Human Autophagins, a Family of Cysteine Proteinases Potentially Implicated in Cell Degradation by Autophagy*

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We have cloned four human cDNAs encoding putative cysteine proteinases that have been tentatively called autophagins. These proteins are similar to Apg4/Aut2, a yeast enzyme involved in the activation of Apg8/Aut7 during the process of autophagy. The identified proteins ranging in length from 393 to 474 amino acids also contain several structural features characteristic of cysteine proteinases including a conserved cysteine residue that is essential for the catalytic properties of these enzymes. Autophagins are broadly distributed in human tissues, being especially abundant in skeletal muscle. Functional studies directed to the functional characterization of these proteins are required for yeast autophagy mutants has revealed that two ubiquitin-like conjugation systems are required for yeast autophagy (15, 16). The Instituto Universitario de Oncología is supported by Obra Social Cajastur-Asturias. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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This article has been withdrawn by the authors upon request from the Journal. The Journal raised questions regarding Figs. 3 and 4A. The authors were able to locate original data for some panels, and for the others, the authors state that new experiments were performed. The authors assert that all of the results reported in this article are valid, some of which have been validated in the literature by different groups (e.g. Choi et al. (2011) Autophagy 7, 1052; Hemelaar et al. (2003) J. Biol. Chem. 278, 51841; Shu et al. (2010) Autophagy 6, 936; Tanida et al. (2004) Int. J. Biochem. Cell. Biol. 36, 2503).

Proteases are initially characterized as nonspecific degradative enzymes associated with protein catabolism, but recent studies have demonstrated that they influence a wide range of cellular functions by processing multiple bioactive molecules. These essential processes initiated, regulated, or terminated by proteases include DNA replication, cell-cycle progression, cell proliferation, differentiation and migration, morphogenesis and tissue remodeling, and angiogenesis and apoptosis (1). An additional process in which proteolytic enzymes have also been implicated in the regulation of cell division is cell autophagy. This cellular process allows the degradation of organelles and has been suggested to be essential for cell survival under starvation conditions. The vacuolar proteases and recycled.

The knowledge of the molecular mechanisms underlying autophagy has considerably improved after the isolation and characterization of autophagy-defective mutants in the yeast Saccharomyces cerevisiae (12, 13). These mutants were derived from screening for starvation-sensitive yeast strains (atg mutants) or for strains defective in the degradation of specific cytosolic proteins (aut mutants). These mutants partially overlap with those isolated in genetic screens for yeast strains defective in the cytoplasm to vacuole-targeting pathway (cvt mutants), a process that shares significant morphological and mechanistic similarities with autophagy (14). A series of elegant studies directed to the functional characterization of these autophagy mutants has revealed that two ubiquitin-like conjugation systems are required for yeast autophagy (15, 16). The

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first one is initiated by Apg12, a modifier protein whose C-terminal Gly residue forms a covalent isopeptide bond with a Lys residue from Apg5. This conjugation process involves an activating E1-like enzyme called Apg7 and a conjugating E2-like enzyme named Apg10 (17, 18). The second ubiquitin-like system requires the participation of Apg8/Aut7 synthesized as a precursor protein, which is cleaved after a Gly residue by Apg4/Aut2, a recently described cysteine proteinase (2, 19, 20). This Gly-terminal residue from the modifier Apg8/Aut7 is also activated by Apg7, but then the modifier protein is transferred to Apg3 and finally conjugated with membrane-bound phosphatidylethanolamine (PE) through an amide bond (16).

The complex Apg8-PE is also deconjugated by the protease Apg4/Aut7, leading to the release of Apg8/Aut7 from membranes. These modification systems are essential components of the membrane rearrangement dynamics taking place during the formation of autophagosomes and execution of autophagy. Recent studies (21–26) have shown that these ubiquitin-like conjugation systems associated with autophagy in yeast are conserved in higher eukaryotes.

In this work, we report the identification and characterization of four human proteins closely related to yeast Apg8/Aut7. We also report the tissue distribution and a preliminary analysis of the enzymatic properties of these proteins, tentatively called autophagins. Finally, we describe a yeast autophagy defect observed in cells lacking one of these proteins, providing further evidence for the existence of a higher eukaryotes of essential components of the autophagy pathway in yeast.

EXPERIMENTAL

Materials—The S. cerevisiae Apg8/Aut7 homologous in the process of autophagy, was kindly provided by Dr. M. J. Mazo (Instituto de Investigaciones Biomédicas, Madrid, Spain). Penta-His monomolecular antibody against His6 tag was purchased from Qiagen (Valencia, CA).

Bioinformatic Screening of the Human Genome and cDNA Cloning—The advanced BLAST program from the National Center for Biotechnology Information was used to search human genome databases looking for regions encoding putative proteins with sequence similarity to yeast Apg4/Aut7. This computer search led us to identify DNA contigs in chromosomes 1, 2, 19, and X containing regions with significant sequence similarity to Apg4/Aut7. To obtain full-length cDNA sequences corresponding to the putative proteins encoded by these DNA contigs, we designed specific oligonucleotides for each of them and performed PCR experiments using a panel of commercially available cDNA libraries (Clontech) and the Expand High Fidelity PCR system (Roche Molecular Biochemicals). All PCR assays were carried out in a GeneAmp 9600 PCR system (PerkinElmer Life Sciences) for 40 cycles of denaturation (94 °C, 15 s), annealing (64 °C, 15 s), and extension (68 °C, 60 s). Full-length cDNAs were cloned into pBluescript vector and characterized by nucleotide sequencing.

Nucleotide Sequence Analysis—Full-length cDNAs were sequenced by the dye terminator chain termination method using the Sequenase version 2.0 kit (U.S. Biochemicals, Cleveland, OH) and the ABI-Prism 310 DNA sequencer (Applied Biosystems). All of the nucleotides were identified in both strands. Computer analysis of DNA and protein sequences was performed with the GCG software package of the University of Wisconsin Genetics Computer Group.

Human Autophagins—The amino acid sequence of the barley Apg8/Aut7 homolog (27) was aligned to the human ortholog sequences in the database using the Clustal W program (28). Phylogenetic relationships were calculated using the neighbor-joining method implemented in the Clustal W program (28) and visualized in a full phylogram using TreeView (29). Amino acid residues conserved in all sequences were determined using the program ConSurf (30) and visualized in a 3D model using the Swiss-PdbViewer software (31). The three-dimensional structure of human Apg8/Aut7 (27) was constructed using the I-TASSER software (32) and visualized using the PyMol software (33).

Immuno blotting and Complementation Studies—Cultures of parental and autophagin-transformed yeast cells were grown in complete YPD or selective medium (synthetic medium/Leu−1 or -Trp−1) for 16 h at 28 °C. For immunoblotting studies, Apg8/Aut7 was normalized to 3.0 and extracts were obtained by lysis with 1.85 M NaOH and 7.4% β-mercaptoethanol. Proteins were precipitated with 25% trichloroacetic acid followed by centrifugation at 14,000 × g. The pellets were resuspended in urea buffer (5% SDS, 8 M urea, 200 mM Tris/HCl pH 6.8, 0.1 mM EDTA, and bromophenol blue), incubated for 10 min at 60 °C, and loaded onto 13% SDS-PAGE gels. Western blots were probed in 5% milk in PBS (PBS containing 0.1% Tween 20) and then incubated for 1 h with rabbit anti-His antibody against API dilution of 1:1000 in PBS. After three washes in PBS, blots were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG at a 1:20,000 dilution and developed with the Renaissance chemiluminescence kit (PerkinElmer Life Sciences). For complementation studies, cells grown overnight were subjected to starvation for 4 h in a medium containing 0.1% potassium acetate and 1 mM PMSF, collected, fixed, and visualized in a phase-contrast microscope.

Autophagin-3 Expression and Purification—Autophagin-3 cDNA was cloned in the expression vector pCEP-Pu provided by Dr. E. Kohfeldt (Max-Planck-Institut fur Biochemie, Martinsried, Germany) and expressed in the E. coli expression strain XL1-Blue (34). The constructed plasmid was transformed into competent E. coli strain XL1-Blue using the GenePilot II system (Qiagen). The recombinant cells were grown overnight in LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol and the isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. Cells were harvested by centrifugation and the inclusion bodies were resuspended in buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM PMSF, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) and treated with 0.5% Triton X-100 to disrupt the inclusion bodies. The solution was incubated for 1 h at 4 °C and then centrifuged at 13,000 × g for 20 min. The supernatant was loaded onto a HisChrom 600 Protein A (Qiagen). The affinity-purified protein was dialyzed against buffer B (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM PMSF, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) and a nickel column (Qiagen), washed with 15 column volumes of buffer B, and eluted with buffer C. Fractions containing the active protein were pooled, concentrated, and dialyzed against buffer D (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM PMSF, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) and stored at −80 °C. The purity of the recombinant protein was assessed by SDS-PAGE and western blotting using a mammalian cell lysate (35) or recombinant E. coli lysate as a control. The catalytic activity of Autophagin-3 was determined using the substrate 4-methylumbelliferyl-β-D-glucuronide (36) in the presence of 1 mM MgCl2 and 1 mM PMSF.
Enzymatic Assays—Enzymatic activity of recombinant autophagin-3 was tested using the fluorogenic peptide Mca-Thr-Phe-Gly-Met-Dpa-NH2 synthesized by Dr. C. G. Knight (University of Cambridge, Cambridge, United Kingdom). Enzyme assays were performed with the purified protein at 30°C and at substrate concentration of 5 μM in an assay buffer of 50 mM Tris/HCl, pH 7.5, 125 mM NaCl, 1 mM dithiothreitol, 10 mM EDTA, and 2 mM AEBSF. The fluorometric measurements were made in a LS 50-B PerkinElmer spectrofluorometer (λex/λem 328 nm and 393 nm, where "ex" stands for excitation and "em" for emission). For the pH profile, assays were performed in assay buffer as noted above but using 50 mM bisTris, pH 6–9, 50 mM sodium acetate, pH 4.5–5.5, or 50 mM glycine, pH 10, as buffer for the indicated pH range and containing 2.5 mM fluorogenic substrate and 10 ng of enzyme. Kinetic studies were carried out using different concentrations of the fluorogenic peptide (0.5–5 μM) in 2 ml of assay buffer containing 10 ng of autophagin-3, and peptide hydrolysis was measured as the increase in fluorescence at 25°C for 15 min. Initial velocities were calculated using the analysis package FL WinLab 2.01 (PerkinElmer), and kcat/Km ratio was calculated as described previously (28). For inhibition experiments, reaction mixture was preincubated for 30 min at 20°C with 1 mM N-ethylmaleimide (Sigma).

RESULTS

Identification and Cloning of cDNAs Encoding Four Novel Human Proteins Similar to Yeast Apg4/Aut2 Cysteine Proteinase—To identify human proteins related to the yeast Apg4/Aut2 protease involved in autophagic processes, we used the BLAST algorithm to screen the human genome databases looking for DNA sequences encoding putative proteins similar to the S. cerevisiae protease. This search allowed us to identify five DNA contigs in chromosomes 1p31.3, 2q37, 19p13.2, Xq13, and Xq22, which contained coding information for putative cysteine proteinases related to yeast Apg4/Aut2. Preliminary analysis of the DNA contig in chromosome Xq13 revealed the presence of several stop codons in different regions of the putative protease coding region present in this contig, indicating that it corresponds to a pseudogene unable to encode a functional enzyme. To generate cDNA clones for the remaining four genes, we carried out PCR amplifications using a panel of human cDNA libraries and specific oligonucleotides derived from the identified genomic sequences. DNA fragments large enough to encode complete Apg4/Aut2-like proteins (~1.4 kb) and containing in-frame initiator and stop codons were amplified from cDNA libraries prepared from human testis, liver, ovary, and brain. After cloning and sequencing the PCR-amplified products, we concluded that the isolated cDNAs coded for proteins of 393, 398, 458, and 474 amino acids (Fig. 1) (GenBank™ accession numbers AJ504652, AJ504651, AJ312234, and AJ312332). Further structural analysis of these amino acid sequences confirmed that they were closely related to yeast Apg4/Aut2 with the percentage of identities ranging from 32 to 25%. Because of the relevance of this yeast protease during the process of autophagy, we have tentatively called autophagins to this family of Apg4/Aut2-related human proteins. Autophagins-1 and -2 matched perfectly with sequences originally predicted from human ESTs and identified because of their homology with yeast Apg4/Aut2 (2). cDNA sequences closely related or identical to those here identified for human autophagins and derived in most cases of large scale sequencing projects have also been recently deposited in databases (GenBank™ accession numbers AL080168, KIAA0943, and AB066215 for autophagin-1; GenBank™ accession numbers AJ320508 and AB066214 for autophagin-2; GenBank™ accession numbers AJ320169, BC008395, and BC033024 for autophagin-3).
Autophagins are BAB88384, BAB88383, Z68302, AL110500, CG6194, and CG4428. It has never been published yet. No report describing the cloning and characterization of any of these human cDNAs or of their encoded proteins has been published yet.

An alignment of the deduced amino acid sequence for human autophagins confirmed the high level of sequence similarity with yeast proteins and with the entire protein sequence with the exception of divergences in both the N- and C-terminal ends of these proteins (Fig. 1). Human autophagins also exhibit structural features characteristic of the yeast protease including a putative active site Cys residue at positions 74, 77, 110, and 134 in autophagins 1–4, respectively (Fig. 1). The amino acid sequences surrounding this Cys residue are highly conserved between human and yeast proteins and include a Gln residue (positions 80, 83, 116, and 140 in autophagins 1–4), which can be part of the exon ion hole present in the structure of cysteine proteases (29). Human autophagins also contain some conserved His and Asp or Asn residues (Fig. 1) that can correspond to the equivalent residues present in other cysteine proteases and found to be essential in the catalytic process (29). The absence in autophagins of a recognizable hydrophobic signal sequence close to the initiator methionine is also remarkable. This indicates that these proteins are cytoplasmic enzymes and therefore distinct from members of the large papain family of secreted cysteine proteases (30). All of these structural features are also absolutely conserved in the amino acid sequence of the mouse orthologues of the four human autophagins whose sequence was deduced from information derived from publicly available ESTs (Fig. 2) (GenBank™ accession numbers AJ504653, AJ504654, AJ312233, and AJ312333 for mouse autophagin-1–4, respectively) as well as in the autophagin-like proteins present in other organisms such as Drosophila melanogaster and Cae...

**Fig. 2.** Phylogenetic tree of the autophagin family. Amino acid sequences of the different human and mouse autophagins and of related proteins identified in Arabidopsis thaliana, C. elegans, and D. melanogaster were aligned using the Phylip program package (version 3.6). Numbers represent reliability values after bootstrapping the data. GenBank™ accession numbers for autophagin-3 and -4 are BAB88384, BAB88383, Z68302, AL110500, CG6194, and CG4428.
expression were detected in the examined fetal tissues (Fig. 3).

Finally, we also addressed the possibility that autophagins could be expressed by human cancer cell lines from different sources. For this purpose, we hybridized a Northern blot containing poly(A)^+ RNAs extracted from different cell lines (HL-60, HeLa, K-562, MOLT-4, Burkitt’s lymphoma Raji, colorectal adenocarcinoma SW480, lung carcinoma A549, and melanoma G361) with probes for the different autophagins. As shown in Fig. 3, autophagin-1 was widely expressed in these cells with the strongest signal observed in A549 and Raji cells. Autophagin-3 was also detected in diverse cell lines such as HeLa, MOLT-4, and Raji, whereas autophagin-4 was strongly expressed in the chronic myelogenous leukemia cell line K-562 and at lower levels in SW480 and HeLa cells.

Complementation Studies with Human Autophagins in Autophagy-defective Yeast Strains

To study the putative implication of the identified proteins in the process of autophagy, we cloned the full-length cDNAs for the four human autophagins in the yeast expression vector pGAD424 (32) under the control of the constitutive ADH1 gene promoter, obtaining four new plasmid constructs pGAD-Autophagin1, pGAD-Autophagin2, pGAD-Autophagin3, and pGAD-Autophagin4. These plasmid constructs were used to transform the *S. cerevisiae* autophagy-defective mutants strains aut2 and aut7, and the properties of the transformed yeasts in terms of restoration of biochemical and morphological markers of autophagy were analyzed.

For this purpose, we first examined the processing of the vacuolar hydrolase API from its inactive precursor, a process that is defective in autophagy yeast mutants. Thus, wild-type cells and aut2 mutants carrying the autophagin cDNAs were grown overnight in YPD or selective medium (synthetic medium/Leu^+^). The cells were collected, lysed in SDS-PAGE sample buffer, and analyzed by Western blot with a polyclonal rabbit antiserum against API. Transformed aut2 mutant cells lacking the endogenous Apg4/Aut2 activity but carrying the autophagin-1 or the autophagin-3 cDNAs were able to complete the processing of proAPI (Fig. 4A). By contrast, the parental aut2 mutant cells were unable to perform the processing of this marker that signals the integrity of the autophagic process. When the same experiments were performed with autophagin-2 and -4 cDNAs, no obvious processing of proAPI was observed, indicating that these human enzymes do not behave as autophagin-1 and -3 in their ability to restore the autophagy deficiency in aut2 mutant yeasts (data not shown). To rule out the possibility that the expression of autophagin-1 or -3 in aut2 cells could complement their autophagy deficiency through a nonspecific effect, aut7 mutant cells that lack the Apg4/Aut2 substrate and are also deficient in autophagy were transformed...
with autophagin-1 or -3 cDNA and analyzed as above. As shown in Fig. 4A, these transformed yeast cells were unable to complete the proAPI processing, confirming that these human autophagins specifically complement the autophagy deficiency derived from absence of the yeast protease.

We also tested the ability of the autophagy-defective yeast mutants carrying autophagin-1 or -3 to accumulate autophagic bodies in the vacuole, a characteristic feature of the autophagic degradation pathway. To do that, wild-type or mutants transformed or not with autophagin-1 or -3 cDNA were grown in YPD or selective medium overnight and then transferred to a medium containing 1 mM PMSF. This serine proteinase inhibitor blocks proteolysis, thereby hampering the autophagy proceeding to the accumulation of autophagic bodies (Fig. 4B). After 4 h of incubation, cells were collected and analyzed. In agreement with the accumulation of proAPI, only aut2 mutants were able to complete autophagy using either autophagin-1 or -3 cDNA were able to complete autophagy in vivo, completing the transport of vesicles to the vacuole (Fig. 4B).

**Enzymatic Assays of Human Autophagin-3**—To test the enzymatic activity of human autophagin-3, we first cloned the full-length cDNA into an expression vector pCEP-Pu containing the BM40 signal peptide to allow protein secretion to the cytoplasm to be directed to the expression vector pCEP-Pu containing the BM40 signal peptide to allow protein secretion to the cytoplasm. For this purpose, we first cloned the full-length cDNA for human autophagin-3 into the expression vector pCEP-Pu containing the BM40 signal peptide to allow protein secretion to the cul

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**Fig. 5. Production of recombinant autophagin-3 in mammalian cells and enzymatic analysis.** A, 50 µl of conditioned medium from 293EBNA cells transfected with pCEP-Autophagin3-His (lane 1) and with pCEP-Pu (lane 2) were analyzed by Western blot using the penta-His monoclonal antibody (Qiagen). The sizes of the molecular mass markers are shown on the left. B, fluorogenic peptide Mca-Thr-Phe-Gly-Met-Dpa-NH2 (5 µM) was incubated with 10 ng of purified autophagin-3 in the absence (○) or presence (●) of 1 mM NEM in 50 mM Tris/HCl, pH 7.5, 125 mM NaCl, 1 mM dithiothreitol, 10 mM EDTA, and 2 mM AEBSF. The fluorometric measurements were made at λem = 528 nm and at λex = 393 nm. Activity is expressed as nanomoles of cleaved substrate per microgram of autophagin-3.

C, rate assays were performed using Mca-Thr-Phe-Gly-Met-Dpa-NH2 as substrate and 50 mM bisTris, sodium acetate, or glycine as buffer (see “Experimental Procedures”).

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**Experimental Procedures**

To test the enzymatic activity of this protease, the pH profile was determined using the fluorogenic substrate Mca-Thr-Phe-Gly-Met-Dpa-NH2. As shown in Fig. 5C, recombinant autophagin-3 exhibited a pH optimum of 7.5, which is identical to that used for analysis of proteolytic activity of yeast Apg4/Aut2 (2). Studies aimed at producing the remaining human autophagin...
family members in this expression system are currently in progress. The future availability of these recombinant proteins will allow us to evaluate the similarities or differences in the pattern of substrates targeted by these enzymes.

**DISCUSSION**

Because of the expanding roles for proteolytic enzymes in the cellular control of multiple biological processes, there has been an increasing interest in the identification and functional characterization of the human degradome, the complete set of proteins related to this process in human tissues (1). In this work, we describe a new family of human proteases called autophagins because of their structural and functional similarity with a yeast cysteine protease involved in the development of autophagy. The approach followed to identify human autophagins was first based on a computer search of the human genome sequence databases looking for regions with similarity to yeast Apg4/Aut2. After identification of several DNA sequences encoding proteins related to this yeast protease and PCR amplification experiments using human cDNA libraries as template, full-length cDNAs coding for four distinct proteins were finally isolated and characterized. A structural analysis of the identified sequences confirmed the close relationship of these four human proteins with their yeast counterpart including an absolutely conserved cysteine residue probably corresponding to the active site residue of cysteine proteases.

Consistent with these structural characteristics, functional analysis of the recombinant autophagin-3 produced in a human expression system revealed that it is a catalytically active cysteine protease. In fact, the recombinant human protease exhibits a significant proteolytic activity toward an artificial substrate designed in this work for the activity of Apg4/Aut2-related mammalian proteases. In analogy to the yeast Apg4/Aut2, the activity of Apg4/Aut2-related proteases is inhibited by N-ethylmaleimide, an inhibitor of Apg4/Aut2p that also blocks the process of autophagy in yeast. Furthermore, this sequence is absolutely conserved in two human proteins, MAP-LC3 and GATE-16, proposed to play essential roles to yeast Apg4/Aut2 in the conjugation cascade as well as Apg8/Aut7. This sequence is also conserved in the human paralogues of this yeast protein (33).

As a preliminary step to evaluate this question, we have performed experiments using human cDNA libraries as template, full-length cDNAs coding for four distinct proteins were finally isolated and characterized. A structural analysis of the identified sequences confirmed the close relationship of these four human proteins with their yeast counterpart including an absolutely conserved cysteine residue probably corresponding to the active site residue of cysteine proteases.

With the exception of autophagins-1 and -3, which complement the autophagy defect in Apg4/Aut2-deficient yeast strains, we do not have evidence yet that the two other autophagins described herein are related to autophagy in human. One possibility is that autophagins-1 and -3 are closely related in functional terms to their yeast homologue, whereas the remaining human autophagins have diverged considerably or possess specific structural or functional constraints because of the need to target different substrates. In fact, the finding that the mammalian autophagin-based proteolytic system is composed of four distinct proteases that may target at least three putative specific substrates compared with the simplified yeast system involving a single protease with a specific substrate clearly indicates that this conjugation system has acquired a high degree of complexity during eukaryote evolution. Therefore, the observation that autophagins-2 and -4 do not complement the autophagy defect in Apg4/Aut2-deficient yeast strains should not be used to rule out their relevance in this process. Interestingly, hApg5 and hApg12, the human homologues of two yeast proteins essential for autophagy, do not complement the autophagy deficiency in Apg5 or Apg12 mutant yeasts (21), providing additional evidence that the complementation experiments may have limitations to extrapolate functional roles from yeast proteins to their human counterparts. It is also remarkable that GABARAP, the third human homologue of Apg8/Aut7, has a sequence around the putative cleavage site by autophagins, which markedly deviates from the consensus sequence found in Apg8/Aut7 as well as in the other human homologues of this yeast protein (33).

In this work and as a previous step to elucidate the physiological role of human autophagins, we have also examined the tissue distribution of these proteins. Similar to other cysteine proteases involved in general degradative processes, the expression of autophagins is detected in a wide variety of human tissues, albeit at low levels in most cases. This finding is consistent with the idea that autophagy is a mechanism for bulk degradation of cytosolic proteins and organelles that takes place in all cells at basal levels (3–6). Nevertheless, the observation of high expression levels of most human autophagins in skeletal muscle suggests that autophagic activity may be especially relevant in this tissue. This finding is also of particular interest in light of previous reports showing the association of autophagy abnormalities with pathological conditions involving skeletal muscle diseases and muscular dystrophies (8, 34, 35). The possibility that MAP-LC3 and GATE-16 may be derived from the two human autophagins and that the human degradome may contain a new family of human proteases related to mammalian autophagins opens the possibility to generate mice deficient in these genes that could contribute to clarifying the role of this autophagy system in physiological and pathological conditions including its specific functions in skeletal muscle.

Previous studies have also shown that the process of autophagy may be of great relevance in cancer. Thus, the finding that the tumor suppressor beclin 1 (Apg6) is an inducer of autophagy has demonstrated that components of the autophagy machinery play a fundamental role in the control of the unregulated cell growth associated with tumor development (7). Autophagy is also linked with type II (non-apoptotic) programmed cell death and may contribute to death in cells in which caspase activity is blocked (37). These findings together with the multiple observations indicating that expression and activity of many proteolytic enzymes are profoundly deregulated in cancer suggest that specific alterations in autophagin-mediated pathways may also be linked to tumor development. As a preliminary step to evaluate this question, we have performed an analysis of autophagin expression levels in human cancer cell lines. The results obtained in these experiments indicate that these proteases are overexpressed in some cancer cells, whereas they appear to be completely absent in other tumor cells. It is also worthwhile mentioning that the regions...
containing the autophagin genes are frequently altered in several human tumors (38–40). It will be of great interest to examine the possibility that autophagins may play specific roles in tumorigenesis in a similar way to that reported for other cysteine proteases, such as Unp, HAUSP, Tre-2/USP6, DUB-1, BAP1, and ubiquitin C-terminal hydrolase 1, associated with protein modification pathways that are related to those mediated by autophagins in autophagy and whose unregulated expression may be linked to cancer (41–46).

Finally, we would like to emphasize that the description of four distinct human and mouse autophagins confirms and extends previous findings proposing the widespread occurrence of this proteolytic system originally described in yeast but also found in mammals, insects, nematodes, and plants (2). Nevertheless, the complexity of the human autophagin system compared with that present in other eukaryotes provides an additional example of the impressive diversity of cysteine proteases mediating a variety of modification reactions in human tissues. To date, four different families of enzymes capable of conjugate/deconjugate protein or lipid adducts through cleavage adjacent to the C terminus of a Gly residue have been described (47, 48). These cysteine proteases include ubiquitin C-terminal hydrolases, ubiquitin-specific processing proteases (USPs or UBPs), and 4 autophagins are produced by human genome sequence analysis, at least 5 ubiquitin deconjugating properties has also been identified recently (49, 50). The large and growing number of enzymes belonging to these different families underlines the importance of conjugation/deconjugation systems in multiple biological processes (51–56). Thus, it is clear that the clarification of the functional roles of these enzymes will extend and clarify the regulation and establishment of diverse ubiquitin-related human tissues.

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