above basal. Because the total quantity of protein sequestered in the vacuoles is also a direct function of deprivation-induced proteolysis, the relative specific activity of the marker enzyme in autolysosomes (enrichment) would be expected to remain constant over the full range of deprivation; numerical estimates of enrichment, in fact, did not differ from the mean (43.9) by more than 5%. The close correspondence between cytoplasmic sequestration and fusion was maintained, despite a greater than 3-fold increase in the average volume of individual vacuoles. This suggests the existence of an intrinsic regulatory mechanism which limits the number of fusions per vacuole, on the basis of information derived from vacuolar volume.

INTRODUCTION

Deprivation-induced autophagy is known to play an important role in the turnover of protein and other macromolecules of the cell. In the hepatocyte, the process is initiated by the segregation of discrete portions of cytoplasm within vacuoles known as autophagosomes. Digestion of the internalized cytoplasm then begins when autophagosomes are transformed into autolysosomes, an event triggered by the fusion of the former with secondary lysosomes (Ericsson, 1969). Since the latter are end-products of autophagy, it can readily be appreciated that, with ongoing sequestration, acid hydrolases are continuously recycled between lysosomes and newly formed autophagosomes.

Much has been learned about the quantitative aspects of cytoplasmic sequestration by autophagy in liver (Mortimore & Ward, 1981; Schworer *et al.*, 1981; Hutson & Mortimore, 1982), but there is little comparable information on the associated fusion and its regulation. These previous studies have shown, for example, that the quantity of sequestered protein correlates directly with rates of proteolysis in the intact liver and the breakdown of degradable protein in lysosomal-vacuolar particles over the full range of deprivation. This correlation raises two alternative possibilities regarding the regulation of fusion. First, if the amount of acid hydrolase activity within the vacuoles greatly exceeds the quantity of substrate present, it might mean simply that sequestration is the rate-determining step in proteolysis. Hence, alterations of fusion would not necessarily play a role in its control. Although generally accepted, this notion has not been experimentally tested, presumably because it has not been possible to vary substrate and

enzyme independently. On the other hand, if substrate is not actually limiting, one must consider the alternative, namely that the balance between sequestered protein and its degradation is actively maintained by the regulation of fusion. If true, one would expect to find a close relationship between the aggregate volume of autophagic vacuoles and the amount of associated lysosomal marker over the range of deprivation-induced proteolysis.

In designing experiments to test this possibility, we took advantage of the fact that secondary lysosomes and autophagic vacuoles can be effectively separated in self-generating gradients of colloidal silica/PVP (Surmacz *et al.*, 1983a,b); autophagic vacuoles (autophagosomes and autolysosomes) are largely retained by a group of mixed organelles within the central region of the gradient, whereas secondary lysosomes pass unhindered to the bottom. We show below that, as autophagy is enhanced by graded amino acid depletion during liver perfusion, lysosomal marker shifts progressively from the bottom to centre fractions of the gradient. This fusion-induced transfer was found to correlate directly with the increased rates of proteolysis and aggregate volumes of autophagosomes and autolysosomes over the full range of deprivation. When considered together with related evidence, these findings support the hypothesis that fusion is actively regulated by information derived from vacuolar volume.

EXPERIMENTAL

Liver perfusion

Liver donors were male rats (125–140 g) of the Lewis strain (Microbiological Associates, Walkersville, MD,

Abbreviations used: PVP, polyvinylpyrrolidone or povidone; β -acetylglucosaminidase, *N*-acetyl- β -D-glucosaminidase; a number followed by $\times$ denotes multiples or fractions of the amino acid concentrations in normal plasma.

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U.S.A.) maintained on pelleted laboratory chow and water *ad libitum* in an environmentally controlled room (lights off 19:00–07:00 h). Livers were perfused *in situ* as described by Schworer *et al.* (1981). The procedure was started in the single-pass or non-recirculating mode and continued for 40 min. When protein degradation was determined, flow was immediately switched to a second-stage cyclic perfusion (see below). Alternatively, when liver samples were to be put over gradients, perfusion was stopped at 40 min and the tissue homogenized as described below.

Perfusion medium

The perfusate consisted of Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932), 3% bovine albumin (Fraction V, Pentex; Miles Laboratories), 10 mM-glucose, and freshly washed bovine erythrocytes (27%, v/v) (Schworer *et al.*, 1981). Various amounts of a concentrated stock solution of plasma amino acids in 0.85% NaCl, pH 7.4, were added (Schworer *et al.*, 1981); final perfusate concentrations, which simulated those in normal rat plasma, were the same as values reported previously (Schworer *et al.*, 1981; Pösö *et al.*, 1982).

Protein degradation

The breakdown of long-lived or resident protein was determined in a second-stage cyclic perfusion (see above) from the release of valine in the presence of 18 μ M-cycloheximide (Schworer *et al.*, 1981). Perfusate samples were taken between 5 and 15 min, plasma valine was measured chromatographically (Mortimore & Mondon, 1970), and the rate of proteolysis was calculated by least-squares regression of the total accumulation of free valine in perfusate and liver water, as detailed by Schworer *et al.* (1981). When corrected for small amounts of valine generated from the breakdown of endocytosed protein and short-lived components, the rates of valine accumulation have been shown to provide a valid measure of resident protein degradation existing in the liver at the moment cycloheximide was added (Schworer *et al.*, 1981; Hutson & Mortimore, 1982).

Colloidal-silica/PVP gradients

Livers were excised and homogenized in 9 vol. of ice-cold 0.25 M-sucrose/1 mM-EDTA, pH 7.4 (Neely *et al.*, 1974). Mitochondrial-lysosomal fractions, containing 75–80% of the lysosomal marker, were separated from complete homogenates by differential centrifugation (Surmacz *et al.*, 1983a) and resuspended. The sampling was assumed to be representative of the total lysosomal population, since the small losses of particles by breakage during homogenization and by entrapment in cell debris and unbroken cells within the nuclear fraction are believed to be relatively non-selective (Pertoft *et al.*, 1978; Mortimore & Ward, 1981). Samples (1 ml; 28.4 mg of protein) were layered on a colloidal-silica/PVP medium, containing 8.04% Ludox HS-40 (E. I. du Pont de Nemours), 4% PVP (Thomas) and 0.25 M-sucrose (Schwarz–Mann), pH 7.2, in 2.54 cm $\times$ 8.89 cm Sorvall polyallomer tubes (Surmacz *et al.*, 1983a). PVP appears to be necessary for the effective separation of dense bodies and autophagic vacuoles under these conditions. In a previous study, for example, lysosomal bimodality was not observed in gradients of Percoll alone, but was seen when 4% PVP was added (Surmacz *et al.*, 1983a). The samples were centrifuged at 18000 rev./min for

150 min in a Sorvall RC-5B centrifuge equipped with an SS-90 vertical rotor (Surmacz *et al.*, 1983a). The densities and β -acetylglucosaminidase in the gradient fractions were determined as described previously (Surmacz *et al.*, 1983a).

RESULTS AND DISCUSSION

Association between gradient shifts of β -acetylglucosaminidase and deprivation-induced autophagy

The induction of autophagy by amino acid deprivation is accompanied by a redistribution of the lysosomal marker, β -acetylglucosaminidase, from the lower to central and upper regions of the gradient (Surmacz *et al.*, 1983b), a shift that reflects increased fusion of secondary lysosomes to autophagosomes. In agreement with our earlier findings (Surmacz *et al.*, 1983b), Figs. 1(a) and 1(b) show that stringent amino acid depletion during liver perfusion produced a substantial movement of β -acetylglucosaminidase from fractions 1–6 to the more buoyant central and upper regions. Most of the shifted enzyme activity appeared in fractions 8–15, with a distinct peak in fractions 10–11 associated with a visible central band. Fraction 7 (Fig. 1a) represents the crossover point in the shift, and thus far it has been highly consistent in position. The proportion of total enzyme transferred during maximally induced deprivation ($0 \times$ versus $10 \times$ normal plasma amino acid concentrations) appears to be equally reproducible: 22.9% in our previous report (Surmacz *et al.*, 1983b), 22.4% in the present study.

Despite the ability of the gradient to discriminate between autophagic vacuoles and secondary lysosomes, previous studies have shown that the separation is not complete (Surmacz *et al.*, 1983a,b). Electron-microscopic examination of pellets from the dense and buoyant peaks after the induction of moderate autophagy indicated that 96–97% of the lysosomes were confined to a zone lying adjacent to and below the central band, whereas most of the autophagic vacuoles were within and above the band. A somewhat better quantitative estimate of the distribution was obtained by determining alterations in the sensitivity of lysosomes to osmotic shock (Surmacz *et al.*, 1983b). More than 85% of the enhancement in osmotic sensitivity with autophagy was found in the central fractions, and the remainder was observed in the dense peak. Undoubtedly a few vacuoles passed through the central region in the early stages of centrifugation before the band was consolidated. Because vacuoles formed in livers from fed rats, with normal glycogen contents, are generally heavier than the average lysosome (Schworer *et al.*, 1979), most of the autophagic vacuoles reaching fractions 1–7 would have moved downward and away from the crossover point. This very probably explains, in part at least, the small increase of marker enzyme in fraction 1 that was observed in most of the experiments (Fig. 1a).

The above enzyme shift is not an exclusive feature of autophagy, but would probably occur in any condition associated with lysosomal enlargement or enhanced buoyancy. NH_4^+ and many amines are capable of inducing swelling as a consequence of their intralysosomal accumulation and associated water uptake (Seglen & Reith, 1976; Ohkuma & Poole, 1981). This is illustrated in Fig. 1(d) by the effect of chloroquine. Here

Fig. 1. Effects of amino acid deprivation and chloroquine on the distribution of β -acetylglucosaminidase in gradients of colloidal silica/PVP

In (b), livers from fed rats were perfused in the single-pass mode with $10\times$ (—) or $0\times$ (----) normal plasma amino acids. Particles from lysosome-enriched fractions of the liver were then separated in colloidal-silica/PVP gradients as described in the Experimental section. (a) represents the mean difference in marker-enzyme distribution between the two lower gradients (12–18 observations in each group). In (d), livers from fed rats were perfused as described above with $1\times$ normal plasma amino acids and in the absence (—) or presence (----) of 0.2 mM-chloroquine; the conditions used for particle separation were also similar, and each group comprised five to six experiments. (c) depicts average densities for all the gradients used in Fig. 2 and Table 1.

Table 1. Effects of plasma amino acids on hepatic protein degradation, the gradient distribution of β -acetylglucosaminidase, and calculated pools of lysosomal protein

Experiments were the same as those in Fig. 2. Values in column A were corrected for a nominal 15% loss of vacuole trapping efficiency in the central band (see the text). For total lysosomal protein in column C, degradable protein was first calculated by dividing the rates in column B by the rate constant of vacuole turnover, 0.087 min^{-1} , and expressed as a percentage of the valine content of liver protein, $465\text{ }\mu\text{mol}/100\text{ g rat}$ (Schworer *et al.*, 1981); an estimate of constitutive proteins then was added by multiplying the degradable pool by 1.153 as described in the text. Experimental data are given as means $\pm$ S.E.M., with numbers of experiments in parentheses.

Plasma amino acids	β -Acetylglucosaminidase		Resident protein degradation		Total lysosomal protein (C)	A/C
	(% of gradient activity in fractions 1–7)	(A) [decrease from $10\times$ ($\times 1/0.85$)]	(nmol of valine/min per liver of a 100 g rat)	(B) (increase above $10\times$)		
$0\times$	41.0 ± 0.9 (18)	26.4	328 ± 15 (15)	217	0.618	42.7
$0.5\times$	51.3 ± 3.0 (6)	15.3	240 ± 10 (8)	129	0.368	41.6
$1\times$	56.3 ± 1.5 (9)	9.4	182 ± 11 (11)	71	0.203	46.3
$2\times$	60.9 ± 1.0 (3)	4.0	142 ± 10 (11)	31	0.089	44.9
$4\times$	63.7 ± 1.4 (5)	0.7	124 ± 15 (13)	13	0.037	18.9
$10\times$	64.3 ± 1.3 (12)	0	111 ± 15 (11)	0	0	0

we see a striking, almost complete, transfer of β -acetylglucosaminidase from the dense peak to the centre of the gradient. Most of this shift would have been the direct result of the aforementioned chloroquine and water uptake. However, because proteolysis is inhibited, the resulting decrease in intracellular amino acids would also tend to stimulate autophagy (Furuno *et al.*, 1982; Kovács *et al.*, 1982; Kominami *et al.*, 1983; Glaumann

et al., 1986). But regardless of the mechanism, buoyant shifts generated by lysosomotropic inhibitors can readily be distinguished from primary alterations in autophagy by the fact that protein degradation is decreased rather than increased.

Our primary objective was to examine quantitatively the relationship between deprivation-induced autophagy and the above-mentioned enzyme distribution. Because

Fig. 2. Correlations between the distribution of β -acetylglucosaminidase, accelerated proteolysis, and the fractional cytoplasmic volumes of autophagosomes and autolysosomes over the full range of amino acid deprivation

Livers from fed rats were perfused as described in Fig. 1. (a) Values for the gradient shift of β -acetylglucosaminidase (○) and the increase of resident protein degradation above basal (●) were taken from Table 1 (columns A and B) and expressed as percentages of the respective maxima. (b) Normalized values of the enzyme shift (○) and increases in the fractional cytoplasmic volumes of autophagosomes (△) and autolysosomes (▲) were plotted against corresponding values of accelerated protein degradation. The stereologic data and associated proteolytic rates were taken from Schworer *et al.* (1981).

increases in the aggregate volume of autophagic vacuoles are known to correlate directly with the induction of resident protein breakdown (Schworer *et al.*, 1981), the latter was measured as a means of monitoring the extent of autophagy in the present study. A comparison of these two parameters over the full range of amino acid deprivation is presented in Table 1 (absolute numbers) and in Fig. 2(a) (normalized values). As would be expected from the agreement in Fig. 2(a), the percentage values of the shift in β -acetylglucosaminidase and induced proteolytic responses correlated closely when plotted as a linear regression in Fig. 2(b). For additional comparison, we computed the increases above basal in autophagosomes and autolysosomes, reported previously by Schworer *et al.* (1981), and plotted them as percentages of their respective maxima against the normalized rates of accelerated protein degradation (Fig. 2b). Both the enzyme shift and the aggregate volumes of autophagosomes and autolysosomes correlated well with proteolysis and appeared to be closely related functions.

It is important to point out that both rates of formation of autophagosomes and their rates of fusion with dense bodies attain equilibrium by 40 min of perfusion. We have shown, for example, that the decrease in the aggregate or total fractional volume of secondary lysosomes with maximal induction of autophagy averaged 8.0 (6.3–9.7)% of the respective increase in autophagosomes+autolysosomes in two groups of 40 min perfusions (Mortimore & Schworer, 1977; Schworer *et al.*, 1981) and 8.2 (6.8–9.6)% in a comparable series of 90 min experiments (Schworer *et al.*, 1979). Thus

one may conclude that vacuolar assembly is complete within the duration of experiment employed here.

Structural uniformity of autophagic vacuoles

The proportionality that was observed in Fig. 2(b) between the aggregate volume of autophagic vacuoles and their acquisition of β -acetylglucosaminidase suggests that these vacuoles, despite their apparent heterogeneity, exhibit considerable uniformity in basic structure. With regard to their protein content, we have shown previously that the pool of degradable protein within the lysosomal–vacuolar system correlates directly with the rate of deprivation-induced proteolysis and can be accurately predicted from the latter (Mortimore & Ward, 1981; Hutson & Mortimore, 1982; Mortimore & Pösö, 1984). Total vacuolar protein then would represent this pool plus constitutive proteins acquired from secondary lysosomes by fusion. Although there is no information currently available on the quantity of this contribution, it would probably not be very large. In four groups of perfusion experiments with maximally induced autophagy, the mean decrease in the aggregate volume of secondary lysosomes resulting from fusion was $15.3 \pm 0.7\%$ of the corresponding increase in autophagosomes (Mortimore & Schworer, 1977; Schworer *et al.*, 1979, 1981). If we assume that the concentration of protein in secondary lysosomes is similar to that in autophagosomes and cytoplasm (Schworer *et al.*, 1981), then protein in the constitutive fraction would average only 15.3% more than that in the degradable pool. Values of vacuolar protein content for the present experiments,

calculated from these considerations, are given in Table 1, column C.

As would be expected from results in Fig. 2(b), the ratio of the enzyme shift to vacuolar protein, A/C, in Table 1 was constant over most of the range of deprivation, averaging 43.9 for values at $0\times$, $0.5\times$, $1\times$ and $2\times$ ($4\times$ could not be evaluated satisfactorily because of its proximity to basal turnover). It is of additional interest that this ratio is the same as the relative specific activity (enrichment) of β -acetylglucosaminidase in autophagic vacuoles, defined as the percentage of total liver enzyme activity in autolysosomes divided by the percentage of liver protein in the same compartment. Enrichments of about 38 for the same enzyme were computed from perfusion experiments in which vacuoles were purified after iron-loading (Ward *et al.*, 1977). Because measurements were based on increases in enzyme and protein above basal values, the resulting enrichments would not have been affected by contaminating organelles. However, some decrease was undoubtedly introduced by the presence of a comparatively stable fraction of ferritin associated with iron-containing granules in lysosomes (Drysdale & Munro, 1966; Arborgh *et al.*, 1974). A more pronounced dilution was reported by Marzella *et al.* (1982), who obtained enrichments of 15 in vacuolar fractions isolated after vinblastine treatment. In this instance, the effect was the result of an inhibition of fusion, which greatly increased the proportion of autophagosomes. Since it is unlikely that the actual protein content of autophagic vacuoles is lower than estimates in Table 1, the true value for enrichment probably lies between 38 and 44.

Regulation of fusion during deprivation-induced autophagy

The correlation in Fig. 2(b) between the shift of β -acetylglucosaminidase and increase in the aggregate volume of autophagic vacuoles might be easily explained if the volume change were solely the result of a numerical increase of vacuoles of similar size. Interactions between secondary lysosomes and autophagosomes would very probably remain constant and, in the simplest case, involve the fusion of only one lysosome per vacuole.

Such a mechanism, however, would not appear to operate over the full range of deprivation-induced proteolysis. On the basis of individual cross-sectional measurements, the average volume of autophagosomes has been shown to increase more than 3-fold over the upper two-thirds of the range of deprivation (Schworer *et al.*, 1981). Because constant ratios of particle interaction would not provide the linearity shown in Fig. 2(b), fusion must be regulated in some way by the volume of individual vacuoles. This could be achieved if new autophagosomes are considered immediate targets for multiple fusions. After one or more events, further fusion might then be switched off as a consequence of the cumulative effects of prior fusion, a form of self-regulation. It is known, for example, that loss of the inner membrane of autophagosomes occurs at the time when they are transformed into autolysosomes (Schworer *et al.*, 1981). This, as well as other changes including particle recognition, could reflect pH-dependent alterations of vacuolar proteins and membranes brought about by acquisition of the proton pump and acid hydrolases. Because the alterations would reside within the matrix as

well as the membrane, vacuolar volume would represent an important modulating factor in determining the number of fusions required to stop recognition.

Deter (1975) has suggested that rates of autolysosome formation might be predicted from a standard model used for the kinetic analysis of the random collision of gas particles. However, preliminary tests with the model indicate that it would be applicable only if amino acid deprivation were associated with unrealistically large increases in particle velocity. Despite the fact that fusion, and quite possibly lysosomal movement, can be decreased by microtubule inhibitors (Marzella *et al.*, 1982; Kovács *et al.*, 1985), to our knowledge there is no evidence that the velocity of cytoplasmic particles is under physiological control. Moreover, any change in the rate of fusion would probably effect the ratio of autophagosomes to autolysosomes, an alteration not associated with amino acid deprivation itself (Schworer *et al.*, 1981).

The advantage of regulating fusion on the basis of the volume of individual vacuoles is that a balance can be maintained between the diversity of internalized cytoplasmic macromolecules and the variety of enzymes required to digest them. It is reasonable to assume that the requisite acid hydrolases are well distributed throughout the lysosomal-vacuolar apparatus, since the total pool of lysosomal β -acetylglucosaminidase is believed to recycle through autophagic vacuoles once every 30 min during maximally induced deprivation (G. E. Mortimore, unpublished work).

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