CONCERNING A POSSIBLE MECHANISM FOR SELECTIVE CAPTURE OF CYTOPLASMIC PROTEINS BY LYSOSOMES

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SUMMARY

Lysosomes seem to be major agents of degradation of intracellular proteins. There is normally little release of intact proteins from lysosomes to cytoplasm, nor accumulation within lysosomes. As the half-lives of cytoplasmic proteins are heterogeneous, their rates of degradation by lysosomes are probably determined by their rates of entry. Therefore, a mechanism for selective uptake of cytoplasmic proteins seems likely. It is suggested that proteins which adsorb to the membranes forming autophagic vacuoles may enter selectively by analogy with the process of adsorptive pinocytosis. Evidence for selective adsorption of rapidly-turning over cytoplasmic proteins to the external membranes of lysosomes, and to lipsomes, consistent with this hypothesis, is presented.

It has been known for some time that lysosomes are responsible for the degradation of endocytosed proteins (1-3). In addition, direct evidence for their involvement in turnover of intracellular proteins has recently been obtained (4). It seems likely that entry of proteins into lysosomes is the rate limiting step in lysosomal protein digestion (2,5-7); nevertheless, the documented mechanisms for uptake of cytoplasmic proteins by lysosomes, the variants of autophagy (1,8), apparently result in non-selective bulk uptake of cytosol material, although the half-lives of cytoplasmic proteins are heterogeneous (9). Therefore, a process conferring selectivity of entry into lysosomes is to be expected.

During endocytosis by cells, some soluble components of the external medium do enter at relative rates which are at variance with their relative concentrations (6,7). This is thought to reflect varying degrees of binding to the membrane forming the endocytic vesicle (6,7), and the kinetic consequences of fluid phase and membrane phase uptake have been analysed (10). The form of autophagy in which invagination of the lysosomal membrane occurs, producing a lysosome with two membranes and containing cytosol (8) is analogous to endocytosis, and the other sequestration mechanisms are quite similar (1). Thus rates of turnover of cytoplasmic proteins may depend on the extent to which they bind to the external membrane of the lysosome, and to other membranes involved in

autophagy. The experiments described here subject this hypothesis to an initial test.

Methods

Rat liver cytoplasmic proteins were labelled *in vivo* successively with [^{14}C] - leucine and [^{3}H] - leucine (quantities of isotope varied) at the intervals described previously (11), so that rapidly turning over proteins have high ratios of [^{3}H]/[^{14}C] radioactivity while those turning over slowly have low ratios (12). The cytoplasmic proteins were prepared as described previously (13) and dialysed against 20 vol. 10mM - potassium phosphate buffer, pH 7.0, to remove free amino acids and small peptides. Rat liver lysosomes (non-radioactive) containing Triton WR - 1339 ("Tritosones") were obtained by the method of Wattiaux et al. (13,14)and incubated briefly with the cytoplasmic proteins (5 min, 37^{o}C), under several different conditions, (see Table 1), each giving good osmotic protection to lysosomes. The radioactivity bound to the intact lysosomes was determined by centrifuging (40,000 g x 5 min, Sorvall SE 12 rotor) the lysosomes through a layer of 0.4 - sucrose (low ionic strength adsorptions) or 0.15M sucrose containing 0.15 M -NaCl and 1.0 µM $CaCl_2$ (ionic adsorptions). and dissolving the pellets in 1 ml of HCOOH for scintillation counting. It has previously been found that under such *in vitro* conditions no internalisation of material by the tritosomes occurs (13).

SDS-gel electrophoresis (15) and isoelectric focusing (16) were performed by previously described methods. 2mm gel segments were dissolved in H_2O_2 for scintillation counting. Liposomes (20 µmoles phosphatidylcholine, 20 µmoles cholesterol, 10 µmoles phosphatidic acid) were prepared in 2ml 0.9% NaCl, as described preciously (4).

Results and Discussion

Substantial binding was observed in low ionic strength media, and the [^{3}H]/[^{14}C] ratio of bound material was slightly higher than that of the starting material. Thus there was preferential binding of rapidly turning over proteins. At physiological ionic strength this preferential binding was much more marked (Table 1).

The bound proteins from the latter adsorptions showed a correlation between subunit size and isotope ratio when analysed by SDS-gel electrophoresis, with large subunits having high ratios, as observed previously for proteins from many cellular sources, including cytoplasmic proteins (9). When the [^{3}H] or [^{14}C] radioactivity in each size range from the absorbed material was expressed as a fraction of the available [^{3}H] or [^{14}C] radioactivity in the cytoplasmic protein sample in the same size range, rough correlations between fraction taken up and subunit size were obtained; larger fractions of the radioactivities of large subunit materials were bound. Thus the increased isotope ratio was due to a selective binding of large subunit proteins, which themselves had high [^{3}H]/[^{14}C] ratios. This binding process may be responsible for the varied degradation rates of cytoplasmic proteins, and perhaps be modulated by ligand-induced conformational and dissociative changes in

TABLE I

Binding of cytoplasmic proteins to the outer surface of "intact" tritosomes

	Total Radioactivities (d.p.m.)		$[^3H]/[^{14}C]$ ratio
	$[^3H]$	$[^{14}C]$	(factorial increase over cytoplasmic proteins in parentheses)
Cytoplasmic proteins used	1,340,840	170,360	7.87
Material retained by tritosomes at low ionic strength	15,470	1,900	8.14 (1.03)
Material retained by tritosomes at physiological ionic strength	12,600	1,020	12.35 (1.56)

Tritosomes (3mg protein) were incubated at 37°C, for 5 min., with cytoplasmic proteins (200 mg, labelled in vivo). Both materials were adjusted to 0.25 M - sucrose before mixing for the low ionic strength adsorption (final volume: 25 Ml). For the adsorption at physiological ionic strength, NaCl (0.15M final) and $CaCl_2$ (1μM final) were added to each, and sucrose was diluted to approx. 50mM in the final mixture (volume 25ml). Similar results were obtained in two other experiments with different preparations of tritosomes and cytoplasmic proteins: one such repeat is included in Table 2.

cytoplasmic proteins, such as are known to affect rates of turnover of individual proteins (9).

The mechanism of binding is not clear. A gross electrostatic basis seems unlikely for two reasons. Firstly, specificity was greater in the presence of 0.15M - NaCl, which should suppress such interactions. Secondly, Goldberg has recently shown that isoelectric point is a determinant of turnover rates, proteins which turnover rapidly tending to have low isoelectric points (17); and this was confirmed for the rat liver cytoplasmic proteins, by demonstrating the correlation between isotope ratio and isoelectric point. At pH 7 therefore, proteins of high $[^3H]/[^{14}C]$ would be mainly negatively charged, and thus repelled by the negative tritosome surface (18).

Bohley and collaborators have suggested that denaturability may be correlated with turnover rate, and obtained some evidence for this (17). However, when rat liver cytosol proteins were denatured by various means (Table 2), the precipitated material (presumably highly denatured)

TABLE 2

Denaturation of cytoplasmic proteins, and adsorption of denatured and of native proteins by "intact" tritosomes at physiological ionic strength.

Denaturation	Radioactivity (d.p.m./ml used)		$[^3H]/[^{14}C]$ ratio (Factor of increase over cytoplasmic proteins used in parentheses)
	$[^3H]$	$[^{14}C]$	
Native cytoplasmic proteins (A)	134,084	17,036	7.87
Native cytoplasmic proteins (A) adsorbed to tritosomes	6,546	484	13.50(1.71)
Material precipitated by SDS treatment (I) of (A)	81,204	9,121	8.90 (1.13)
Material precipitated by pH-treatment (2) of (A)	130,977	16,577	7.90 (1.00)
Native cytoplasmic proteins (B)	52,615	28,365	1.84
Material precipitated by freezing and thawing (3) cytoplasmic proteins (B)	16,465	9,904	1.66 (0.90)
Adsorption	d.p.m. total used		
	$[^3H]$	$[^{14}C]$	
Material soluble after SDS-treatment (1) of (A)	158,580	23,740	6.67
Material soluble after SDS-treatment of (A) adsorbed to tritosomes	71,307	10,515	6.78 (1.02)
Material soluble after pH treatment of (A)	27,963	4,131	6.76 (1.01)
Material soluble after pH treatment of (A), adsorbed to tritosomes	3,807	498	7.64 (1.14)

Cytoplasmic proteins were denatured: 1) by addition of SDS (sodium dodecyl sulphate) to 1%, and dialysis against 10mM - Tris - HCl pH 7.8, 50 vol, for 24 h ; or 2) by titration (with conc. HCl) to pH 1.5, and retitration with 2M - Tris - HCL, pH 9.0, to pH 7.0 after 15 min; or 3) by freezing, storing at - 20°C for 7 days, and thawing. This denaturation was presumably due to pH changes during freezing, as well as to freezing per se. After denaturation, precipitated material was removed by centrifugation (10,000 g x15 min) and the supernatants sampled for scintillation counting, and used for adsorptions. All adsorbtions were under ionic conditions (as in Table 1) and contained 40mg cytoplasmic protein (in the case of native proteins), or material soluble either after SDS-treatment of 60mg of cytoplasmic protein, or after pH treatment of 180 mg of cytoplasmic proteins. 2.5 mg tritosomal protein was used in each case.

was hardly or not at all elevated in $[^3H]/[^{14}C]$ ratio. The proteins which remained soluble (probably also denatured to some extent) were adsorbed on tritosomes: there was little selectivity of binding (Table 2). Thus violent denaturation prevents specific uptake; nevertheless, susceptibility to mild denaturation or to submit dissociation, perhaps actually on the membrane surface, may well determine the degree of

TABLE 3

Surface binding of cytoplasmic proteins by multilamellar liposomes.

	Total d.p.m.		
	[^{3}H]	[^{14}C]	[^{3}H]/[^{14}C] ratio (factorial increase overstarting material in parentheses)
Cytoplasmic protein sample	361,500	184,610	1.96
Material adsorbed to liposomes	12,446	3,611	3.45 (1.76)

The adsorptions were performed three times with the cytoplasmic protein sample illustrated here, and twice with a different preparation: all gave similar results. Liposomes (as in methods) and cytoplasmic proteins (140mg) were incubated (5 min, 37°C) in 0.15 M - NaCl, 1μM-$CaCl_2$ (final concentrations, in a volume of 1.5 ml). Subsequently they were sedimented at 100,000 g, 30 min, 4°C, and washed once with 0.15M - NaCl, 1 μM $CaCl_2$. The resedimented pellet was dissolved for scintillation counting as before.

retention of individual proteins on the membrane. Other factors such as detree of surface hydrophobicity (17) may also be important.

Although it has been argued forcibly (8) that lysosomal invagination is the main route of entry of cytoplasmic proteins of liver into the lysosomal system, endoplasmic reticulum membranes may also contribute to autophagy of cytoplasm. Therefore specificity of binding on several membranes may be required. That this is feasible is suggested by experiments on adsorption by negatively charged multilamellar liposomes (Table 3), consisting of small numbers of concentric lipid bilayers with entrapped aqueous phases (19). Selective retention of fast turning over proteins was observed.

The results support the possibility that binding of cytoplasmic proteins to the surfaces of membranes forming autophagic vacuoles may allow selective entry of cytosol proteins into the lysosomal system, and thus be a determinant of turnover rate of such proteins. More subtle tests should now be applied, in conjunction with the further study of *in vitro* internalisation by lysosomes (13,20,21).

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