Autophagosome-associated variant isoforms of cytosolic enzymes

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In a search for autophagosome-associated proteins, two-dimensional gel separations of proteins from purified autophagosomes, postnuclear supernatant, cytosol, lysosomes, mitochondria, endosomes and a cytomembrane fraction (mostly endoplasmic reticulum) were compared. Three proteins, with monomeric molecular masses of 43, 35 and 31 kDa, were enriched in total or sedimentable fractions of autophagosomes relative to the corresponding fractions of postnuclear supernatant, suggesting an association with the autophagosomal delimiting membrane. These proteins were also present on lysosomal membranes, but they were absent from mitochondria, and detected only in small amounts in the cytomembrane fraction and in endosomes, indicating that they were not associated with organelles sequestered by autophagy. However, all three proteins were present in the cytosol, suggesting that they were cytosolic proteins binding peripherally to the delimiting membrane of autophagosomes, probably to its innermost surface as indicated by their resistance to treatment of intact autophagosomes with proteinase or protein-stripping agents. Amino acid sequencing identified these proteins as an isoform of argininosuccinate synthase, an N-truncated variant of glyceraldehyde-3-phosphate dehydrogenase, and a sequence variant of short-chain 2-enoyl-CoA hydratase.

Key words: argininosuccinate synthase, autophagy, enoyl-CoA hydratase, glyceraldehyde-3-phosphate dehydrogenase, lysosome.

INTRODUCTION

Autophagy is the major pathway by which cells degrade their cytoplasmic macromolecules in response to changes in the nutritional status of the cell (reviewed in [1]). In the initial sequestration step of the process, cytoplasmic components become enclosed by double or multiple membranes, forming vacuoles known as autophagosomes. Autophagosomes can fuse with endosomes to form amphisomes [2]; the amphisomes (and possibly the autophagosomes) eventually fuse with lysosomes, whereupon the sequestered macromolecules (proteins, lipids and polysaccharides) are degraded by the lysosomal enzymes [3]. The degradation products are reused as energy substrates or metabolic building materials. This cellular pathway has important roles in the control of metabolic homoeostasis, cellular growth and protein metabolism. Under certain conditions, such as during amino acid starvation, autophagy can account for 70–80 % of the total intracellular protein degradation [4].

The gross morphological (ultrastructural) features of autophagy have been reasonably well outlined [4]. However, the nature and origin of the sequestering membrane, which forms the wall of the autophagosome, are poorly understood, despite repeated attempts to characterize it [5–9]. The presence of certain rough endoplasmic reticulum (rough ER) markers in the autophagosomal delimiting membrane has been reported [7], but a recent study of purified liver autophagosomes revealed no enrichment in ER markers [10]. Although it is likely that the autophagic sequestering membrane, like other cytomembranes, is ultimately derived from the ER, it seems to have sufficiently distinct properties to be regarded as a unique organelle, called a phagophore [4].

Considerable progress has been made during recent years in identifying signalling mechanisms that participate in the control of autophagy. In addition to the regulation by amino acids [4], there is now good evidence for an involvement of protein phosphorylation [11], cyclic nucleotides [12], AMP [13], GTP and trimeric GTPases [14], and lipid kinases and phosphatidylinositides [15]. In yeast, in which the autophagic process shares many similarities with autophagy in mammalian cells [16,17], several genes have been identified as being essential for autophagy [18]. These include a Ser/Thr protein kinase [19], a phosphofructokinase subunit [20] and a novel ubiquitin-like protein conjugation system [21,22], but none of the proteins identified so far have been shown to be associated with autophagosomes or to be directly involved in the autophagic sequestration process.

In the present study we have characterized the protein composition of purified rat liver autophagosomes by two-dimensional (2D) gel electrophoresis. The results suggest that the autophagosomal delimiting membrane is virtually devoid of organelle-specific transmembrane proteins. However, three identifiable cytosolic proteins associated with autophagosomal membranes with some selectivity: these were variant forms of argininosuccinate synthase (ASS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and short-chain 2-enoyl-CoA hydratase (SCEH).

MATERIALS AND METHODS

Biochemicals

Nycodenz and iodixanol (Optiprep®) were purchased from Nycomed Pharma AS (Oslo, Norway). Percoll was from Pharmacia AB (Uppsala, Sweden). Anti-(superoxide dismutase) antibody was a gift from Dr Ling-Yi Chang (Durham, NC, U.S.A.).

Abbreviations used: 2D, two-dimensional; ASS, argininosuccinate synthase; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPN, glycyl-L-phenylalanine 2-naphthylamide; GRP78, glucose-regulated protein of 78 kDa; LDH, lactate dehydrogenase; PNS, postnuclear supernatant; SCEH, short-chain 2-enoyl-CoA hydratase; aASS, aGAPDH and aSCEH represent autophagosome-associated variants of the respective enzymes.

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Antibodies against protein disulphide isomerase and glucose-regulated protein of 78 kDa (GRP78) were from Affinity Bio Reagents (Golden, CO, U.S.A.). Anti-(lactate dehydrogenase) (anti-LDH) antibody was from Chemicon International (Temecula, CA, U.S.A.). All other biochemicals, including vinblastine, Amido Black, cytochrome *c* and a protease inhibitor cocktail containing 4-(2-aminoethyl)benzenesulphonyl fluoride, bestatin, pepstatin A, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) and phosphoramidon, were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell preparation and incubation

Hepatocytes were prepared from 18 h starved male Wistar rats (250–300 g) by a two-step collagenase perfusion [23]. The cells were incubated (as 0.4 ml aliquots in shaking centrifuge tubes, 70–80 mg/ml wet mass) for 2 h at 37 °C in suspension buffer [23] fortified with pyruvate (20 mM) and Mg^{2+} (2 mM). Vinblastine (50 μM) or leupeptin (0.3 mM) was added to accumulate autophagosomes or amphisomes respectively; for isolation of lysosomes, the autophagy inhibitor 3-methyladenine (10 mM) was included along with vinblastine [2,10].

Subcellular fractionation

Purification of autophagosomes, lysosomes, mitochondria and a cytomembrane fraction (mostly ER) from electrodisrupted hepatocytes [24] was performed (at 0–4 °C) as described previously [2,10] with minor modifications. After homogenization of the disrupted mixture, the nuclei were removed by centrifugation for 2 min at 4000 rev./min (2000 ***g***). For purification of autophagosomes, the postnuclear supernatant (PNS) from cells treated with vinblastine (50 μM) was incubated with 0.5 mM glycyl-L-phenylalanine 2-naphthylamide (GPN) and 0.33 % (v/v) DMSO (GPN solvent) for 6 min at 37 °C to disrupt the lysosomes [25]. For the preparation of lysosomes, the cells were pretreated with vinblastine (50 μM) and the autophagy inhibitor 3-methyladenine (10 mM); the GPN step was omitted.

Mitochondria were recovered from the heavy Nycodenz fraction, and cytomembranes from the upper interface of the Percoll gradient [10]. Cytosol was prepared from PNS, by centrifugation for 22 min at 40000 rev./min (285000 ***g***). For purification of endosomes, 32 ml of PNS was centrifuged for 9 min at 13500 rev./min (21780 ***g***) in the SS34 rotor of a Sorvall RC5B centrifuge to sediment mitochondria, peroxisomes, lysosomes and autophagosomes. In each of two tubes, 15 ml of the supernatant was layered on top of 20 ml of metrizamide/sucrose [8 % (w/v) metrizamide/50 mM potassium phosphate/1 mM dithiothreitol/1 mM EDTA/0.05 % sucrose (pH 7.5)] and centrifuged at 20000 rev./min (47800 ***g***) for 60 min in an SS34 rotor to remove cytosol. Each cytosol-free pellet was resuspended in 9 ml of homogenization buffer [10], of which 7 ml was layered on top of a three-layered discontinuous sucrose gradient [14 ml of 15 % (w/v) sucrose, 12 ml of 37 % sucrose and 4 ml of 48 % sucrose] and centrifuged for 6 h in an SW28 rotor at 28000 rpm (141000 ***g***). The band at the upper interface was used as an endosome preparation; it was found to be highly enriched in radioiodinated tyramine-cellobiose-asialo-orosomucoid when hepatocytes had been allowed to take up this endocytic marker during preincubation at 37 °C for 25 min.

For disruption of organelles and preparation of sedimentable fractions, isotonic fractions of purified autophagosomes (1.4 mg/ml protein), mitochondria (1.7 mg/ml protein), lysosomes (0.7 mg/ml protein), endosomes (0.3 mg/ml protein), cytomembranes (1.4 mg/ml protein) or PNS (7.0 mg/ml protein) were mixed with three parts of hypotonic potassium phosphate buffer (10 mM) in an Eppendorf tube and frozen (for 5 min) to −70 °C in isopentane/solid CO_2. After thawing at 37 °C, the tube was mixed with a Whirlimixer for a few seconds and centrifuged for 2 h at 20000 rev./min (47800 ***g***). The quantity of organelles disrupted was adjusted to obtain 0.2 mg of protein in the final sediment (calculated as the difference between the measured protein concentrations in the supernatant and in the whole organelle preparation). In some experiments, a mixture of proteinase inhibitors was included at all steps of the procedure for isolation of autophagosomes and autophagosomal membranes.

Assays and analyses

Enzymic activities and protein concentration were determined spectrophotometrically in a Technicon RA-1000 autoanalyser. LDH was measured by the method of Bergmeyer and Berndt [26], and acid phosphatase by the method of Ames [27]. Protein was measured by the method of Bradford [28], with a kit from Bio-Rad (Munich, Germany). Cytochrome *c* oxidase was measured as described by Cooperstein and Lazarow [29], and neutral esterase by the method of Beaufay et al. [30].

2D protein gel electrophoresis

For 2D-gel electrophoretic analysis of the total protein content of different isolated fractions, purified organelles were mixed with an equal volume of 10 mM potassium phosphate-buffered, isotonic (0.25 M) sucrose and sedimented by centrifugation for 15 min at 20000 rev./min (47800 ***g***). The pellets from total or sedimentable fractions (see above) were dissolved in a sample buffer containing 8 M urea, 65 mM dithiothreitol, 2 % (w/v) Pharmalyte (Pharmacia AB) and 0.5 % (v/v) Triton X-100. PNSs and cytosol were mixed directly with the sample buffer. The material was subjected to isoelectric focusing on linear Immobiline Dry Strips, pH 3–10 (Amersham Pharmacia Biotech, Uppsala, Sweden), with the Pharmacia Multipor II apparatus and procedure. The protein sample was loaded in the low-pH area of the isoelectric gel strips. To facilitate comparisons, 0.2 mg of protein was routinely loaded on all gels. After equilibration in 2 % (w/v) SDS, the strips were placed on top of a vertical SDS/PAGE (10 %, w/v) gel and subjected to electrophoresis with a Bio-Rad Protean II apparatus. Finally, proteins in the gel were detected by silver staining as described by Heukeshoven and Dernick [31].

Amino acid sequencing

Before amino acid sequencing, proteins were separated by 2D gel electrophoresis and transferred to a microporous PVDF membrane (Immobilon P^{SQ}; Millipore Corp., Bedford, MA, U.S.A.) with a 10 mM Caps/NaOH buffer, pH 11. The PVDF membrane was wetted in methanol before transfer. After detection with Amido Black, the proteins of interest were excised from the PVDF membranes and subjected to amino acid sequencing. For this preparative purpose, the protein sample was loaded in both the low-pH and high-pH areas of the isoelectric gel strips. N-terminal amino acid sequences were obtained with automated Edman degradation chemistry, followed by phenylthiohydantoin analysis with HPLC. Internal amino acid sequences were obtained by sample digestion with trypsin, followed by HPLC purification and matrix-assisted laser desorption ionization–time-of-flight MS analysis, before N-terminal sequence analysis [32].

Freeze-fracture electron microscopy

Organelles were fixed by adding an equal volume of 4 % (w/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. After 1 h the organelles were sedimented in an Eppendorf table centrifuge at 13000 ***g*** for 30 min, then resuspended in 20 % (v/v) glycerol. Most of the supernatant was removed, leaving a small volume with a high concentration of organelles. A droplet was applied on a gold-alloy specimen carrier and frozen in propane cooled in liquid nitrogen. The specimens were stored in liquid nitrogen until freeze-cleavage, which was performed at −100 °C; they were then etched for 60 s on a freeze-etching apparatus (model BAF 400; Balzers AG, Balzers, Liechtenstein). The specimens were unidirectionally shadowed with a 2 nm thick Pt/C layer (at 45°) and a 20 nm carbon layer (at 90°). The replicas were cleaned in 10 % (v/v) sodium hypochlorite and distilled water, picked up on grids and examined in the Jeol CM 10 transmission electron microscope operated at 60 kV. Pictures were taken with a Kodak Megaplus camera model 1,6 i.

RESULTS

Morphological characteristics of purified autophagosomes

The purified autophagosomes used in the present study have been characterized previously and shown to have an ultrastructural appearance and a contents composition comparable to those of autophagosomes *in situ* in intact hepatocytes [10]. The characteristic double unit membrane structure (corresponding to a single membrane cisterna) of autophagosomal delimiting membranes was thus observed in 83 % of the autophagosomes both *in situ* and after isolation; an additional 10–15 % had more than two unit membranes [33,34]. Freeze-fracture electron microscopy revealed that the delimiting membranes of isolated autophagosomes had a uniquely smooth appearance, being virtually devoid of intramembrane particles (Figure 1A). In contrast, the delimiting membranes of isolated lysosomes were studded with such particles (Figures 1B and 1C). Because the intramembrane particles are visualizations of transmembrane proteins, the freeze-fracture pictures suggest that autophagosomal delimiting membranes are exceptionally poor in protein [34].

Characterization of autophagosomal proteins by 2D gel electrophoresis

Because autophagosomes are essentially pieces of cytoplasm enwrapped by a protein-poor membrane, the complement of proteins recovered from these organelles by detergent extraction (Figure 2A) is a representative mixture of cellular proteins, not greatly different from that of the cytoplasmic extract (PNS) obtained after centrifugal removal of the nuclei (Figure 2C). Proteins identified by database searching or immunocytochemically in the autophagosome preparation thus included cytosolic enzymes such as LDH, ER-resident proteins such as protein disulphide isomerase and GRP78, and mitochondrial proteins such as the 60 kDa heat shock protein (HSP60) and the β subunit of F1-ATPase (βF1-ATPase) (Figure 2A). All of these were also present, in similar quantities, in the PNS (Figure 2C), which was consistent with the largely non-selective nature of autophagic sequestration [35].

Despite an estimated 24-fold purification of autophagosomes [10], no equivalent enrichment in individual proteins was observed. Any specific structural protein in the autophagosomal delimiting membranes therefore seems to be of such low abundance as to be undetectable in ordinary silver-stained 2D gels, in accordance with the protein-poor nature of these membranes as

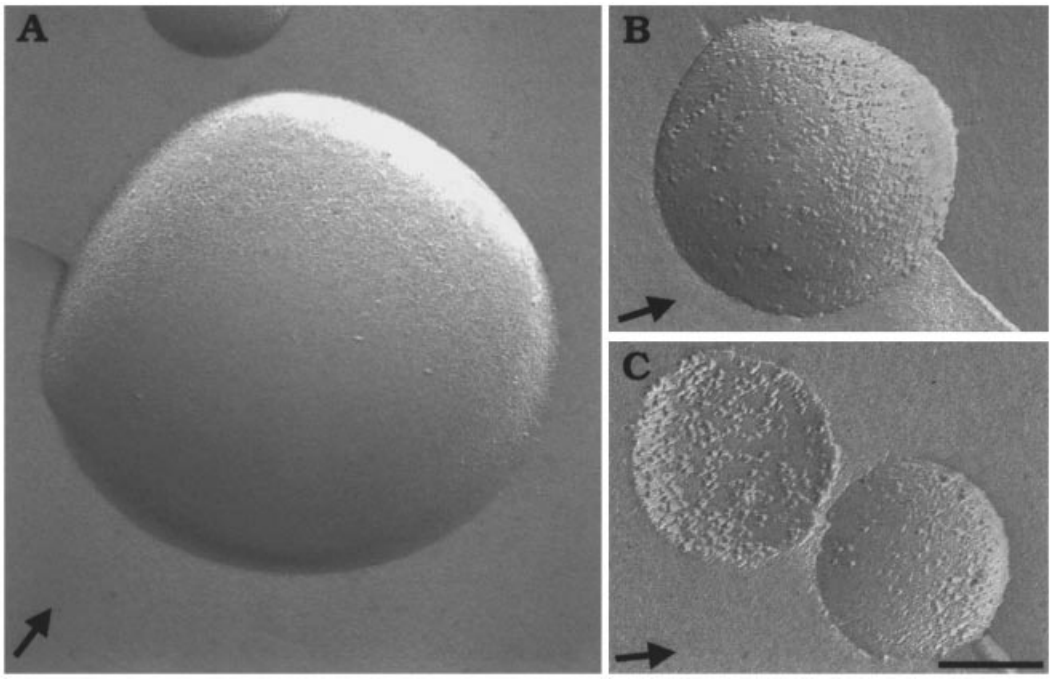

Figure 1 Freeze-fracture morphology of autophagosomes and lysosomes

Isolated autophagosomes (**A**) or lysosomes (**B**, **C**) were fixed, freeze-fractured and shadowed with platinum in the direction indicated by the arrows. Notice that the fracture through the autophagosomal delimiting membrane is virtually devoid of intramembrane particles, whereas the lysosomal membrane fracture faces have many such particles, representing transmembrane proteins. Scale bar, 250 nm.

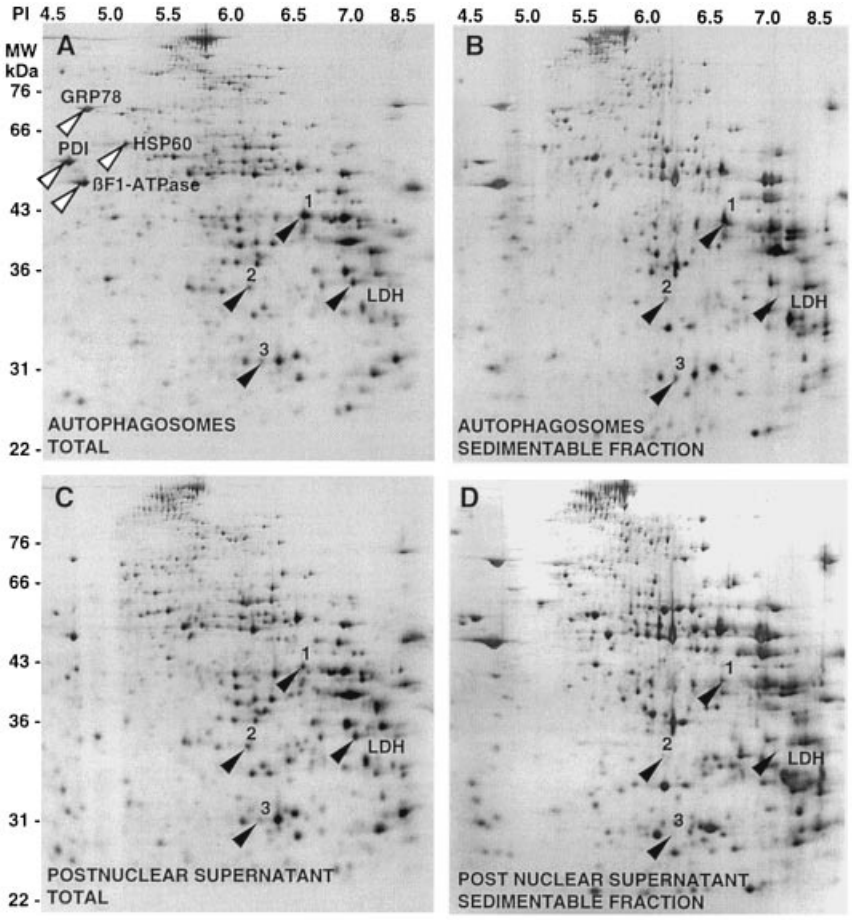

Figure 2 2D gel separation of proteins from total and sedimentable fractions of purified autophagosomes and PNSs

Purified autophagosomes (**A**), the sedimentable fraction from osmotically disrupted autophagosomes (**B**), PNS (**C**) or the sedimentable PNS fraction (**D**) were dissolved in urea-containing electrophoresis sample buffer; samples containing 0.2 mg of protein were subjected to 2D gel electrophoresis and staining with silver. Open arrowheads indicate proteins identified by immunoblotting (LDH, protein disulphide isomerase, GRP78) or by their position in the gel as recorded in the database of the Large Scale Biology Corp. (Rockville, MD, U.S.A.) [60 kDa heat shock protein (HSP60), βF1ATPase]; filled arrowheads indicate three proteins (numbered 1–3) found to be enriched in autophagosomal fractions compared with the corresponding PNS fractions. PI, pI.

indicated by freeze-fracture morphology and surface biotinylation [34]. Only one protein (spot 1 in Figure 2A) was consistently, albeit moderately, enriched in autophagosomes relative to PNS.

Detection of proteins associated with autophagosomal membranes

To improve the detection of proteins that might be associated with the autophagosomal delimiting membrane, purified autophagosomes were disrupted by osmotic lysis and freeze–thawing; the membranes were sedimented by high-speed centrifugation. To facilitate comparison, PNS samples were subjected to the same treatment. The disruption of the autophagosomes caused the loss of 40% of the protein and 90–100% of cytosolic enzymes such as LDH and superoxide dismutase, whereas the mitochondrial membrane protein cytochrome *c* oxidase and the ER protein GRP78 remained completely sedimentable (results not shown). The sedimentable fraction would therefore contain the membranes of autophagically sequestered organelles as well as the autophagosomal delimiting membranes but with very little cytosolic contamination (compare with the disappearance of LDH in Figure 2(B).

Three proteins (spots 1–3 in Figure 2A) were found to be enriched in the sedimentable autophagosome fraction (Figure 2B) relative to the sedimentable PNS fraction (Figure 2D). Raising the polyacrylamide concentration from 10% to 14% (w/v) to detect proteins smaller than 22 kDa did not uncover any additional autophagosome-enriched proteins. Protein 1 (43 kDa, pI 6.7) was always enriched in both total and sedimentable autophagosome fractions, indicating a marked tendency to associate with the autophagosomal delimiting membrane. Proteins 2 (35 kDa, pI 6.2) and 3 (31 kDa, pI 6.3) were enriched in the sedimentable fraction (relative to PNS membranes) but not in the total autophagosome fraction (relative to total PNS), indicating that their tendency to bind preferentially to autophagosomal delimiting membranes was not as strong as for protein no. 1.

Treatment of intact autophagosomes with a proteolytic enzyme (proteinase K) or with protein-stripping agents such as urea (2 M), sodium bicarbonate (0.1 M), Triton X-100 (2%, v/v) or high salt (1 M NaCl), failed to remove proteins 1–3 from autophagosomal membrane fractions prepared subsequently (results not shown). These proteins therefore seem to reside inside the autophagosomes, most probably attached to the innermost surface of the delimiting membrane.

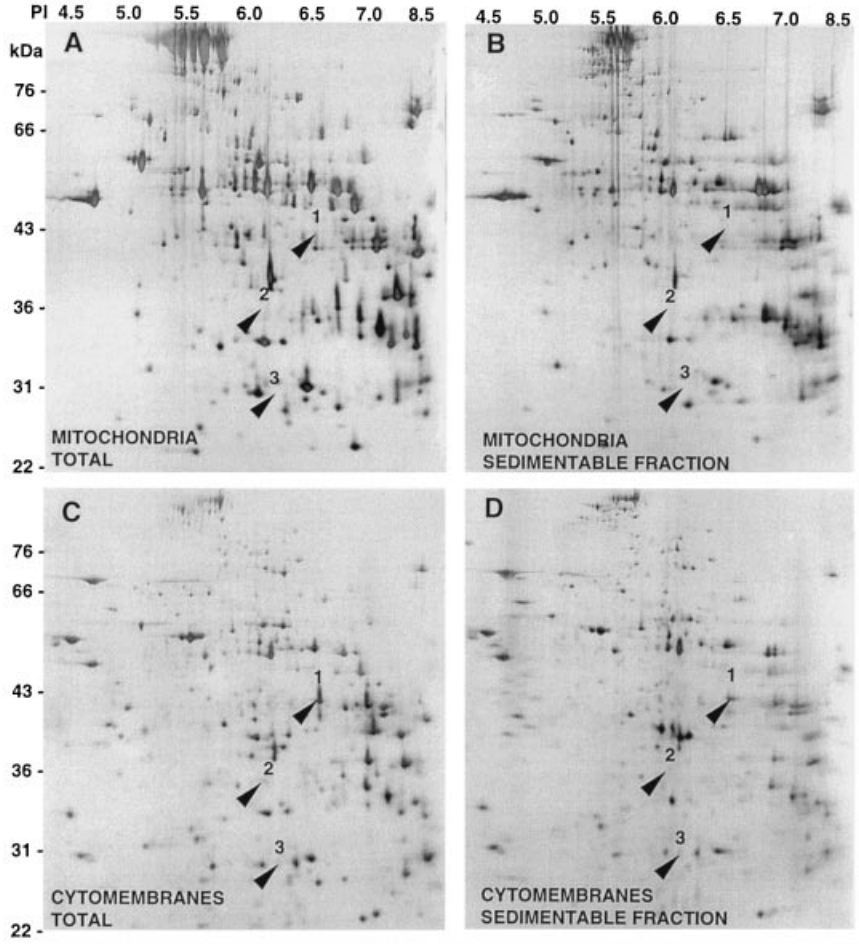

Figure 3 2D gel separation of proteins from mitochondria and cytomembranes

A mitochondrial fraction and a cytomembrane fraction (consisting of microsomes and other small membrane vesicles and cisternae) were isolated at different steps in the autophagosome preparation procedure [10]. Whole mitochondria (**A**), whole cytomembranes (**C**) or sedimentable fractions from mitochondria (**B**) or cytomembranes (**D**) were dissolved in urea-containing electrophoresis sample buffer; samples containing 0.2 mg of protein were subjected to 2D gel electrophoresis and staining with silver. The expected positions of the autophagosomally enriched proteins 1–3 are indicated by arrowheads. PI, pI.

Relation of autophagosome-associated proteins to other organelles

To see whether the autophagosomally enriched proteins might also associate with other organelles, detergent extracts of mitochondria, cytomembranes (mostly ER), lysosomes, endosomes or cytosol were analysed by 2D-gel electrophoresis (Figures 3–5). The autophagosome-associated proteins 1–3 were clearly absent from mitochondria (Figures 3A and 3B) and only negligible amounts were found in the cytomembrane (Figures 3C and 3D) and endosome (Figures 4C and 4D) fractions. It is therefore unlikely that the autophagosomally enriched proteins are bound to the membranes of organelles sequestered autophagically. These proteins could, however, be detected in lysosomal membranes (albeit in smaller amounts than in the autophagosomal membranes) (Figures 4A and 4B), indicating that autophagosomal and lysosomal membranes might have related protein-binding properties. All three proteins were clearly present in cytosol (Figure 5), suggesting that they are cytosolic proteins with a specific ability to associate with autophagosomal (and lysosomal) delimiting membranes.

Identification of autophagosome-associated proteins by amino acid sequencing

For molecular identification of the autophagosome-associated proteins, the 2D gels were electroblotted to PVDF membranes; spots 1–3 stained with Amido Black were excised and analysed by N-terminal Edman microsequencing. As protein 1 was found to be N-blocked, several spots of this protein were pooled, cleaved with trypsin and subjected to internal amino acid sequence analysis. The major sequence thus obtained (Table 1) was identical with an internal amino acid sequence (residues 26–41) in the urea-cycle enzyme ASS. Rat ASS is a homotetrameric enzyme with a predicted monomeric molecular mass of 46.5 kDa [36]. Several ASS isoforms have been reported from rat liver, with the same molecular mass but with different pI values, all in the alkaline range [37]. Because our protein 1 had a smaller mass (43 kDa) and a more acidic pI (6.7), it seems to be a novel autophagosome-associated isoform (aASS).

The N-terminal sequence obtained from spot 2 (Table 1) matched exactly residues 17–26 of rat GAPDH, a cytosolic glycolytic enzyme. Protein 2 therefore seems to represent a novel

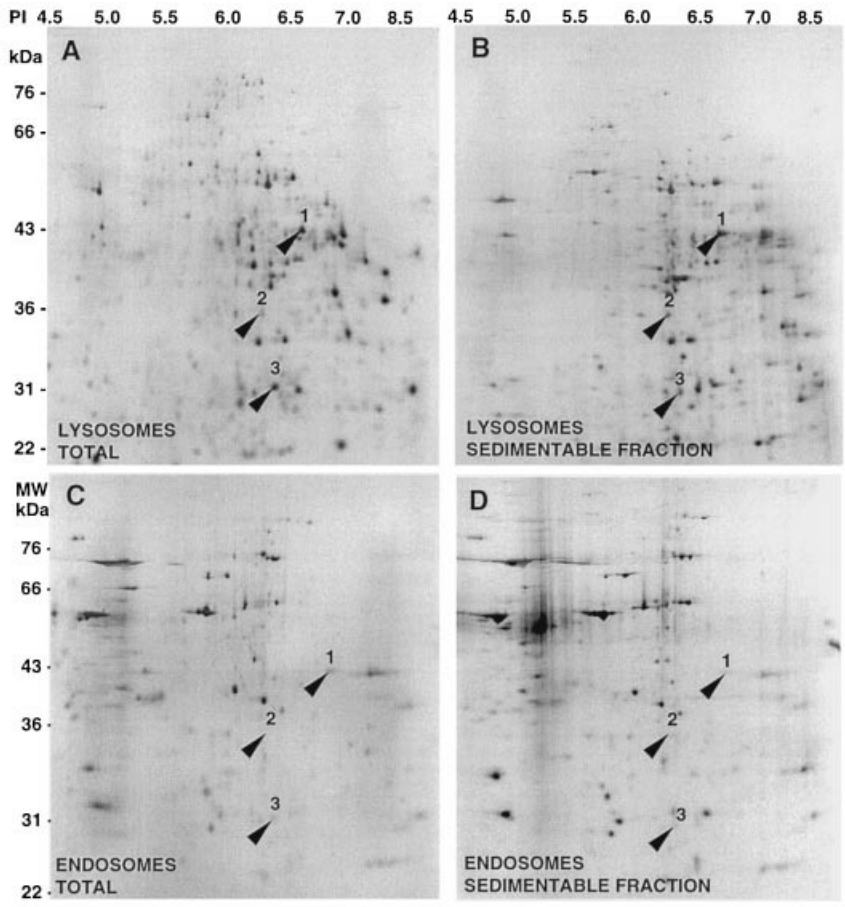

Figure 4 2D gel separation of proteins from lysosomes and endosomes

Lysosomes were prepared from hepatocytes in which autophagy had been blocked by 10 mM 3-methyladenine [10]; endosomes were prepared from vinblastine-treated hepatocytes as described in the Materials and methods section. Whole lysosomes (**A**), whole endosomes (**C**) or sedimentable fractions from lysosomes (**B**) or endosomes (**D**) were dissolved in urea-containing electrophoresis sample buffer; samples containing 0.2 mg of protein were subjected to 2D gel electrophoresis and staining with silver. The expected positions of the autophagosomally enriched proteins 1–3 are indicated by arrowheads. PI, pI.

autophagosome-associated, N-truncated form of GAPDH (aGAPDH), lacking the first 16 residues of the normal enzyme. Only a single functional GAPDH gene is known to be expressed in rat tissues; however, several isoenzymes have been described, with probable functions in for example vesicle fusion, cytoskeletal interactions, transcription and glycolysis (reviewed in [38]). The monomeric molecular mass reported for rat GAPDH is 36 kDa [39], i.e. slightly higher than the 35 kDa observed for the N-truncated aGAPDH. The autophagosomal isoform would therefore most probably be a proteolytic derivative of the functional GAPDH enzyme.

Because a previous study showed that a membrane-binding, N-truncated fragment of betaine homocysteine methyltransferase could be generated proteolytically during the isolation of lysosomal membranes [9], we performed experiments in which a mixture of proteinase inhibitors was included throughout the subcellular fractionation procedure. The inhibitors did not alter the detectability of aGAPDH or of the other two autophagosome-associated proteins (results not shown), suggesting that none of these were proteolytic artefacts of the isolation method.

The N-terminal sequence obtained from spot 3 (Table 1) matched residues 28–37 of the SCEH proenzyme, except that the serine residue in position 29 was replaced with valine. The autophagosome-associated enzyme therefore seems to be a variant or isoform of SCEH, an important enzyme in mitochondrial fatty acid β-oxidation. The sequence in the first 29 residues of pro-SCEH is a mitochondrial import signal that is cleaved off to form the mature 28 kDa mitochondrial enzyme [40]. Our autophagosome-associated enzyme variant (aSCEH) lacked only the first 27 residues, indicating that the presence of a valine residue in the normal cleavage position induced an alternative processing of the enzyme. Although aSCEH is therefore two residues longer than the normal, mature rat enzyme, it is nevertheless, at 31 kDa, somewhat larger than would be expected. The full sequence of aSCEH would be needed to determine its relationship to normal SCEH.

DISCUSSION

A major problem in the study of autophagosomal proteins is that these organelles have a content that includes all the proteins present in normal cytoplasm. Attempts have been made to compare the membrane protein composition of autophagy-engaged lysosomes (autolysosomes) with that of passive, dense lysosomes [8,9], but it is not clear whether the differences observed were due to proteins from autophagosomal delimiting mem-

Table 1 Amino acid sequences of autophagosome-associated enzymes

Autophagosome-associated proteins were separated by 2D gel electrophoresis and transferred to a PVDF membrane. After detection with Amido Black, the proteins of interest were excised from the PVDF membrane and subjected to amino acid sequencing. The sequence from autophagosomal ASS (aASS) is an internal sequence obtained after enzymic digestion; the sequences from autophagosomal GAPDH (aGAPDH) and autophagosomal SCEH (aSCEH) were obtained by N-terminal Edman degradation. The sequences of the autophagosomal proteins are compared with the known sequences of rat [36] and human [49] ASS, rat [50] and human [39] GAPDH, and rat [40] and human [46] SCEH respectively.

Enzyme	Partial sequence
Rat aASS	..................EQGYDVIAYLANIGQK..........
Rat ASS	.........(21)LVWLKEQGYDVIAYLANIGQKEDFE(45)..
Human ASS	..(21)LVWLKEQGYDVIAYLANIGQKEDFE(45)..
Rat aGAPDH	(1)RAAFSCDKVD........
Rat GAPDH	(1)VKVGVNGFGRIGRLVTRAAFSCDKVD(26)..
Human GAPDH	(1)VKVGVNGFGRIGRLVTRAAFNSGKVD(26)..
Rat aSCEH	(1)AVGANFQYII......
Rat SCEH	(1) GANFQYII......
pro-SCEH	(1)MAALRALLPRACNSLLSPVRCPEFRRFASGANFQYII(37)..
Human pro-SCEH	(1)MAALRVLLSCARGPLRPPVRCPAWRPFASGANFEYII(37)..

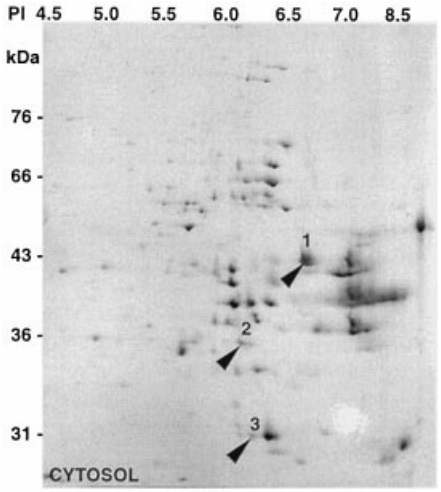

Figure 5 2D gel separation of cytosolic proteins

Cytosol was prepared as a high-speed supernatant from vinblastine-treated hepatocytes, then mixed with an equal volume of urea-containing electrophoresis sample buffer; a sample containing 0.2 mg of protein was subjected to 2D gel electrophoresis and staining with silver. The expected positions of the autophagosomally enriched proteins 1–3 are indicated by arrowheads. PI, pI.

branes or from autophagocytosed membranous organelles. In purified autophagosomes, proteins specific to the delimiting membranes would be expected to be enriched approx. 24-fold [10]; however, if such proteins were of low abundance they might still not be detectable on 2D gels amid the multitude of more abundant proteins from other organelle membranes. Our ultrastructural freeze-fracture studies have in fact revealed that isolated rat liver autophagosomes have nearly uniformly smooth delimiting membranes, suggesting that very few transmembrane proteins (revealed as intramembrane particles) are present [34]. The lack of autophagosomal surface proteins susceptible to biotinylation similarly indicates that the membranes of these organelles are exceptionally poor in protein [34]. The detection of specific membrane proteins in autophagosomes will therefore probably require purification of their delimiting membranes, together with highly sensitive analytical methods.

The three autophagosome-associated proteins (isoenzymes) identified in the present study were additionally present both in the cytosol and in lysosomal membranes; they were accordingly only moderately enriched in the autophagosome preparation. Their resistance to treatment of intact autophagosomes with proteolytic enzymes and surface-stripping agents suggests an internal localization, for example at the inner surface of the autophagosomal delimiting membrane, where they could fulfil a guiding role during autophagic sequestration. However, their presence in lysosomal membranes could also indicate a dual attachment capacity and a role in autophagosome–lysosome fusion, or simply reflect a transfer of membrane-associated proteins from autophagosomes to lysosomes as a natural part of the autophagic process.

One of the autophagosome-associated proteins was identified as a novel N-truncated isoform of the glycolytic enzyme GAPDH. Previously described GAPDH isoforms have been reported to have masses similar to the major form; they are believed to represent post-translational modifications such as ADP-ribosylation [41]. GAPDH is encoded by a single, highly conserved, functional gene (the numerous pseudogenes found both in rodent and human genomes are generally regarded as non-functional [39]), yet it seems to be an extremely versatile multifunctional protein. GAPDH can bind to nucleic acids as well as to other proteins; different isoforms have been shown to inhibit DNA replication, stimulate RNA synthesis, control mRNA degradation, regulate cytoskeletal interactions and promote apoptosis [38]. Of particular interest to the present study is the ability of a non-glycolytic GAPDH isoform to catalyse membrane fusion through a phosphatidylserine-binding motif [42,43]. Our N-truncated GAPDH isoform (aGAPDH) might similarly be able to bind to lipids in the autophagosomal delimiting membrane to serve a role in autophagic sequestration. In the yeast *Pichia pastoris*, another glycolytic enzyme, phosphofructokinase, has been shown to be involved in the initial steps of the autophagic pathway [20]. Alternatively, the binding of aGAPDH and other abnormal proteins to autophagosomal and lysosomal membranes could be a prelude to their eventual intralysosomal degradation [44]; compare this with the ability of even normal GAPDH to bind to the surface of rat liver lysosomes for subsequent internalization and proteolytic breakdown [45].

A second autophagosome-associated protein was identified as a sequence variant of SCEH, which catalyses the second step of mitochondrial short-chain fatty acid β-oxidation [46]. The auto-

phagosomal isoform (aSCEH) has the N-terminal sequence AVGANFQ, which differs from both the GANFQ-terminus of normal mature SCEH and the ASGANFQ sequence of the normal proenzyme, suggesting that a Ser-29 → Val substitution has induced an abnormal and premature cleavage of the mitochondrial targeting propeptide (aSCEH is not found in the mitochondrial fraction). Although no variant SCEH isoforms have been described previously, other members of the low-similarity enoyl-CoA hydratase/isomerase family are multifunctional proteins with roles in lipid metabolism as well as in RNA degradation [47]; it therefore seems reasonable that some family members could have evolved to serve a role in autophagy.

A third autophagosome-associated protein was identified, by internal sequencing (residues 26–41), as the urea-cycle enzyme ASS. There seems to be only a single functional ASS gene (plus a number of pseudogenes) that encodes a 46.5 kDa monomer [36], i.e. with a size somewhat larger than the 43 kDa autophagosomal isoform (aASS). The (at least) five ASS isoforms previously described from rat liver all have a pI in the alkaline range [37], whereas the pI of aASS (6.7) is clearly acidic, like the kidney isoforms [48]. Because aASS was N-blocked, its N-terminus could not be sequenced by the methods available to us; whether it is a truncated variant isoform, like aGAPDH and aSCEH, will therefore have to be clarified by future studies. It is intriguing that the three autophagosome-associated enzymes represent three different major metabolic pathways: amino acid (aASS), carbohydrate (aGAPDH) and lipid metabolism (aSCEH). Considering the important role of autophagy in the degradation of macromolecules to supply metabolites to all of these pathways, it seems reasonable that variants of representative enzymes might have evolved to serve a role in the integration of autophagic activity with energy metabolism in general. Such integration could conceivably take place at many levels, as exemplified by the well-established feedback control of autophagy by amino acids [4] and by the recently demonstrated suppression of autophagy by AMP [13].

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