Vol. 151, No. 1, 1988
February 29, 1988
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 40-47

PRELYSOSOMAL CONVERGENCE OF AUTOPHAGIC AND ENDOCYTIC PATHWAYS

Paul B. Gordon and Per O. Seglen

Department of Tissue Culture, Institute for Cancer Research,
The Norwegian Radium Hospital, Montebello, 0310 Oslo 3, Norway

Received January 4, 1988

[^{14}C]Lactose, electroinjected into the cytosol of isolated rat hepatocytes, was sequestered by autophagy, transferred to lysosomes and eventually hydrolysed. Asparagine prevented the fusion between prelysosomal autophagic vacuoles and lysosomes, and caused lactose to accumulate in the former. However, if the hepatocytes were simultaneously allowed to endocytose added β-galactosidase, no lactose accumulation occurred. These results suggest that autophagically sequestered lactose and endocytosed β-galactosidase were delivered to the same prelysosomal vacuole, where the lactose was hydrolysed by the enzyme. The name amphisome is suggested for this new functional compartment, common to the autophagic and endocytic pathways.

Electroinjection of radioactive sugars into isolated hepatocytes has proved to be a useful technique for investigation and characterization of the autophagic-lysosomal pathway (1,2). Inert sugars like [^{14}C]sucrose and [^{3}H]raffinose are sequestered autophagically along with proteins and other cytoplasmic components, and can be used as probes of autophagic sequestration, the first step in the pathway (3,4). [^{14}C]Lactose, on the other hand, is rapidly hydrolysed by lysosomal β-galactosidase once it enters the lysosomes and can, therefore, be used to investigate post-sequestrational events (5).

In previous work, we showed that autophagically sequestered sucrose, accumulating in lysosomes, could be hydrolysed upon endocytosis of added invertase, a sucrose-cleaving enzyme (2). Pre-accumulated lysosomal sucrose was completely hydrolysed even in the absence of ongoing autophagy, indicating that invertase-loaded endosomes could fuse directly with all lysosomes that had received autophagic material (6). Convergence of the autophagic

0006-291X/88 $1.50

and endocytic pathways at the lysosomal level could therefore be inferred (2,6).

However, the above results did not exclude the possibility of a convergence of these pathways even at the prelysosomal level. The latter possibility could be explored by taking advantage of the rapid intralysosomal hydrolysis of autophagically sequestered lactose (5). Agents like vinblastine, which blocks the fusion of lysosomes with endosomes (7) as well as with autophagic vacuoles (8), or asparagine, which specifically inhibits the latter process (9), both cause an accumulation of undegraded lactose in prelysosomal autophagic vacuoles (5,10), thereby providing a marker for that compartment. In the present report we show that such prelysosomal lactose can be hydrolyzed by an endocytosed lactose-degrading enzyme, β-galactosidase. This indicates that the endocytic and autophagic pathways can meet even at the prelysosomal level.

MATERIALS AND METHODS

[^{14}C]Lactose (57 mCi/mmol; 200 μCi/ml) was purchased from Amersham Int. plc., Bucks, U.K.; Metrizamide was from Nycomed, Oslo, Norway; and all other biochemicals from Sigma Chemical Co., St. Louis, Mo, USA.

Hepatocytes were isolated from the liver of 18-h starved male Wistar rats, approx. 250 g, by collagenase perfusion (11). During experimental incubations cells were incubated as 0.4 ml aliquots in suspension buffer (11) fortified with 15 mM pyruvate, in 15 ml glass centrifuge tubes shaking at 215 rpm for up to 120 min at 37^{0}C.

Cells were electroloaded with [^{14}C]lactose essentially as described in detail previously (1,12). Routinely, 15 x 2 ml aliquots of cell suspension were given 5 pulses from a 1.2 μF capacitor at 2 kV/cm (1 cm^{2} chamber, 5 cm high), pooled, concentrated to approx. 15 ml, and 5 μCi/ml [^{14}C]lactose added. After 1 h on ice, cells were resealed by incubation at 37^{0}C on a tilting platform (30 min at 10 rpm), and then washed three times in ice-cold wash buffer (11) to remove extracellular isotope.

Following experimental incubation, cells were electrodisrupted and centrifuged through Metrizamide cushions (to separate cell corpses containing organelle-associated lactose from the remaining cytosolic sugar) and treated with digitonin (to selectively extract autophagically sequestered lactose) as described in detail elsewhere (12,13).

RESULTS AND DISCUSSION

Asparagine causes accumulation of [^{14}C]lactose in an autophagic compartment which is accessible to endocytosed β-galactosidase.

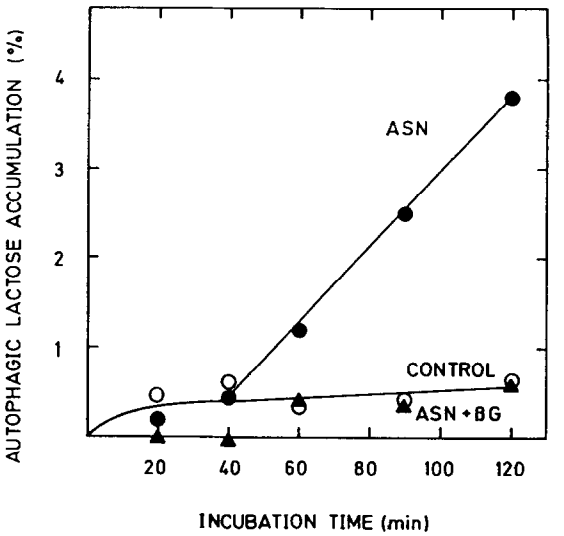

Fig. 1. Effects of asparagine and endocytosed β-galactosidase on prelysosomal accumulation of autophagically sequestered lactose. Hepatocytes were electroloaded with [^{14}C]lactose and incubated at 37° C without additions (○); with 25 mM asparagine (ASN) (●); or with asparagine plus β-galactosidase (βG), 250 units/ml (▲). At the indicated time points, cell samples were electrodisrupted and the amount of digitonin-extractable radioactivity in sedimentable organelles measured (0 min background subtracted). Each value is the mean of three cell samples, expressed as % of the total radioactivity in intact cells at that time point.

In previous work, summarized in ref. 10, we demonstrated that the strong inhibition of autophagic-lysosomal protein degradation by high concentrations of asparagine was due more to an inhibition of fusion between autophagic vacuoles and lysosomes than to an inhibition of autophagic sequestration. This amino acid would not appear to inhibit endosome-lysosome fusion, since it did not interfere with the degradation of endocytosed protein (14). Asparagine may therefore be regarded as a post-sequestrational, prelysosomal inhibitor of autophagic degradation. This effect of asparagine has been investigated further and found to be maximal at 25 mM (unpublished results).

Following electroloading with [^{14}C]lactose, hepatocytes were incubated for various time periods (0-120 min) in the absence or presence of asparagine (25 mM), plus or minus β-galactosidase (approx. 250 units/ml). In control hepatocytes, autophagically sequestered [^{14}C]lactose was, as previously observed (5), rapidly hydrolysed in the lysosomes and only a low, steady-state level was maintained – presumably in a prelysosomal, autophagic compartment (Fig. 1). Addition of asparagine caused, after a brief lag, a considerable accumulation of sequestered

Table 1. Vinblastine protects prelysosomal lactose against degradation by endocytosed β-galactosidase

	Accumulation of autophagically sequestered [^{14}C]lactose (%)	
	No β-galactosidase	+ β-galactosidase
Control	0.46 ± 0.28 (4)	0.41 ± 0.31 (4)
Asparagine	4.16 ± 0.30 (4)	0.44 ± 0.22 (4)
Vinblastine	5.05 ± 0.44 (4)	6.32 ± 0.25 (4)

Hepatocytes electroloaded with [^{14}C]lactose were incubated for 2 h at 37^{0}C without additions, or in the presence of asparagine (25 mM), vinblastine (0.05 mM) and/or β-galactosidase (250 units/ml) as indicated. The amount of digitonin-extractable radioactivity in sedimentable organelles at 2 h (0 h value subtracted) was measured and expressed as % of the total radioactivity in intact cells at that time. Each value is the mean ± S.E. of four experiments.

lactose, indicating that entry of the sugar into lysosomes and its hydrolysis by the lysosomal β-galactosidase was prevented. However, when exogenous β-galactosidase was added to the system in addition to asparagine, the accumulation of lactose was completely abolished (Fig. 1). Apparently, β-galactosidase, ingested by endocytosis, was able to reach the lactose-accumulating compartment and degrade the lactose under these conditions. Convergence of the endocytic and autophagic pathways at a prelysosomal level would, therefore, seem to be indicated.

It is noteworthy that the basal steady-state level of sequestered lactose was unaffected by the addition of β-galactosidase (Fig. 1 and Table 1). Most of this steady-state lactose would thus seem to be present in a compartment which was not reached by endocytosed enzyme. The initial sequestering vacuoles, the autophagosomes, may represent such a compartment.

Entry of endocytosed β-galactosidase into the prelysosomal autophagic compartment is prevented by vinblastine.

Like asparagine, vinblastine prevents the entry of sequestered [^{14}C]lactose into lysosomes and causes its accumulation in a prelysosomal autophagic compartment (5). However, lactose accumulation in the presence of vinblastine (0.05 mM) was not affected by endocytosis of β-galactosidase (Table 1). Unlike asparagine, vinblastine apparently blocked

the access of endocytosed enzyme to the lactose-accumulating autophagic compartment. The ability of vinblastine to inhibit fusion – as evidenced by it causing accumulation of lactose (5), as well as by its inhibitory effect on endosome-lysosome fusion (7,8) – would therefore appear to extend to the fusion of endosomes with prelysosomal autophagic vacuoles.

The experiments described were performed in the presence of continuous autophagic sequestration. The possibility would, therefore, have to be considered that endosomes filled with β-galactosidase might be sequestered into the same autophagosome as [^{14}C]lactose, thereby (following endosome rupture) allowing contact between the enzyme and the substrate without any real, functional convergence of pathways. However, the ability of vinblastine to completely prevent such contact would seem to rule out that possibility, since, at the concentration used, vinblastine has very little effect on sequestration (5). Furthermore, analogous experiments with autophagically sequestered sucrose and endocytosed invertase (6), have shown that sugar pre-accumulated in the prelysosomal compartment is reached by endocytosed enzyme even in the absence of autophagy, but not in the presence of vinblastine (unpublished experiments). It would therefore seem most likely that the autophagic and endocytic pathways meet by a vinblastine-sensitive fusion process.

In the light of recent evidence linking the movements of endosomes and lysosomes to an intact microtubular network (15), it is tempting to interpret the vinblastine effect in terms of a perturbation of microtubular function. It should be noted, however, that the concentration of vinblastine needed to block lactose degradation is considerably higher than the concentrations usually employed to effect microtubular depolymerization.

Prelysosomal meeting place of autophagic and endocytic pathways: the amphisome

The ability of endocytosed β-galactosidase to degrade autophagically sequestered [^{14}C]lactose under conditions where the endogenous lysosomal β-galactosidase does not, provides strong evidence for a prelysosomal site of degradation. The lactose accumulation which is affected is known to take place in

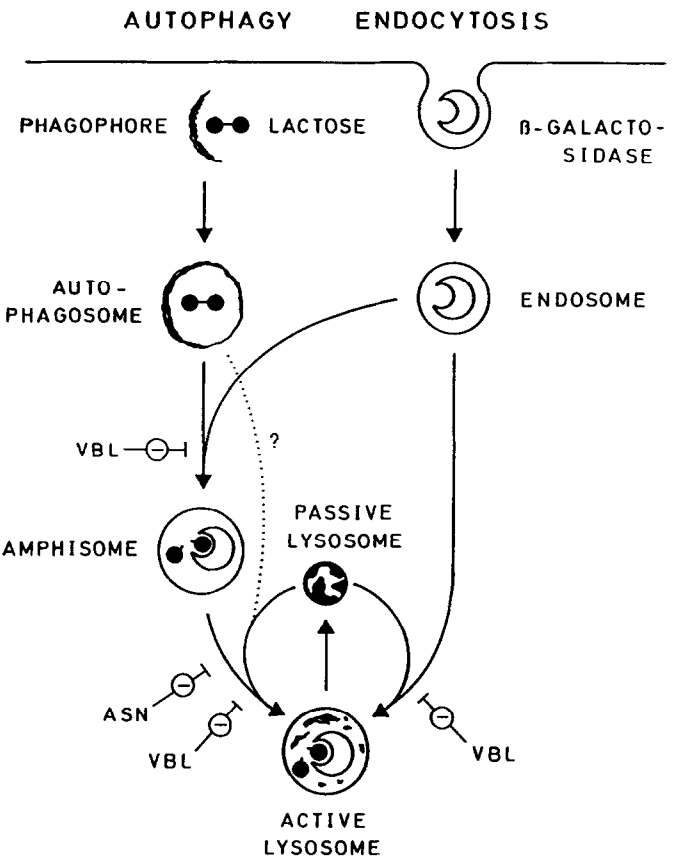

Fig. 2. Prelysosomal and lysosomal convergence of the autophagic and endosomal pathways. ASN, asparagine; VBL, vinblastine.

sedimentable, digitonin-extractable vacuoles, and is prevented by the autophagy inhibitor 3-methyladenine (5); it is, therefore, clear that the lactose must be in some kind of autophagic vacuole. The only known prelysosomal vacuole of this kind is the autophagosome, usually defined as the initial vacuole formed upon sequestration (10). By definition, autophagosomes would not yet have engaged in any other activity such as, for example, fusion with endosomes or lysosomes; the presence of both endocytosed and autophagocytosed material in the same prelysosomal vacuole would, therefore, delineate a new functional compartment. We propose the name amphisome for this compartment, and define it as a non-lysosomal vacuolar organelle which does not contain the lysosomal marker enzyme β-galactosidase, but which receives and mixes material both from endocytosis and from autophagic sequestration.

It is most likely that amphisomes are generated upon fusion of autophagosomes with endosomes (Fig. 2). The fact that the

amphisome provides a functional environment for an enzyme with such a low pH optimum as β-galactosidase (5) suggests that this organelle has an acidic interior. The acidity could well be provided by the endosomal partner, since it is firmly established that endosomes are acidic (16), due to a proton pump which lowers the intravacuolar pH within minutes after endocytic internalization has taken place (17).

Since lactose accumulation is totally abolished by endocytosed β-galactosidase, it is evident that all sequestered lactose enters amphisomes, at least when fusion with lysosomes is blocked. However, it is not clear to what extent amphisome formation is an obligatory step in the autophagic pathway under normal conditions. Endosomes can fuse directly with lysosomes in the absence of autophagy (6); whether autophagosomes can, similarly, fuse directly with lysosomes, independently of endocytosis, remains to be investigated.

ACKNOWLEDGEMENT

This project is generously supported by the Norwegian Cancer Society.

REFERENCES

1. Gordon, P.B., and Seglen, P.O. (1982) Exp. Cell Res., 142, 1-14.
2. Seglen, P.O., Gordon, P.B., and Høyvik, H. (1986) Biomed. Biochim. Acta, 45, 1647-1656.
3. Gordon, P.B., Tolleshaug, H., and Seglen, P.O. (1985) Exp. Cell Res., 160, 449-458.
4. Seglen, P.O., Gordon, P.B., Tolleshaug, H., and Høyvik,H, (1986) Exp. Cell Res., 162, 273-277.
5. Høyvik, H., Gordon, P.B., and Seglen, P.O. (1986) Exp. Cell Res., 166, 1-14.
6. Høyvik, H., Gordon, P.B., and Seglen, P.O. (1987) Biochem. Soc. Trans., 15, 964-965.
7. Oka, J.A., and Weigel, P.H. (1983) Biochim. Biophys. Acta, 763, 368-376.
8. Kovacs, A.L., Reith, A., and Seglen, P.O. (1982) Exp. Cell Res., 137, 197-201.
9. Grinde, B., and Seglen, P.O. (1981) Exp. Cell Res., 134, 33-39.
10. Seglen, P.O. (1987) in Lysosomes: Their Role in Protein Breakdown, (Glaumann,H., and Ballard,F.J., eds.) pp 371-414, Academic Press, London.
11. Seglen, P.O. (1976) Meth. Cell Biol., 13, 29-83.
12. Seglen, P.O., and Gordon, P.B. (1984) J. Cell Biol., 99, 435-444.
13. Gordon, P.B., Tolleshaug, H., and Seglen, P.O. (1987) Biochem. J., 232. 773-780.

14. Seglen, P.O., Gordon, P.B., and Poli, A. (1980) Biochim. Biophys. Acta, 630, 103-118.
15. Matteoni, R., and Kreis, T.E. (1987) J. Cell Biol., 105, 1253-1265.
16. Tycko, B., and Maxfield, F.R. (1982) Cell, 28, 643-651.
17. Mellman, I., Fuchs, R., and Helenius, A. (1986) Ann. Rev. Biochem., 55, 663-700.