Ap8γ is a ubiquitin-like protein involved in autophagy in yeast. Ap8γ is covalently but transiently attached to membrane lipids through the actions of activating, conjugating, and processing/deconjugating enzymes. The mammalian Ap8γ homologues GATE-16, GARARAP, and MAP1-LC3 have been implicated in intra-Golgi transport, receptor sorting, and autophagy, respectively. All are served by a single set of activating and conjugating enzymes. Here we identify a novel mammalian Ap8γ homologue, which we name Ap8γL, and describe the synthesis of electrophilic probes based on the GATE-16, GARARAP, MAP1-LC3, and Ap8γL proteins. These probes not only form specific adducts in crude cell lysates, but also allow identification of the cellular proteases specific for the C termini of these Ap8γ homologues. We find a single protease, Apg4B/autophagin-1, capable of acting on GATE-16, GABARAP, MAP1-LC3, and Ap8γL. The Ap8γβ/autophagin-1 protease thus serves as a processing/deconjugating enzyme for these four highly divergent mammalian Ap8γ homologues.

Autophagy is a process via which both yeast and mammalian cells degrade and recycle cytoplasmic components. Upon nutrient starvation a so-called isolation membrane engulfs a portion of the cytoplasm, resulting in the formation of a double-membrane structure called an autophagosome. The outer membrane of the autophagosome subsequently fuses with the vacuole/lysosome, releasing a single-membrane-bound vesicle (also called autophagic body) into this compartment. The membrane is then broken down and its cytosolic contents degraded (Fig. 1D) (1).

Elegant work by Ohsumi, Klionsky, and colleagues (2) has implicated in membrane processes (10–12). GATE-16 (Golgi-association with membrane protein of 16 kDa) is an Apg8 homologue, which we name GATE-16, capable of acting on GATE-16, GABARAP, MAP1-LC3, and Ap8γL. The Apg8β/autophagin-1 protease thus serves as a processing/deconjugating enzyme for these four highly divergent mammalian Ap8γ homologues.

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nate t-SNARE syntaxin 5 (10, 13). These GATE-16 interactions are thought to modulate Golgi membrane transport.

GABARAP (GABA receptor-associated protein) is another Apg8-like protein that reportedly interacts with NSF and the y2 subunit of the receptor for the neurotransmitter γ-aminobutyric acid (GABA) (12, 14). GABARAP localizes to intracellular membranes, including the Golgi apparatus and post-synaptic cisternae. Accordingly, it has been proposed that GABARAP is involved in intracellular membrane trafficking, in particular in receptor sorting and/or targeting in neurons (15). GABARAP may also link GABA receptors to the cytoskeleton via its interactions with microtubules and microfilaments (12, 14, 16).

The Apg8 homologue MAP1-LC3 (microtubule-associated protein 1-light chain 3) has been implicated in autophagy in mammalian cells (11). Rat MAP1-LC3 is processed by proteolysis C-terminally of its conserved glycine residue to produce an active cytosolic form, LC3-I. LC3-I is then further modified to generate LC3-II, which is electrophoretically distinct from MAP1-LC3-I and is localized to the autophagosomal membrane. The amount of LC3-II is correlated with the extent of autophagosome formation induced by starvation. Although the molecular nature of the modification is not known, LC3-II behaves like an integral membrane protein, consistent with its modification involving a lipid moiety, similar to Apg8 in yeast (11). Recently, three human isoforms of MAP1-LC3 have been cloned and characterized (MAP1-LC3A, -B, and -C), which differ in their post-translational modifications (17). MAP1-LC3A and MAP1-LC3C exist in two forms, a higher molecular weight cytosolic form (-I) and a lower molecular weight membrane-associated form (-II), like rat MAP1-LC3. MAP1-LC3A and MAP1-LC3C are proteolytically processed C-terminally of the conserved glycine residue. MAP1-LC3B, on the other hand, does not undergo C-terminal cleavage and exists as a single modified form. Instead, the essential site for post-translational modifications is not known. LC3-II is tightly associated with membrane, although the nature of the modification remains unknown. MAP1-LC3A and MAP1-LC3C largely colocalize with GFP-ratLC3, indicating autophagosomal localization and function, whereas MAP1-LC3B localizes to some other subcellular compartment (17).

The enzymatic pathways that mediate yeast Apg8 processing, conjugation, and deconjugation are conserved for the mammalian Apg8 homologues GATE-16, GABARAP, and MAP1-LC3. These three Apg8 homologues all possess a conserved glycine residue followed by a single variant amino acid at the C-terminal end (17). Mammalian Apg8 homologues GATE-16, MAP1-LC3, and GABARAP, as well as a novel Apg8 homologue, Apg8L, were found by searching the NCBI data bases. The data base accession numbers of the sequences used are: A097810 (GATE-16), A1181000 (MAP1-LC3), BF300927 (GABARAP), and BG244294 (Apg8L). Clones containing the desired sequences were obtained from Research Genetics and ATCC. Open reading frames of the predicted processed forms of the proteins, omitting the C-terminal codon (GATE-161-116; MAP1-LC31-116, GABARAP1-116, Apg8L1-116; see Fig. 1A), were amplified by polymerase chain reaction (PCR). The PCR primers used had extensions at their 5’ ends to generate a Ndel restriction site at the N-terminal coding end of the PCR product and a Sapl restriction site at the C-terminal coding end of the PCR product. To generate the epitope-tagged versions of the MAP1-LC3 fusion, coding sequences for the HA epitope were included in the 5’ PCR primer, between the NdeI and SapI restriction sites. These active site-directed probes were used to study the cellular proteases specific for the C termini of these Apg8-like proteins and to identify the identification of Apg8L as a protease specific for GATE-16, GABARAP, MAP1-LC3, and Apg8L. A single protease thus serves this family of ubiquitin-like proteins, which diverge in sequence from each other by as much as 71%.

EXPERIMENTAL PROCEDURES

Cloning of GATE-16, MAP1-LC3, GABARAP, and Apg8L into ptTYB1—Sequences of mouse GATE-16, MAP1-LC3, GABARAP, and Apg8L were found by searching the NCBI data bases. The data base accession numbers of the sequences used are: A097810 (GATE-16), A1181000 (MAP1-LC3), BF300927 (GABARAP), and BG244294 (Apg8L). Clones containing the desired sequences were obtained from Research Genetics and ATCC. Open reading frames of the predicted processed forms of the proteins, omitting the C-terminal codon (GATE-161-116; MAP1-LC31-116, GABARAP1-116, Apg8L1-116; see Fig. 1A), were amplified by polymerase chain reaction (PCR). The PCR primers used had extensions at their 5’ ends to generate a Ndel restriction site at the N-terminal coding end of the PCR product and a Sapl restriction site at the C-terminal coding end of the PCR product. To generate the epitope-tagged versions of the MAP1-LC3 fusion, coding sequences for the HA epitope were included in the 5’ PCR primer, between the NdeI and SapI restriction sites. The fragment was cloned into the ptTYB1 vector (New England Biolabs), generating an in-frame fusion with the intein and chitin binding domain (29). All constructs generated were sequence verified.

Synthesis of Vinyl Sulfone Derivatives of GATE-16, MAP1-LC3, GABARAP, and Apg8L—The synthesis of vinyl sulfone derivatives of GATE-16, MAP1-LC3, GABARAP, and Apg8L was identical for all. These GATE-16, MAP1-LC3, GABARAP, and Apg8L were modified with a C-terminal electrophilic vinyl sulfone moiety and shown to form specific adducts in crude cell lysates. These active site-directed probes were used to study the cellular proteases specific for the C termini of these Apg8-like proteins and to identify the identification of Apg8L as a protease specific for GATE-16, GABARAP, MAP1-LC3, and Apg8L. A single protease thus serves this family of ubiquitin-like proteins, which diverge in sequence from each other by as much as 71%.
(PMSF) and lysed by French press (1500 p.s.i.). After centrifugation, the supernatant was loaded onto a 15-mL chitin bead column (New England Biolabs) at a flow rate of 0.5 mL/min, to allow binding of the Apg8-intein-chitin binding domain fusion protein. The column was washed with 80 mL of lysis buffer, followed by 50 mL of lysis buffer containing 50 mM β-mercaptoethanesulfonic acid (MESNa; Sigma). The column, containing buffer with MESNa, was incubated overnight at 37 °C to allow on-column cleavage. The Apg8-MESNa thiostere was eluted with 25 mL of lysis buffer, and the fractions containing Apg8-MESNa product were concentrated using a Centrerpel (M, 3000 cut-off; Millipore). Overall yield was 1–5 mg of Apg8-MESNa/liter of bacterial culture.

Apg8-MESNa was converted to the vinyl sulfone derivative in a chemical ligation reaction with a large excess of glycine vinyl sulfone (31). Vinyl sulfone was added to an aliquot of concentrated Apg8-MESNa (~2 mg/mL) to a final concentration of 0.25 mM, followed by addition of 75 μL of 2 N hydroxysuccinimide and 30 μL of 2 M NaOH. The mixture was incubated at 37 °C for ~1 h, and the reaction was monitored by liquid chromatography/mass spectrometry, using an LCZ electrospray mass spectrometer instrument (Micromass) with an LCZ electrospray mass spectrometer instrument (Micromass) to allow identification. Samples were analyzed by LC-electrospray ionization-MS/MS using a quadrupole time-of-flight (QTOF) microtandem mass spectrometer (Micromass/Waters) as previously described (30).

RESULTS

A Novel Mammalian Apg8 Homologue, Apg8L—GATE-16, MAP1-LC3, and GABARAP are mammalian homologues of the yeast Apg8 protein (10–12). To find additional Apg8 homologues, we used the yeast Apg8 amino acid sequence to search the NCBI data base. A new mouse sequence of 117 amino acids with 54% identity to yeast Apg8 was identified, which we named Apg8L, for Apg8-like (Fig. 1A). Apg8L has 33, 60, and 86% sequence identity to MAP1-LC3, GATE-16, and GABARAP, respectively (Fig. 1B). In addition to an overall similarity in size and amino acid sequence to the other three Apg8 homologues, Apg8L shares the conserved glycine at the C terminus followed by a single lysine residue (Fig. 1A). These four Apg8 homologues are between 29 and 86% identical to each other (Fig. 1B). A phylogenetic tree constructed with the mammalian Apg8 homologues reported to date revealed a clear divergence of the four types of Apg8 homologues from the yeast Apg8 and each other (Fig. 1C). This suggests that the mammalian Apg8 homologues may have evolved to play roles in divergent cellular processes. However, the human orthologues of the mouse GATE-16, MAP1-LC3, GABARAP, and Apg8L proteins have amino acid sequences identical to their mouse counterparts. This indicates a function of each of these proteins that is evolutionarily conserved in mammalian cells.

Generation of GATE-16, MAP1-LC3, GABARAP, and Apg8L with a C-terminal Vinyl Sulfone—The mammalian activating (E1) and conjugating (E2) enzymes of GATE-16, MAP1-LC3, and GABARAP have been identified as Apg7 and Apg3 (21–24). However, at least four mammalian homologues of the yeast Apg8-specific protease Apg4 exist in the data bases (28), and their specificity for mammalian Apg8 homologues has remained unknown. We have previously described the synthesis of C-terminally modified derivatives of ubiquitin, SUMO-1, Nedd8, and ISG15, which could covalently modify processing and deconjugating enzymes specific for these proteins (41). By this method, active Ub/UBL-specific proteases were targeted and identified based on their specificity for and reactivity toward the C terminus of Ub and the UBLs (41). To target and identify mammalian proteases specific for GATE-16, MAP1-LC3, GABARAP, and Apg8L, we synthesized these proteins modified with a C-terminal electrophile trap, a vinyl sulfone moiety, using an intein-based method (Fig. 2). Apg8-like proteases were radiolabeled with 125I-Labeled probe (5 × 106 cpm) was incubated with 20–40 μg of cell lysate for 1 h at 37 °C. Where indicated, non-radioactive competing Apg8-like probes were added to radiolabeled probes prior to addition of SDS sample buffer with β-mercaptoethanol and boiling for 5 min. Polypeptides were resolved by 11% SDS-PAGE. Gels were analyzed by Coomassie Brilliant Blue staining and autoradiography using standard procedures, as indicated.
Conversion reactions were monitored by mass spectrometry (LC-electrospray ionization-MS). The masses determined for Apg8-like vinyl sulfone derivatives were in good agreement with predicted values (data not shown; see "Experimental Procedures").

GATE-16-VS, MAP1-LC3-VS, GABARAP-VS, and Apg8L-VS Each Form a Thiol-dependent 70-kDa Adduct in EL-4 Cell Lysates—We have previously shown that radioiodination of vinyl sulfone derivatives of Ub and UBLs allowed the detection of specific adducts in cell lysates. These species were identified either genetically or by tandem mass spectrometry as adducts of the probes with deconjugating enzymes (30, 32, 41). To examine the labeling profiles of the prepared Apg8-like probes, we incubated EL-4 cell lysates with radio-iodinated Apg8-like probes (Fig. 3). EL-4 cells were chosen as a model cell line, allowing a direct comparison with labeling profiles obtained with the other UBL probes (30, 32, 41). Incubation of EL-4 cell lysates with [125I]GATE-16-VS, [125I]MAP1-LC3-VS, [125I]GABARAP-VS, or [125I]Apg8L-VS resulted in the detection of a single specific adduct at 70 kDa for all probes used (Fig. 3, lanes 1 and 2 in each panel). The nonspecific species at 30–40 kDa most probably represent dimeric forms of the probes. The isolation and 125I labeling of GABARAP proved the most variable of the probes used here, and may account for the somewhat weaker labeling observed with [125I]GABARAP-VS. Formation of the 70-kDa adducts observed with each of the four probes was resistant to pre-incubation with the serine protease inhibitor PMSF, but was completely abolished by pre-incubation with the thiol alkylating reagent NEM, indicating dependence of adduct formation on a thiol group, likely an active site cysteine residue (Fig. 3).

Cytoplasmic Localization of the Polypeptides Labeled by GATE-16-VS, MAP1-LC3-VS, GABARAP-VS, and Apg8L-VS—

Fig. 1. Apg8L, a novel mammalian Apg8 homologue. A, a ClustalW sequence alignment of Saccharomyces cerevisiae (Sc) Apg8 (data base accession no. Z35839), Mus musculus (Mm) Apg8L (BG24294), GATE-16 (AA097810), MAP1-LC3 (AI181000), and GABARAP (BF30927). The gray arrow indicates the last amino acid included in the Apg8-intein-chitin binding domain expression constructs (see Fig. 2). The black arrow indicates the conserved glycine residue present at the C terminus of the processed forms of the Apg8-like proteins. B, percentage of amino acid identity of Apg8 homologues. Percentages of identity are pairwise alignment scores from the ClustalW alignment in panel A. C, phylogenetic tree of mammalian Apg8 homologues. An alignment of the mammalian Apg8 homologues was generated using the MEGA2 program of the DNAStarTM software package (using a ClustalW algorithm). A neighbor-joining phylogenetic tree was then generated based on the alignment. Data base accession numbers of the sequences used, unless indicated in the legend of panel A, are as follows (including references, if applicable): Mm_GABARAP1L, AF180518.1 (38); Cp_gcc, AI012920.2 (33); Hs_GABARAP1L, AF087847.1 (38); Hs_Apg8L, AA476809; Hs_GABARAP2, AF180519.2 (38); Hs_GABARAP, NP_098209 (18); Hs_GABARAP, AF161588 (16); Hs_GABARAP2, AF087848.1 (38); Hs_GATE-16, O08765; Mm_GABARAP2, AF190644.1 (38); Hs_MAP1-LC3 (Mm_MAP1-LC3A), NP_115903; Mm_MAP1-LC3-1, AK003122.1 (17); Mm_MAP1-LC3-2, AK003106.1 (17); Hs_MAP1-LC3, U35748.1 (17); Hs_GABARAP, AF087847.1 (17); Hs_GABARAP, AF276659.1 (17). Hs, Homo sapiens (human); Mm, M. musculus (mouse); Rn, Rattus norvegicus (rat); Cp, Cavia porcellus (guinea pig). Red circles mark the proteins used in this study. D, schematic representation of Apg8 conjugation and deconjugation during autophagy in yeast. Apg8 is conjugated to a lipid moiety (phosphatidylethanolamine) by the action of an activating enzyme (E1, Apg7) and a conjugating enzyme (E2, Apg3). The cysteine protease Apg4 is responsible for processing of the Apg8 precursor (removing the C-terminal Arg residue to expose the glycine) as well as Apg8 deconjugation. Both functions of Apg4 are essential for the progression of autophagy in yeast (8).
targets are expressed in a tissue-specific fashion. We therefore labeled lysates prepared from various mouse tissues to examine the tissue distribution of active target enzymes (Fig. 5). A single adduct of ~70 kDa was formed with each of the four Apg8-like probes in all tissues examined. Some additional species were observed at lower molecular mass, but these were nonspecific, as they were also observed without lysate, albeit at lower intensity (Fig. 5, lane 1 in each panel). For each of the four probes, labeling of the ~70-kDa adduct was strongest in spleen, thymus and brain. Labeling was less efficient in kidney extract and weakest, but still detectable, in liver. We therefore conclude that the proteins targeted by GATE-16-VS, MAP1-LC3-VS, GABARAP-VS, and Apg8L-VS, and resulting in a ~70-kDa adduct, are ubiquitously and constitutively expressed and active. No additional specific targets for any of the probes were identified in these tissues. However, the slight variations of labeling could reflect subtle differences in the target enzyme modified, or could indicate multiple labeled target species of the same molecular mass.

GATE-16-VS, MAP1-LC3-VS, GABARAP-VS, and Apg8L-VS Target the Same Polypeptide in EL-4 Cell Lysates—Using active site-directed probes derived from four different Apg8-like proteins, we labeled a single ~70-kDa adduct in EL-4 lysate with each probe (Fig. 3). In all cases this activity was inhibited by NEM and localized in the soluble cytoplasmic fraction (Figs. 3 and 4). In addition, highly similar labeling profiles in tissue extracts were observed for the four probes (Fig. 5). These similarities in labeling raised the possibility that the four probes target the same polypeptide. To address this, we performed competition experiments, in which increasing amounts of non-radioactive probes were added to labeling reactions in EL-4 lysates. As expected, labeling of the ~70-kDa target by each radiolabeled probe was diminished in a dose-dependent manner by inclusion of increasing amounts of the unlabeled version of the same probe (Fig. 6, across from top left to bottom right). We then used the same amounts of unlabeled competitor probe in a cross-competition experiment on EL-4 lysates for each of the radiolabeled probes. Labeling by each radiolabeled probe was effectively diminished by inclusion of any of the other Apg8-like probes (Fig. 6). The competition profiles with the different Apg8 probes were very similar, indicating that the affinity of each of the probes for the target is roughly comparable. Competitions for labeling are specific, as formation of a ~95-kDa adduct with [125I]SUMO-1-VS2 was unaffected by inclusion of the unlabeled Apg8-like probes (Fig. 6). As GATE-16-VS, MAP1-LC3-VS, GABARAP-VS, and Apg8L-VS cross-competitive in these labeling experiments, we conclude that they target the same polypeptide in EL-4 cell lysates.

Identification of Apg4B as the Target Protein of MAP1-LC3-VS in EL-4 Cell Lysates—Having demonstrated by competition experiments that the four Apg8-like probes target the same polypeptide in EL-4 cell lysate, it was sufficient to identify the modified target for only one of the probes. To identify the protein targeted by MAP1-LC3-VS, we prepared a version of this probe with an N-terminal hemagglutinin epitope tag, HA-MAP1-LC3-VS. We first established that HA-MAP1-LC3-VS could effectively inhibit formation of the ~70-kDa adduct by [125I]MAP1-LC3-VS, indicating that HA-MAP1-LC3-VS targets the same protein as [125I]MAP1-LC3-VS and that the N-terminal epitope tag does not interfere with target recognition (data not shown). A large scale labeling reaction of HA-MAP1-LC3-VS with EL-4 cell lysates was performed, followed by immunopurification of the probe and its covalently attached targets. We conducted the reaction and immunopurification with an active probe (HA-MAP1-LC3-VS) as well as

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**Fig. 2. Synthesis of Apg8-like proteins with a C-terminal vinyl sulfone.** Step 1, the Apg8-like proteins (Apg8; i.e. GATE-16, GABARAP, MAP1-LC3, or Apg8L), minus the 2 C-terminal amino acids (~2aa) (see Fig. 1A), are expressed as a fusion protein with a intein and a chitin binding domain (Chitin BD). Soluble fusion protein binds to a chitin affinity column. Step 2, N-S acyl rearrangement resulting in an intermediate in which the peptide bond is replaced by a thioester linkage. Step 3, the Apg8-like protein is released from the column by a transthioesterification reaction induced by incubation with MESNa, resulting in the Apg8-MESNa product. Step 4, the MESNa group is removed by glycine vinyl sulfone (Gly-VS) in a chemical ligation reaction, producing Apg8-VS. Step 5, nucleophilic active site residues of enzymes can covalently react with the VS group.

GATE-16, MAP1-LC3, and GABARAP have been implicated in membrane transport and fusion processes such as intra-Golgi transport, autophagy, and receptor transport/targeting (10–12). We therefore wondered whether the enzymatic activity targeted by the Apg8-like probes is associated with membranes or present in the soluble cytoplasmic fraction. To address this question, we lysed cells in the absence of detergent and removed the unlysed cells and nuclei by centrifugation at low speed. The resulting supernatant was subsequently centrifuged at 100,000 g for 1 h, resulting in a pellet consisting of membrane compartments and a soluble cytoplasmic fraction. Separation of both fractions was confirmed by Western blotting with antibodies specific for calnexin (membrane fraction) and aldolase (cytoplasm) (data not shown). Equal proportions of the fractions generated were incubated with the Apg8-like probes (Fig. 4). For each of the probes, the ~70-kDa adduct was formed in the low speed supernatant and the 100,000 × g/1-h supernatant, but not in the low speed pellet and 100,000 × g/1-h pellet fractions (Fig. 4). The cellular activity that gives rise to the formation of the ~70-kDa adduct is thus a soluble, cytoplasmic factor not tightly associated with membranes.

Ubiquitous Expression and Activity of the Polypeptides Labeled by GATE-16-VS, MAP1-LC3-VS, GABARAP-VS, and Apg8L-VS—In EL-4 cells, a single polypeptide was labeled with each of the Apg8-like probes, resulting in adducts of ~70 kDa (Fig. 3). It is possible that EL-4 cells do not express all existing target proteins for these probes and that some of these
with its inactive counterpart (HA-MAP1-LC3–119) as a control for nonspecific binding. Comparison of the profiles obtained showed the presence of a specific adduct at ~70 kDa (Fig. 7A, arrowhead). This band was excised from the gel and processed for sequence analysis by tandem mass spectrometry. Analysis of the peptide sequence data and searches of the available data bases revealed the target to be the putative cysteine protease Apg4B/autophagin-1. The identification was based on five unique matching peptide sequences, which correspond to 22% sequence coverage of the 393 amino acid sequence of Apg4B (Fig. 7B). The matched peptide sequences are unique to Apg4B and not present in Apg4A, Apg4C, and Apg4D (data not shown; Ref. 28). In addition, a peptide corresponding to a sequence of MAP1-LC3 was identified (Fig. 7B). The observed ~70-kDa species therefore consists of Apg4B as a covalent adduct with HA-MAP1-LC3-VS. As the competition experiments had indicated that GATE-16-VS, MAP1-LC3-VS, GABARAP-VS, and Apg8L-VS target the same polypeptide in EL-4 cell lysates, we conclude that all four Apg8-like probes target Apg4B.

**DISCUSSION**

The reactivity of the Apg8-like probes with a single polypeptide in crude EL4 lysates immediately distinguishes this labeling pattern from that obtained with similarly modified ubiquitin derivatives, which label up to 23 polypeptides in the same lysates (30, 32). The protein modified by the Apg8-like probes was identified by mass spectrometry as mouse Apg4B/autophagin-1. The identification of Apg4B/autophagin-1 in no way relied on any previous assumptions as to its specificity. Apg4B is one of four mammalian Apg4 homologues (named Apg4A-D or autophagin1–4), identified on the basis of sequence homology to yeast Apg4 (28). The Apg4 homologues have been annotated as members of the C54 family of cysteine proteases in the MEROPS protease data base (28). Apg4B consists of 393 amino acids and has a predicted molecular mass of 44.4 kDa and a pl of 4.93. In yeast, Apg4 has been characterized as a novel cysteine protease that cleaves Apg8 after its C-terminal glycine residue (8, 9). Apg4 functions both as a processing and deconjugating protease, and both functions are required for efficient progression of autophagy (8). The catalytic cysteine of Apg4 was identified (8), indicated for mouse Apg4B in Fig. 7B. Cys and His boxes similar to those of the Ub-specific protease family were tentatively assigned based on sequence conservation and invariant Cys and His residues (Ref. 8; Fig. 7B). Indeed, we observed complete abrogation of labeling of Apg4B by inclusion of the thiol-alkylating agent NEM in labeling reactions (Fig. 3), consistent with the requirement of an active-site cysteine for labeling and therefore activity. Apg4B is a soluble, cytoplasmic protein, as demonstrated by subcellular fractionation (Fig. 4). This finding is in agreement with reports in yeast, where Apg4 was also found in the soluble fraction after centrifugation at 100,000 × g for 1 h (8, 9). Apparently, Apg4 does not require strong association with membranes to deconjugate Apg8 from the lipid to which it is attached. Although this is the first report on the subcellular...
localization of a mammalian Apg4 homologue, further investigations, by immunocytochemistry, are needed to further define its localization. The probes used can only result in formation of covalent thioether adducts upon nucleophilic attack by active site residues on the correct carbon atom in the C-terminal position (Fig. 2, steps 4 and 5). Therefore, the observed reac-
Fig. 6. GATE-16-VS, MAP1-LC3-VS, GABARAP-VS, and Apg8L-VS cross-compete for the same target protein. Radio-iodinated and unlabeled probes were mixed together before incubation with EL-4 cell lysate. 80 µg of EL-4 lysate and 1–2 × 10^6 cpm 125I-labeled probe per reaction were used. Amounts of unlabeled probe added increased in 3-fold increments. For each unlabeled probe, the same amounts were used for competition with all different iodinated probes, although amounts used differed slightly between unlabeled probes (see “Experimental Procedures” for details). The reactions were terminated as described before. Polypeptides were resolved by 11% SDS-PAGE and visualized by autoradiography. The radiolabeled probes are indicated on the left, and the molecular weights of the adducts examined are indicated on the right. The unlabeled competing probes, as well as the other components added, are indicated on the top: [125I]GATE-16-VS (A), [125I]MAP1-LC3-VS (B), [125I]GABARAP-VS (C), and [125I]Apg8L-VS (D).

Fig. 7. Isolation and identification of polypeptides reactive with HA-MAP1-LC3-VS. A, 8 mg of EL-4 lysate was incubated with 100 µg of HA-MAP1-LC3-VS or its inactive counterpart HA-MAP1-LC3 1–119. Probes and probe-enzyme adducts were immunopurified using Sepharose beads conjugated to anti-HA antibody 12CA5. Precipitates were extensively washed and proteins eluted with 100 mM glycine, pH 3.0, and evaporated to dryness. The pellet was solubilized in 1× reducing SDS sample buffer and resolved by 11% SDS-PAGE. The position of the free probes as well as the antibody light chain (LC) are indicated on the right. The position of the band identified by tandem mass spectrometry is indicated by a black arrowhead on the gel. B, the amino acid sequences of the proteins identified in the band indicated in panel A, Apg4B/autophagin-1 and MAP1-LC3, are shown. The peptides identified by mass spectrometry are highlighted in red. In Apg4B, the Cys and His boxes, similar to those of the Ub-specific protease (UBP) family, are underlined (residues 69–77 and 251–280, respectively). The putative catalytic cysteine residue in the Cys box (Cys-74) is highlighted in blue (8).
tivity of Apg4B with the Apg8-like probes demonstrates that a mammalian member of the Apg4 family can cleave Apg8 homologues after their C-terminal glycine. Our probes do not allow us to distinguish between processing and deconjugating activity of this class of proteases, although they are likely to be one and the same.

Members of the Ub C-terminal hydrolase, Ub-specific protease, and SUMO-specific protease families show remarkable structural conservation of their active site Cys, His, and Asp residues, as determined by x-ray crystallographic analysis, in the absence of sequence similarities (34–36). Whether the Apg4 proteases also conform to this active site configuration will have to await their structural analysis.

A remarkable finding is the observation that a single protease, Apg4B, can act on the C terminus of four different mammalian Apg8 homologues. These Apg8 homologues differ from each other in amino acid sequence by as much as 71%. Nevertheless, the competition experiments with the Apg8-probes indicated that the affinity of each of the probes for Apg4B is roughly comparable (Fig. 6). In addition, GATE-16, MAP1-LC3, and GABARAP are served by a single mammalian E1 activating and E2 conjugating enzyme, Apg7 and Apg3, respectively (21, 22). Thus, the entire conjugating and deconjugating machinery has been evolutionarily conserved, whereas Apg8 itself has given rise to a set of homologues that diverged considerably.

An important clue to understand how a single set of enzymes can act on such a diverse set of substrates comes from structural analysis (19). When the positions of identical and homologous residues of the multiple Apg8 homologues are mapped onto the surface of the structure of GABARAP, one face of the molecule, with many charged residues, was highly conserved among Apg8 homologues, whereas the other face shows almost no sequence conservation (19). The conserved surface is thus likely to be involved in protein-protein interactions common to all Apg8 homologues, such as interactions with Apg7, Apg3, and Apg4B. In contrast, the unique face of each Apg8-like protein may allow interactions specific and relevant for the function of each individual homologue.

In all cell lysates tested, only a single adduct of ~70 kDa was detected with all four Apg8-like probes (Figs. 3 and 5). In EL-4 cell lysates this band was identified as Apg4B (Fig. 7). No peptides that matched Apg4B-related proteins were identified in the excised polypeptide. The adducts observed in tissue extracts had apparent molecular masses indistinguishable from the adduct in EL-4 cell lysate, and may therefore also correspond to an Apg4B adduct (Fig. 5). This we consider the most likely interpretation. However, the predicted molecular mass of Apg4A is very similar to Apg4B (45.2 and 44.4 kDa, respectively), and we can therefore not exclude that the adducts in tissue extracts contain Apg4A, or even yet other proteins. The slight labeling differences observed between the probes could probably be explained by the presence of a target of a molecular mass identical to that of Apg4B, in which case Apg4A might be such a candidate. However, analysis by mass spectrometry did not reveal the presence of this enzyme. It seems unlikely that the adducts observed in tissues involve Apg4C or Apg4D, as these proteins are considerably larger than Apg4B, having predicted masses of 52.1 and 52.9 kDa, respectively.

The role of the other Apg4 homologues (Apg4A, Apg4C, and Apg4D) in the modification of Apg8 homologues remains unknown. These Apg4 homologues may not be expressed in the cell line and tissues examined in this study. Instead they might be present in particular cell types or at particular developmental stages, although the transcript of Apg4C was reportedly detected in a broad array of tissues (28). Alternatively, targeting of the other Apg4-like proteases might require different electrophilic C-terminal substituents, as observed for ubiquitin-specific proteases of the OTU family (30). It remains possible therefore that these Apg4 homologues can also cleave some or all of the Apg8-like proteins used in this study. Human Apg4A has indeed been reported to be able to process the GATE-16 C terminus in vitro (37). Alternatively, these Apg4 homologues might be specific for other members of the Apg8 family in mammals (Fig. 1C), or for yet other (ubiquitin-like) proteins. Generation of probes based on other Apg8 homologues is required to assess this. However, some of these Apg8-like proteins might not be processed at their C-terminal glycine in vivo, as was recently discovered for MAP1-LC3B (17). In addition, some Apg4 homologues might be responsible for processing of Apg8-like proteins, whereas others are responsible for their deconjugation.

Although GABARAP has been implicated in GABA receptor sorting and/or targeting in neurons, it is expressed in all tissues, including brain (15). GATE-16 protein is found in spleen, kidney, liver, and brain (10). MAP1-LC3 and Apg8L transcripts were also broadly detected, and were present in spleen, kidney, brain, and liver (17, 38). Therefore, for each of these proteins, detection of protease activity specific for their C termini in tissues coincides with the expression pattern of the respective Apg8-like proteins. In general, the broad expression patterns of the Apg8 homologues (10, 15, 17, 38), their activating and conjugating enzymes (22, 23), as well as their cognate active proteases (Fig. 5) implies an involvement of these modification pathways in the control of important constitutive cellular processes.

The precise role of the Apg8 homologues in mammalian cells remains to be established. These Apg8-like proteins function as ubiquitin-like modifying proteins and share conserved conjugation and deconjugation enzymes. Their conjugation targets remain to be identified, although it is tempting to speculate that these are lipids. Their limited sequence homology implies a divergence in cellular function for each of these modifiers, whereas the high level of conservation for each of these Apg8-like modifiers in mammals indicates evolutionarily conserved functions (Fig. 1B). They might be redundant in a tissue-specific fashion or have specialized functions and be subject to coordinate regulation by the modifying enzymes (thereby altering their membrane binding state). This might occur at different stages of the same pathway (such as autophagy), or in different pathways altogether (such as different membrane sorting processes). MAP1-LC3, GATE-16, and GABARAP have so far all been implicated in membrane processes (10–12). MAP1-LC3 is involved in autophagy in mammalian cells and seems to play a role very similar to Apg8 in yeast (11). GATE-16 has been proposed to regulate intra-Golgi transport, through interactions with NSF, SNAP, and GOS-28, a Golgi v-SNARE (10, 13). However, in yeast NSF, SNAP, and SNARE factors regulate fusion of the autophagosome with the vacuole, but not autophagosome formation itself (39). It remains possible, therefore, that GATE-16 plays a role at a later stage of autophagy in mammalian cells, namely in autophagosome/lysosome fusion. GABARAP localizes to intracellular membranes and reportedly interacts with NSF, a GABA receptor subunit and tubulin (12, 14, 16). This has led to proposals for the role of GABARAP role in intracellular membrane transport, in particular in receptor sorting and/or targeting in neurons. However, as indicated earlier, GABARAP is ubiquitously expressed, suggesting a broader role outside neuronal tissue. In fact, GABARAP, as well as GATE-16, have been detected on autophagosomal structures (40). Nothing is known about the sub-
cellular localization of Apg8L. It may well be therefore that all mammalian Apg8 homologues play a role in autophagy.

Human Apg4B and Apg4C are able to complement the autophagy defect in an Apg4 deficient yeast strain (28). The active site-directed probes described here are mechanism-based inhibitors, and therefore labeling intensity of the target enzymes is proportional to the amount of active enzyme. To investigate whether the activity of Apg4B was affected by the induction of autophagy in mammalian cells, we labeled lysates prepared from cells under starvation conditions. No difference in labeling intensity of the ~70-kDa adduct was observed for any of the Apg8-like probes used (data not shown). Apg4B activity might therefore not be regulated during autophagy, which would be consistent with data obtained in yeast, where Apg8 is the only gene for which expression is enhanced during starvation (3).

The differential roles of the Apg8 homologues clearly need further study, and these proteins must be examined in parallel to assess differences in subcellular distribution and function. The roles of Apg4B and the other Apg4 homologues also need further clarification. A determination of their subcellular distribution in relation to the Apg8 homologues may prove informative. If the Apg8-like probes described in this study can be rendered cell-permeable, they could be used to study the effect of specific inhibition of Apg4 proteases on cellular physiology.

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