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Leupeptin-Induced Appearance of Partial Fragment of Betaine Homocysteine Methyltransferase during Autophagic Maturation in Rat Hepatocytes[1]

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A cytosolic enzyme, betaine homocysteine methyltransferase (BHMT), and its partial fragments were discovered as autolysosomal membrane proteins from rat liver in the presence of leupeptin [Ueno *et al.* (1999) *J. Biol. Chem.* 274, 15222–15229]. The present study was undertaken to further characterize the transport and processing of BHMT from cytosol to autolysosome and to test if the fragment can be used as an *in vitro* probe for the maturation step of macroautophagy. Upon subcellular fractionation, BHMT (p44) was found in all fractions, while its 32-kDa fragment (p32) was found only in the mitochondrial-lysosomal (ML) fraction. Incubation of isolated hepatocytes with leupeptin induced time-dependent accumulation of p32 in the ML fraction from 30 to 90 min after the start of incubation. However, chloroquine completely inhibited the appearance of p32, indicating that the processing from p44 to p32 is lysosomal. Incubation with Bafilomycin A_1, a vacuolar H^+-ATPase inhibitor, together with leupeptin, led to linear accumulation of p44, but not of p32. The p44 accumulation rate was calculated to be 4.9%/h, which was comparable to autophagic sequestration rate. The distribution of p44 within the ML fraction turned out to be dual, *i.e.*, the membrane-surface attached and luminal/sedimentable forms. Amino acids and 3-methyladenine, both of which specifically suppress macroautophagy, inhibited the accumulation of p32 as well as of p44. Finally, energy-dependent appearance of p32 was demonstrated during incubation of postnucler supernatant fractions, making it possible to establish an *in vitro* assay system. All the results strongly support the idea that BHMT is taken up and degraded to p32 through the macroautophagic pathway, and that p32 could be a novel probe for the maturation of macroautophagy.

Key words: autophagic maturation, autophagosome-lysosome fusion, betaine homocysteine methyltransferase, leupeptin, macroautophagy.

Autophagy plays a major role in the bulk degradation of intracellular proteins and organelles *via* a vacuolar/lysosomal system and is critically important for protein balance in the cell (*1–4*). In mammalian cells, the lysosomal degradation system of intracellular proteins is mainly categorized into three classes: macro- and micro-autophagy, and selective lysosomal transport (*4*, *5*). A macroautophagic pathway is classically well-known and characterized as follows: it is initiated by nonselective sequestration of cytoplasmic components into a double membrane structure, an autophagosome (AVi). The lumen of AVi is acidified by vacuolar H^+-ATPase and fuses with lysosome to form an autolysosome (AVd), in which sequestered molecules are degraded by lysosomal hydrolases. Microautophagy is a less well defined mechanism by which a mixed population of cytosolic proteins or small organelles is sequestered by small vesicles derived from smooth endoplasmic reticulum (*6*), or taken up directly by invagination of the lysosomal membrane (*7*). It is not influenced by extracellular amino acids, although macroautophagy is strictly controlled by them. Selective lysosomal transport is a system of direct protein uptake into lysosomes mediated by the heat shock cognate protein of 73 kDa (hsc73), and it is thus called hsc73-mediated transport. Protein with a specific KFERQ-like motif is recognized by this chaperone hsc73 and the pathway is activated by nutrient deprivation (*5*).

While the initial AVi formation step of the macroautophagic pathway has been extensively studied, there are very few reports dealing with the maturation step (*8–11*). AVi matures into AVd in a stepwise fashion: (a) acquisition of

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Abbreviations: ATP-DS, ATP depleting system; ATP-RS, ATP regenerating system; AVd, autolysosome (or degradative autophagic vacuole); AVi, autophagosome (or initial autophagic vacuole); BHMT, betaine homocysteine methyltransferase; β-hexosaminidase, *N*-acetyl-β-D-glucosaminidase; 3-MA, 3-methyladenine; ML fraction, mitochondrial-lysosomal fraction; PNS, postnuclear supernatant; Reg-AA, regulatory amino acids plus alanine.

lysosomal membrane proteins, *e.g.*, lgp120 or vacuolar ATPase, by fusing with a vesicle deficient in hydrolytic enzymes; (b) vacuole acidification by vacuolar ATPase; and (c) acquisition of hydrolases by fusing with preexisting secondary lysosomes (*9*). As an *in vitro* system to analyze this maturation step, streptolysin O-permeabilized hepatocytes were successfully reconstituted in our laboratory (*12*). Other essential tools that have been developed include methods to monitor the fusion of AVi and lysosomes, a major concern in autophagic maturation, such as the degradation of electroinjected ^{14}C-lactose by lysosomal β-galactosidase (*13*, *14*), and the intravacuolar ratio of cytosolic enzymes, carbonic anhydrase and CuZn-superoxide dismutase, based on their different susceptibility to lysosomal proteolysis (*10*, *15*). However, in order to establish an *in vitro* reconstituted assay system, it is critical to develop more pertinent probes.

Betaine homocysteine methyltransferase (BHMT; EC 2.1.1.5), a cytosolic enzyme abundant in the liver and kidney, catalyzes the transfer of a methyl group from betaine to homocysteine to produce dimethylglycine and methionine (*16*, *17*). Recently, during an attempt to identify specific proteins localized in autolysosomes, BHMT and its proteolytic fragments were discovered as autolysosomal membrane proteins in the presence of leupeptin or E64, thiol protease inhibitors, in rat hepatocytes (*18*). If BHMT is sequestered by macroautophagy and processed by lysosomal enzymes, its proteolytic fragments, especially the 32-kDa peptide (p32), could provide a probe of AVd, and thus of fusion between AVi and lysosomes. Therefore, the present study was undertaken to ascertain whether BHMT is actually sequestered and processed by macroautophagy, not by other lysosomal routes, and to test whether p32 is detectable in *in vitro* incubation. It was proved that p32 is an intermediate peptide of the native cytosolic protein BHMT, probably produced by AVi-lysosome fusion and located within AVd. Evidence was also obtained by using this probe that autophagic maturation including AVi-lysosome fusion takes place *in vitro*.

MATERIALS AND METHODS

Materials—Bafilomycin A_1, chloroquine, Triton X-100, bovine serum albumin, ATP, phosphocreatine, and creatine phosphokinase were purchased from Sigma-Aldrich, and leupeptin was from Peptide Institute (Osaka). Collagenase and hexokinase were obtained from Roche Diagnostics. ECL Western blot detection kit and horseradish peroxidase conjugated anti-rabbit IgG (from donkey) were purchased from Amersham Pharmacia Biotech.

Isolation of Fresh Hepatocytes—Male rats of Wistar strain (250–300 g) were meal-fed a 35% casein diet for 4 h (14:00–18:00) to synchronize their feeding pattern. Hepatocytes were isolated by the collagenase method of Seglen (*19*), modified by Venerando *et al.* (*20*). After preincubation for 45 min, these fresh hepatocytes were suspended at a density of 1×10^7 cell/ml in Krebs-Ringer bicarbonate buffer containing 6 mM glucose and 0.5% albumin. Oxygenation and pH (7.4 at 37°C) were maintained by gassing with O_2:CO_2 (95:5, v/v). At the start of incubation, leupeptin (1 mM, final conc.) was added to the cell suspension. When necessary, various inhibitors, *i.e.*, chloroquine (1 mM), bafilomycin A_1(1 μM), 3-methyladenine (3-MA, 10 mM), and a regulatory amino acid mixture containing Leu, Tyr, Pro, Met, His, Trp, and Ala (RegAA) at 10 times (×) normal plasma levels (*21*) were added.

Preparation of Mitochondrial-Lysosomal Fraction from Hepatocytes—After incubation, hepatocytes were resuspended in 0.25 M sucrose–1 mM EDTA (pH 7.4) for cell disruption, and homogenized with a tightly-fitting Dounce homogenizer (7 ml size, 100 strokes, Wheaton Science Products, NJ, USA) on ice. Homogenates were spun at 500 ×*g* for 10 min in a Kubota KM-15200 centrifuge with an RA-150AM rotor to remove nuclei and unbroken cells and obtain the postnuclear supernatant (PNS) fractions. The fractions were then spun at 17,500 ×*g* for 10 min. To avoid cytosolic contamination, these pellets were resuspended with 0.25 M sucrose-1 mM EDTA and spun at 17,500 ×*g* for 10 min. The pellets were washed twice and designated as a mitochondrial-lysosomal (ML) fraction. This ML fraction included all of the autophagy-related vacuoles, *i.e.*, AVi, AVd, and secondary lysosomes, the majority of which were AVd in leupeptin-treated cells (*22*, Goto, M., Niioka, S., and Kadowaki, M., manuscript in preparation).

Distribution of BHMT and Its p32 Fragment in ML Fraction—Fresh ML fractions prepared from hepatocytes incubated with leupeptin for 90 min were exposed to various treatments at 0°C for 30 min as described in the legend of Fig. 5, then centrifuged at 17,500 ×*g* for 10 min. The pellet and supernatant were subjected to immunoblot analyses.

In Vitro Incubation of PNS Fractions—Hepatocytes were preincubated with leupeptin for 30 min, then homogenized to obtain their PNS fractions. These fractions were incubated at 37°C for 60 min with leupeptin in order to determine whether autophagic maturation takes place *in vitro*. To examine the energy dependency of the reaction, an ATP-regenerating system (ATP-RS: 1.5 mM ATP, 5 mM phosphocreatine, and 5 U/ml creatine phosphokinase) and an ATP-depleting system (ATP-DS: 2 mM glucose, 30 U/ml hexokinase) were employed.

Immunoblotting of BHMT and Its p32 Fragment—ML fractions, their subfractions after various treatments or PNS fractions were boiled for 5 min in 10 mM Tris-HCl (pH 6.8), 20 mM dithiothreitol, 20% (v/v) glycerol, 1.0% SDS (as final conc.), and subjected to SDS-PAGE (10%) according to the method of Laemmli (*23*). Separated polypeptides were transferred to PVDF membrane. To detect BHMT and its p32 fragment, an anti–rat p32 antibody raised against a synthetic peptide corresponding to the 10 amino-terminal residues of p32 (α-p32-10R, from rabbit), which recognized both p44 and p32, but much less p35, was used as a primary antibody (*18*). An ECL Western blot detection kit was used as the substrate for horseradish peroxidase conjugate of the secondary antibody. Blots were exposed on X-ray films, which were quantitated by use of a Fast Scan Personal Scanning Imager (Molecular Dynamics).

Analytical Procedures—Lysosomal marker enzyme β-hexosaminidase activity was assayed according to Peters *et al.* (*24*) in the presence of 0.2% Triton X-100. Protein concentration was measured by the Bradford method (*25*). All data were expressed as means ± SE. Statistical significance was evaluated by the Student's *t*-test; *p* values greater than 0.05 were considered not significant.

RESULTS

Subcellular Localization of BHMT and Its p32 Fragment—To identify the subcellular localization of BHMT and its fragments, cell fractionation was performed. Since freshly isolated hepatocytes were known to be resistant to homogenization, the conditions necessary for cell disruption were first tested (Fig. 1). Cell disruption was not possible with ionic buffers such as KRB, but it was possible with non-ionic, isotonic 0.25 M sucrose–1 mM EDTA. A negligible number of unbroken cells remained after about 50 strokes, but more than 80 strokes were needed for the maximal recovery of a lysosomal marker β-hexosaminidase in PNS fraction. Thus, we decided to employ 100 strokes to obtain complete cell disruption and the maximal recovery of ML fraction, which was about 50% recovery.

After incubation with leupeptin for 90 min, hepatocytes were homogenized and fractionated into a 500 ×*g* precipitate (N), 17,500 ×*g* precipitate (ML), and 17,500 ×*g* supernatant (PS), each of which was subjected to immunoblot analysis (Fig. 2b). The primary antibody α-32p-10R reacts with intact BHMT (p44) as well as its proteolytic fragment p32 (*18*). In spite of its cytosolic origin, p44 was detected in all fractions, although mostly in PS (microsome and cytosol) fraction. The p44 in the N fraction seemed to be a contaminant from autolysosomes and cytosol, judged from the distribution of β-hexosaminidase (Fig. 2a). p32 was detected only in the ML fraction, which strongly supports the notion that this fragment is generated and accumulated in the ML fraction.

Time Course of p32 Appearance in Response to Leupeptin—Changes in BHMT and its fragment p32 in the ML fraction were followed for 90 min in the presence of leupeptin. As depicted in Fig. 3, BHMT (p44) tended to decrease slightly, but not significantly so, whereas p32 was accumulated time-dependently after 30 min. The amount of p32 at time zero (in the absence of leupeptin) was small and variable, probably reflecting a variation in cell preparations. On the contrary, the bands at and after 30 min showed a quite constant linearity. This accumulation of p32 clearly demonstrated that p32 degradation to smaller peptides was inhibited by leupeptin, but the initial degradation from p44 to p32 was not.

Effects of Inhibitors on BHMT Accumulation and Its Partial Degradation—To ascertain whether p44 is partially degraded to p32 by lysosomal hydrolase(s), the lysosomotropic agent chloroquine was tested in the presence of leupeptin. ML fractions from chloroquine-treated cells were prepared in the same way as from leupeptin-treated cells (Fig. 4a). p44 tended to accumulate slightly, while the increase in p32 fragment was completely suppressed (Fig. 4c). Although it is not clear whether chloroquine affects the AVi formation step (*13*), we can conclude that BHMT is degraded to p32 by leupeptin-resistant lysosomal proteases, and that non-lysosomal proteases are almost certainly not involved.

Next, the effect of bafilomycin A_1 was investigated in the presence of leupeptin (Fig. 4b). Unlike chloroquine, bafilomycin A_1, an inhibitor of vacuolar H^+-ATPase (*26*), inhibits not only protein degradation (*27*) but also AVi-lysosome fusion (*11*), by inhibiting the acidification of acidic compartments. As shown in Fig. 4d, p44 accumulated time-dependently, while the increase in p32 was blocked after the first 30 min, which seemed to reflect a delay in the inhibitor's effectiveness. These results indicated that bafilomycin inhibited partial degradation of BHMT to p32 by inhibiting proteolysis or AVi-lysosome fusion, or both. Based on the morphological findings that bafilomycin does not affect AVi

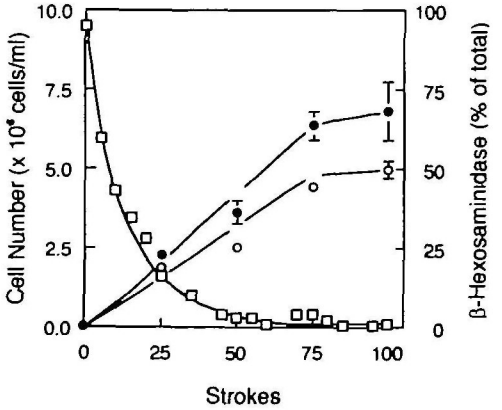

Fig. 1. **Disruption of isolated rat hepatocytes and recovery of lysosomes.** Hepatocyte suspension (10^7 cells/ml) in 0.25 M sucrose–1 mM EDTA was homogenized with various numbers of strokes with a Dounce homogenizer. Cell numbers (□) were counted microscopically, and the recovery of β-hexosaminidase in PNS (●) and ML fraction (○) was measured simultaneously as in "METERIALS AND METHODS" (n = 3).

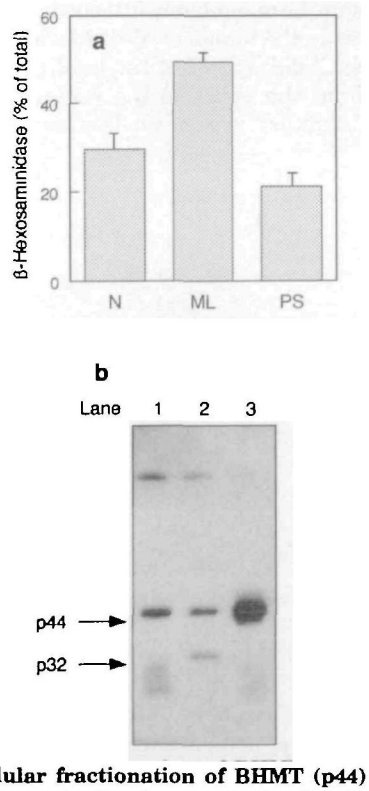

Fig. 2. **Subcellular fractionation of BHMT (p44) and its p32 fragment.** Homogenates from hepatocytes incubated with leupeptin for 90 min were fractionated into N (500 ×*g* precipitate), ML (17,500 ×*g* precipitate), and PS (17,500 ×*g* supernatant) fractions. Each fraction was subjected to lysosomal marker β-hexosaminidase assay (a) (n = 4) and to immunoblot analysis (b): lane 1, N; 2, ML; 3, PS.

formation at all (Goto, M. *et al.*, manuscript in preparation), the net accumulation of p44 was calculated as the fractional rate as shown in Fig. 4e, with the assumption that p44 in the cytosol is taken up by AVi (ML fraction) and with correction for the recovery of ML fraction in the homogenate (50%, from Fig. 2). The rate was found to be 4.9%/h, which was quite similar to AVi formation rate measured by other methods (see "DISCUSSION").

Distribution of BHMT and p32 in ML Fraction—Based on the previous findings (*18*), we had initially thought that most of the p44 in the ML fraction would be located inside the lumen of AVi and be a substrate for lysosomal degradation. However, compared with p32, the change in p44 by the above treatments was much smaller than expected. Substantial amounts of p44 were observed even without inhibitors (at zero time). To elucidate how it exists in the ML fraction, the interaction of p44 with membranes was tested by centrifugation of ML fractions at 17,500 ×*g* for 10 min after various treatments (Fig. 5). The absence of p44 in the supernatant after washing the ML fraction with 0.25 M sucrose confirmed that a majority of p44 is not a contaminant from the cytosol, but is attached to or inside autophagic vacuoles, especially AVi. Triton X-100 treatment solubilized 60% of β-hexosaminidase (Fig. 5b) and shifted about 70% of p44 from the pellet to the supernatant. Hypotonic treatment with 0.05 M sucrose, which disrupts osmotically sensitive vacuolar particles but cannot solubilize membranes, caused a slight shift of p44. Although band intensities varied depending on conditions, p32 was located only in the pellet fraction even after Triton X-100 or hypotonic treatment, implying that it exists as a heavy sediment or remnant form, probably attached to denatured protein aggregates in the lumen of AVd (*18*, *28*). The treatment with 1.0 M NaCl did not affect the localization of p32, but shifted p44 from the pellet to the supernatant, although membrane disruption was much less as evident from β-hexosaminidase assay. Urea treatment showed a similar but smaller effect on the release of p44. These results strongly indicate that about half of p44 in the ML fraction [~55%; averaged from NaCl treatment, 66 ± 4% (n = 4), and urea treatment, 43 ± 8% (n = 4)] is located on the outer surface of membraneous components in a loosely-bound form. This result was obtained with samples not only at 90 min but also at 0 min (data not shown), which suggested that this average fraction (55%) is constant. It is not clear whether the membrane-bound form of p44 is a normal localization in the cell or an artifact of manipulations such as homogenization. Anyway, the p44 remaining inside the vacuoles, accounting for less than half of the total, would be a form sequestered by autophagy and susceptible to lysosomal proteloysis.

Effects of Amino Acids and 3-Methyladenine on BHMT Accumulation and Processing to p32—To ascertain whether a macroautophagic pathway is involved in these processes, we tested the effect of 3-MA and amino acids, inhibitors of macroautophagy. Excess levels of regulatory amino acids (RegAA, 10×) are known to inhibit AVi formation completely in perfused liver (*29*) and isolated hepatocytes (*21*). As depicted in Fig. 6, a and b, hepatocytes were incubated with leupeptin for 90 min. 3-MA was added at 0 min to completely inhibit macroautophagy for estimating its minimum level. On the other hand, RegAA were added at 60

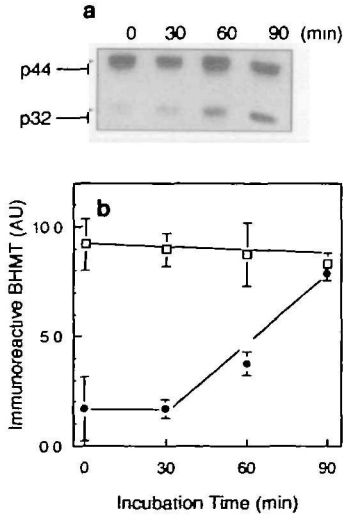

Fig. 3. **Time course of leupeptin's effect on p32 appearance.** ML fractions were prepared from hepatocytes incubated with leupeptin for 0, 30, 60, and 90 min. Each sample (50 μg protein per lane) was subjected to immunoblot analysis. The same amounts of protein were applied in all the following experiments (a). p44 (□) and p32 (●) were quantitated by densitometric scanning, expressed as arbitrary unit (AU) (b, n = 4).

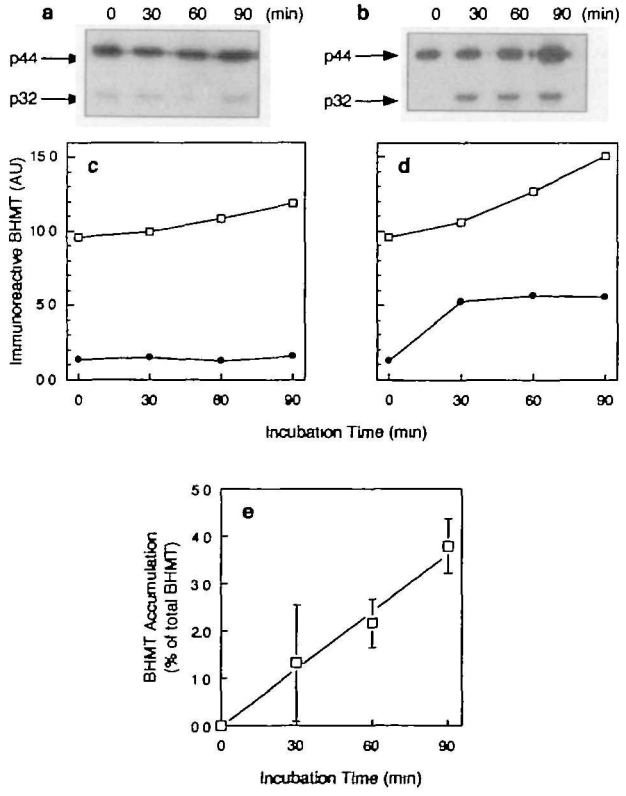

Fig. 4. **Effect of chloroquine and bafilomycin A_1 on BHMT processing.** ML fractions from chloroquine- (a) or bafilomycin A_1- (b) treated hepatocytes in the presence of leupeptin at 0, 30, 60, and 90 min were subjected to immunoblot analysis. Representative data (p44, □; p32, ●) of (a) and (b) were depicted in (c) and (d), respectively. Net accumulation of p44 with bafilomycin was calculated as a percentage of total BHMT in the cytosol, and plotted in (e) (n = 4).

min, when p32 started to accumulate, especially to see their acute effect on the accumulation of p44 and the processing to p32. Band intensities of p44 were subtracted by the fraction of p44 attached to membraneous components (~55%), which is irrelevant to autophagic sequestration. As shown in Fig. 6, c and d, 3-MA markedly inhibited the calculated autophagically-sequestered p44 and accumulation of p32. RegAA also showed similar levels of suppression to 3-MA of the sequestration of p44 and appearance of p32. Since the hepatocytes include a complete and sequential set of autophagic processes, it was not clear whether the suppression of p32 by RegAA was due to a direct effect or a secondary effect to their effect on AVi formation. Whatever the case, these results demonstrate that p44 is processed to p32 *via* the macroautophagic pathway.

In Vitro Incubation Study of p32 Appearance—To investigate whether p32 can be employed as a novel probe for autophagic maturation *in vitro*, the possibility that energy-dependent p32 production takes place in the PNS fractions was tested. After preincubation with leupeptin for 30 min (refer to Fig. 3), hepatocytes were homogenized to obtain PNS fractions, which were then incubated with leupeptin. As shown in Fig. 7, a time-dependent increase in p32 was observed up to 30 min, which reached a plateau thereafter in the presence of ATP regenerating system. This increase was reduced to 60% by ATP depletion, and inhibited to less than 20% by chloroquine, which indicates that autophagic

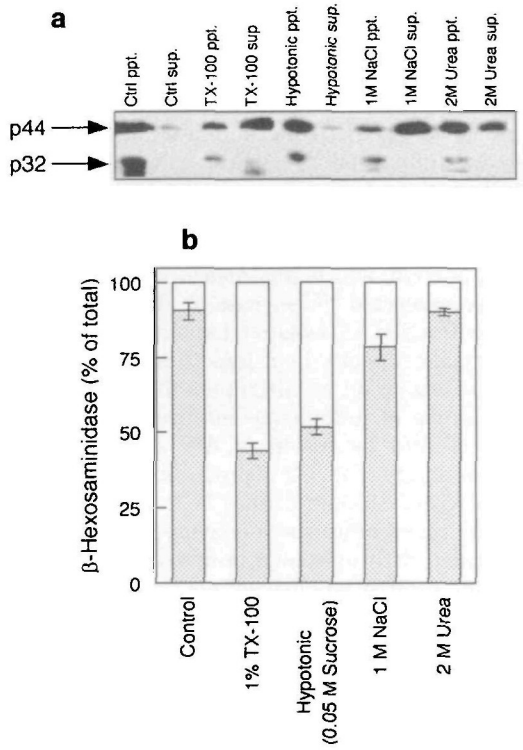

Fig. 5. **Effect of various treatments on distribution of p44 and p32 in ML fraction and lysosomal latency.** (a) ML fractions prepared from hepatocytes incubated with leupeptin for 90 min were exposed to various treatments at 0°C for 30 min as follows: 0.25 M sucrose as a control, 1.0% Triton X-100 (TX-100) for solubilization, 0.05 M sucrose as a hypotonic treatment, 1.0 M NaCl, and 2.0 M urea. Samples were centrifuged at 17,500 ×*g* for 10 min, and both the pellet, resuspended in 0.25 M sucrose, and the supernatant were electrophoresed, transferred to PVDF membrane, and probed by immunoblotting as described in "MATERIALS AND METHODS." The immunoblotting bands are representative of 3–4 experiments. (b) To monitor membrane disruption of vacuoles, lysosomal latency was estimated by measuring β-hexosaminidase activities in the pellet (dotted bar) and supernatant (open bar) fractions under the conditions described in (a). The data are percentages of total enzyme activities and means ± SE (n = 3). Treatments with NaCl and urea were also tested with samples at 0 min (without leupeptin), which confirmed the results at 90 min with leupeptin.

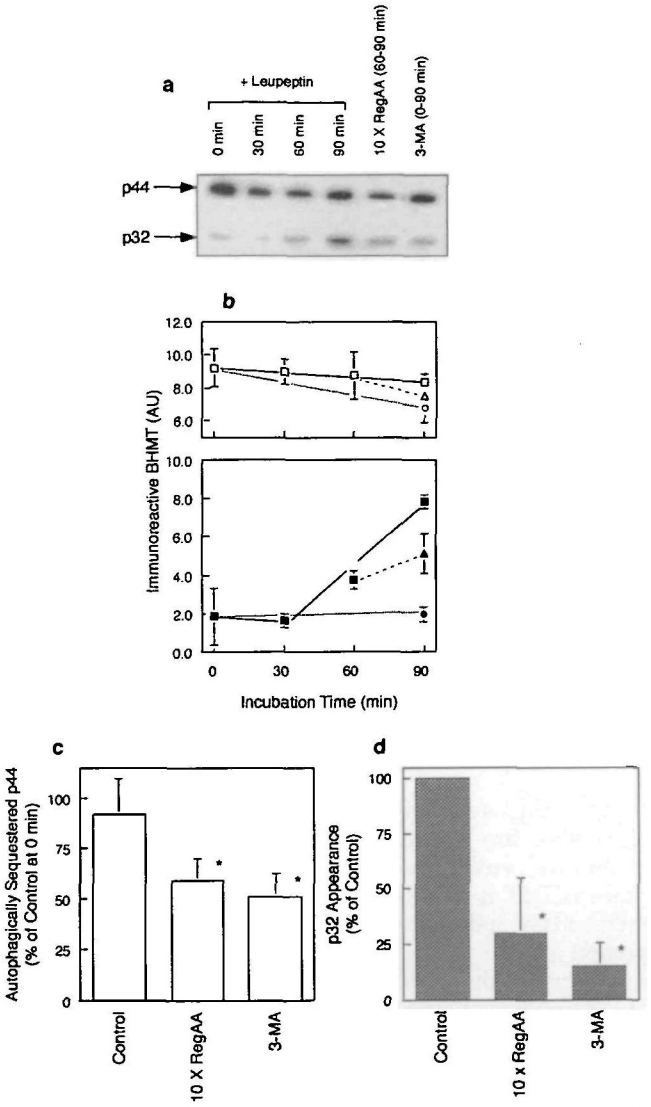

Fig. 6. **Effect of regulatory amino acids and 3-MA on BHMT partial degradation.** (a) ML fractions from leupeptin-treated hepatocytes at 0, 30, 60, and 90 min (lane 1–4, respectively) were subjected to immunoblot analysis. RegAA (10×) was added at 60 min and cells were taken at 90 min (lane 5). 3-MA was added for 0–90 min (lane 6). (b) p44 (open symbols) and p32 (closed symbols) in the control (□, ■), RegAA (△, ▲), and 3-MA (○, ●), respectively, were quantitated by densitometric scanning (n = 4). (c) Since p44 consists of autophagically-sequestered and membrane-bound forms as demonstrated in Fig. 5, the data in (b) were subtracted by an averaged fraction of the membrane-bound form (55% at 0 min), and the autophagically-sequestered p44 was reperesented as a percentage of the control at 0 min. Statistical significance was evaluated between the data at 90 min; $^*p < 0.05$ *vs.* the control. (d) Rates of p32 appearance were calculated from (b): the control, 30–90 min; RegAA, 60–90 min; 3-MA, 0–90 min, respectively. $^*p < 0.05$ *vs.* the control.

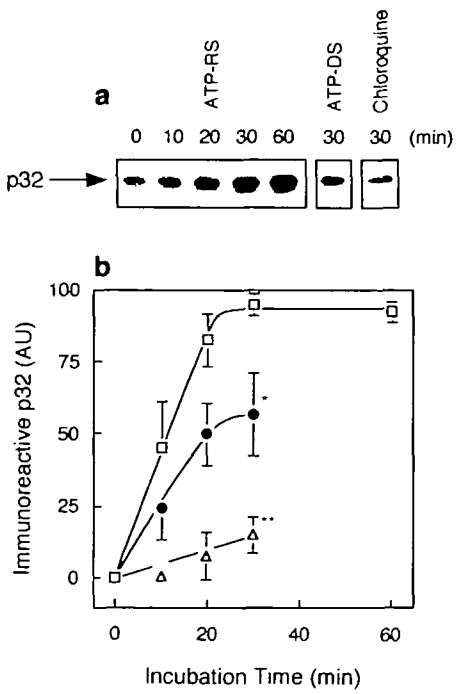

Fig. 7. ***In vitro* production of p32 in PNS fractions.** PNS fractions from hepatocytes preincubated with leupeptin for 30 min were incubated at 37°C for 60 min with leupeptin in the presence of ATP-RS (□) together with chloroquine (△), or in the presence of ATP-DS (●). p32 was detected by immunoblotting (a), and quantitated by densitometric scanning, expressed in arbitrary units (AU) (b). Data are means ± SE ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* ATP-RS.

maturation takes place during incubation of these fractions.

DISCUSSION

In the macroautophagic pathway, cytosolic proteins and organelles are sequestered non-selectively into double membrane structures called autophagosomes (*30*, *31*). Since BHMT is a cytosolic enzyme involved in amino acid metabolism and is expressed up to 1.6% of total soluble protein in the liver, macroautophagy is the most likely route for its digestion. Ueno *et al.* (*18*) discovered BHMT and its proteolytic fragments as autolysosome membrane proteins in the presence of leupeptin or E64d in rat hepatocytes. The two fragments, p35 and p32, were identified as partial proteolytic fragments of BHMT, the former being attached to the outer surface of AVd, and the latter being present at the inner surface and in the lumen of AVd. Both fragments derive from a common precursor form, the intact BHMT, but p32 is more appropriate as a proteolytic intermediate of BHMT than p35, beacause the production of p35 may be an artifact irrelevant to autolysosomal proteolysis (*18*).

It is noteworthy that, although leupeptin inhibits bulk proteolysis completely as do other general lysosomal inhibitors (*32*), it does not necessarily block initial or intermediate degradation of some proteins, such as the processing of BHMT to p32. On the contrary, chloroquine, which inhibits lysosomal function by raising lysosomal pH, completely blocked the appearance of p32 (Fig. 4, a and c). These results clearly indicate that initial degradation of BHMT to p32 is catalyzed by leupeptin-resistant lysosomal protease(s). Accumulation of p32 in a time-dependent manner (Fig. 3) suggested that BHMT is steadily supplied to AVd by fusion with secondary lysosomes. The enzyme(s) involved in the processing to p32 is not known yet, but we obtained preliminary indirect evidence that only hepatocytes which exhibited a higher expression of cathepsin L than cathepsin B could produce p32 fragment in the presence of leupeptin (data not shown). In addition, the processing to p32 has not yet been detected in cultured cells in which BHMT cDNA was transfected. Therefore, this specialized p32 production may be possible only in the hepatocytes in which cathepsin L is a major class of hydrolases expressed in lysosomes.

When the fusion step was blocked by bafilomycin A_1, which is a highly specific inhibitor of vacuolar H^+-ATPase, essential for acidification of lumen within acidic compartments including autophagic vacuoles, p44 was accumulated time-dependently with no increase in p32 (Fig. 4, b and d). In a morphological study, bafilomycin A_1 led to the specific accumulation of AVi and a nearly complete loss of AVd in the cytoplasm (*11*). Based on the cytoplasmic volume, the AVi formation rate was calculated to be 4.2%/h (Goto, M. *et al.*, manuscript in preparation). In the present study, bafilomycin also evoked BHMT accumulation in the ML fraction (Fig. 4e). Assuming that the BHMT accumulated in the ML fraction during incubation is a population sequestered into AVi from the cytoplasm, its accumulation rate was estimated to be 4.9%/h. The value is similar to that of Seglen's group, *i.e.*, 5–6%/h, which was measured by the sequestration of electroinjected ^{14}C-sucrose or ^{3}H-raffinose into the cell corpse fraction in isolated hepatocytes (*33*, *34*). The results strongly supported our hypothesis.

In the present study, we attempted to characterize p32 as a specific probe of autophagic maturation, especially the formation of AVd by fusion of AVi and lysosomes. The unlikely possibility that p32 is produced within AVi prior to the fusion with lysosome cannot be ruled out at present, because AVi may aquire some enzyme(s) for its processing through fusion with unknown prelysosomal vesicles. However, the finding that p32 existed not as a soluble form, but only as an aggregate sedimentable form (Fig. 5), which is typical for leupeptin-treated autolysosomal contents (*18*, *28*), would not support such a possibility. In principle, leupeptin interacts with AVd, but not with AVi, so these granular aggregates could not be formed in AVi. This leads to the notion that the processing to p32 proceeds within AVd. Another possibility is that BHMT is taken up by selective lysosomal transport system. However, BHMT has no KFERQ-like motif in its sequence (*18*, *35*) and the accumulation of p32 was blocked by 3-MA, suggesting that it was taken up by macroautophagy rather than by the hsc73-mediated transport, which is insensitive to 3-MA (*36*). Otherwise, p32 would accumulate in the ML fraction even in the presence of 3-MA.

Although 3-MA and RegAA are both known to inhibit macroautophagy at the step of AVi formation, p44 appeared to decrease to a much smaller extent than expected in response to these agents (Fig. 6). This suggested that BHMT might be taken up by microautophagy or by selective lysosomal transport. However, the appearance of p32 was undoubtedly blocked by these treatments. This clearly indi-

cated that p32 was processed from p44 through macroautophagic sequestration. In addition, Fig. 5 indicates that about half of p44 in the ML fraction was not derived from sequestration, and not located in the lumen of AVi. Considering this dual distribution of p44 in the ML fraction, the corrected results reached the conclusion similar to that obtained by other probes, *e.g.*, ^{14}C-sucrose (*33*).

The *in vitro* fusion assay between AVi and lysosome in the maturation step of macroautophagy has not been reported yet. Similar types of vesicle fusion between endocytic vesicles (*37*), late endosome and lysosome (*38*), lysosome and lysosome (*39*), phagosome and lysosome (*40*), and phagosome and endosome (*41*), have been reported. These types of membrane fusion are, in most cases, monitored by use of extracellularly-induced probes, taking advantage of biotin-avidin conjugates or of DNP and anti-DNP antibody conjugates. However, these probes are, by nature, not applicable to autophagy in intact cells. For autophagy, the methods developed include monitoring of electroinjected ^{14}C-lactose degradation (*13*, *14*), and the intravacuolar ratio of cytosolic enzymes, carbonic anhydrase and CuZn-superoxide dismutase, based on their different susceptibility to lysosomal proteolysis (*10*, *15*). In yeast, a modified alkaline phosphatase assay, which was developed for simple monitoring of autophagy, essentially takes advantages of fusion between autophagosome and vacuole (*42*). The maturation step including AVi-lysosome fusion was reconstitued using streptolysin O-permeabilized hepatocytes, which was monitored by valine release (*12*). The present results of incubation of PNS fractions provide another novel probe showing the autophagic maturation *in vitro* in the presence of ATP, although this system still includes several steps, *e.g.*, AVi-lysosome fusion, processing to p32 through acidification, *etc.* This PNS fractions consist of autophagy-related particles and cytosol together, in which organized structures within the cells are completely disrupted, leading to the *in vitro* reconstituted assay shortly.

In summary, the present results strongly indicate that cytosolic BHMT is taken up by autophagic sequestration and its processing to p32 includes the fusion of AVi and lysosomes. Thus, the detection of p32 in the presence of leupeptin could become a novel, intrinsic probe to monitor the maturation step of macroautophagy. Research is underway to establish an *in vitro* assay system in hepatocytes based on the present study.

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