

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. Northern blot analysis demonstrated that autophagins are broadly distributed in human tissues, being especially abundant in skeletal muscle. Functional and morphological analysis in autophagy-defective yeast strains lacking Apg4/Aut2 revealed that human autophagins-1 and -3 were able to complement the deficiency in the yeast protease, restoring the phenotypic and biochemical characteristics of autophagic cells. Enzymatic studies performed with autophagin-3, the most widely expressed human autophagin, revealed that the recombinant protein hydrolyzed the synthetic substrate Mca-Thr-Phe-Gly-Met-Dpa-NH₂, whose sequence derives from that present around the Apg4 cleavage site in yeast Apg8/Aut7. This proteolytic activity was diminished by N-ethylmaleimide, an inhibitor of cysteine proteases including yeast Apg4/Aut2. These results provide additional evidence that the autophagic process widely studied in yeast can also be fully reconstituted in human tissues and open the possibility to explore the relevance of the autophagin-based proteolytic system in the induction, regulation, and execution of autophagy.

Proteolytic enzymes, through their ability to catalyze irreversible hydrolytic reactions, play crucial roles in the development and maintenance of all living organisms (1). Proteases

were 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 recently implicated is autophagy (2–4).

Autophagy is a biological process involved in the intracellular destruction of endogenous proteins and the removal of damaged organelles and has been suggested to be essential for cell homeostasis as well as for cell remodeling during differentiation, metamorphosis, non-apoptotic cell death, and aging (3–6). In addition, autophagy has also been associated with diverse pathological conditions. Thus, the reduced levels of autophagy have been described in some malignant tumors, and a role for autophagy in controlling the unregulated cell growth linked to cancer has been proposed (7). A deficiency in autophagy has also been found in heart diseases such as Danon cardiomyopathy (8). By contrast, elevated levels of autophagy have also been reported in other human pathologies, especially in neurodegenerative diseases (9). There are four distinct autophagy-related mechanisms: macroautophagy, microautophagy, crinophagy, and chaperone-mediated autophagy (3–6, 10, 11). Macroautophagy, the most widely studied mechanism in this regard and usually referred to as simply as autophagy, is a nutritionally and developmentally regulated process by which a portion of the cytosol is sequestered by an isolation membrane (3–6). This results in the formation of a structure known as autophagosome containing a double membrane, which subsequently fuses with the lysosome/vacuole. The inner membrane of the autophagosome called the autophagic body and its protein and organelle contents are then degraded by lysosomal/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 (*apg* 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 (*cut* 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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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ312234, AJ312332, AJ504651, AJ504652, AJ312233, AJ312333, AJ504653, and AJ504654.

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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 E1¹-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 fact, proteins structurally and functionally related with the diverse yeast Apg/Aut proteins have been described in mammalian cells, and their roles in the process of autophagy have been elucidated in some cases. However, to date, very little is known regarding the putative mammalian homologues of Apg4/Aut2, the yeast cysteine proteinase essential for the proteolytic activation, and subsequent lipidation and delipidation processes of Apg8/Aut7 (2, 19, 20).

In this work, we report the identification and characterization of four human proteins closely related to yeast Apg4/Aut2. We also report the tissue distribution and a preliminary analysis of the enzymatic properties of these proteins that we have tentatively called autophagins. Finally, we demonstrate that human autophagins-1 and -3 are able to complement the autophagy defect observed in yeast strains defective in Apg4/Aut2, providing further evidence on the functional conservation in higher eukaryotes of essential components of the autophagy pathway in yeast.

EXPERIMENTAL PROCEDURES

Materials—The *S. cerevisiae* strains *aut2* and *aut7*, defective in the process of autophagy, were kindly provided by Dr. M. Thumm (University of Stuttgart, Stuttgart, Germany). Yeast strains were grown in either YPD (1% yeast extract, 2% peptone, and 2% glucose) or synthetic medium (0.67% yeast nitrogen base without amino acids, 2% glucose, and auxotrophic amino acids as needed). *Escherichia coli* strain XL-Blue was used for subcloning experiments. The yeast expression vector pGAD424 was obtained from Clontech (Palo Alto, CA). Restriction endonucleases and other reagents used for molecular cloning were from Roche Molecular Biochemicals. Oligonucleotides were synthesized in an Applied Biosystems (Foster City, CA) model 392A DNA synthesizer. Double-stranded DNA probes were radiolabeled with [α -³²P]dCTP (3000 Ci/mmol) from Amersham Biosciences using a commercial random-priming kit purchased from the same company. cDNA libraries from human tissues and nylon filters containing polyadenylated RNAs from different fetal and adult human tissues were from Clontech. All of the media and supplements for cell culture were obtained from Sigma with the exception of calf serum, which was from Roche Molecular Biochemicals. Antiserum against proaminopeptidase-I (proAPI) was kindly provided by Dr. M. J. Mazón (Instituto de Investigaciones Biomédicas,

Madrid, Spain). Penta-His monoclonal 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 dideoxy 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.

Northern Blot Analysis—Nylon filters containing 2 μ g of poly(A)⁺ RNA of a wide variety of human tissues and cancer cell lines were prehybridized at 42 °C for 3 h in 50% formamide, 5 \times saline/sodium phosphate/EDTA (1 \times saline/sodium phosphate/EDTA = 150 mM NaCl, 10 mM NaH₂PO₄, 1 mM EDTA, pH 7.4), 10 \times Denhardt's solution, 2% SDS, and 100 μ g/ml of denatured herring sperm DNA and then hybridized with radiolabeled probes for each full-length clone cDNA. Hybridization was performed for 20 h under the same conditions. Filters were washed with 0.1 \times SSC and 0.1% SDS for 2 h at 50 °C and exposed to autoradiography. RNA integrity and equal loading were assessed by hybridization with an actin probe.

Transformation of Yeast Strains with Plasmid DNA—5 ml of overnight culture of yeast cells were added to 45 ml of fresh medium and incubated for 4 h at 30 °C with shaking. The cells were collected, washed with 50 mM Tris and 1 mM EDTA, and resuspended in 2 ml of 0.1 M lithium acetate. Approximately 1 μ g of plasmid DNA was added to a 200- μ l aliquot of cells and incubated for 10 min at 30 °C. Heat shock treatments were done at 42 °C for 5 min, and then cells were washed with 1 M sorbitol, were spread on selective plates, and incubated for 48 h at 28 °C.

Immunoblotting and Complementation Studies—Cultures of parental and autophagin-transformed yeast cells were grown in complete YPD or selective medium (synthetic medium/Leu⁻) for 16 h at 28 °C. For immunoblotting studies, A_{600} was adjusted 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 \times 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 blocked in 5% milk in PBT (PBS containing 0.1% Tween 20) and then incubated for 1 h with rabbit antiserum against API dilution of 1:1000 in PBT. After three washes in PBT, 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 chemoluminescence 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 für Biochemie, Martinsried, Germany) downstream of the BM40 signal peptide to allow protein secretion to the culture medium (27), and a His₆ tag was placed at the C-terminal end to facilitate purification. The resulting plasmid, pCEP-Autophagin3-His, was transiently transfected into 293EBNA cells (Invitrogen), and 24 h after transfection, culture medium was replaced by serum-free medium. Cells were allowed to secrete the recombinant protein for 48 h, and autophagin-3 was purified from the conditioned medium using a nitrilotriacetic acid-agarose column (Qiagen). Purified His₆-tagged autophagin-3 concentration was determined by Western blot using known amounts of His₆-tagged MMP-26 as standard and analyzed with the software package Phoretix 1D Advanced version 5.10 (Nonlinear Dy-

¹ The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; kb, kilobase; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; *apg/aut* and *APG/AUT*, yeast autophagy mutant and wild-type genes; Apg, expression products of APG genes; Dpa, *N*-3-(2,4-dinitrophenyl)-*L*- α , β -diaminopropionyl]; GABARAP, γ -aminobutyric acid receptor-associated protein; GATE-16, Golgi-associated ATPase enhancer of 16 kDa; MAP-LC3, microtubule-associated protein light chain 3; Mca, 7-(methoxycoumarin-4-yl)acetyl; NEM, *N*-ethylmaleimide; proAPI, proaminopeptidase I; PMSF, phenylmethylsulfonyl fluoride; USP, ubiquitin-specific processing proteases.

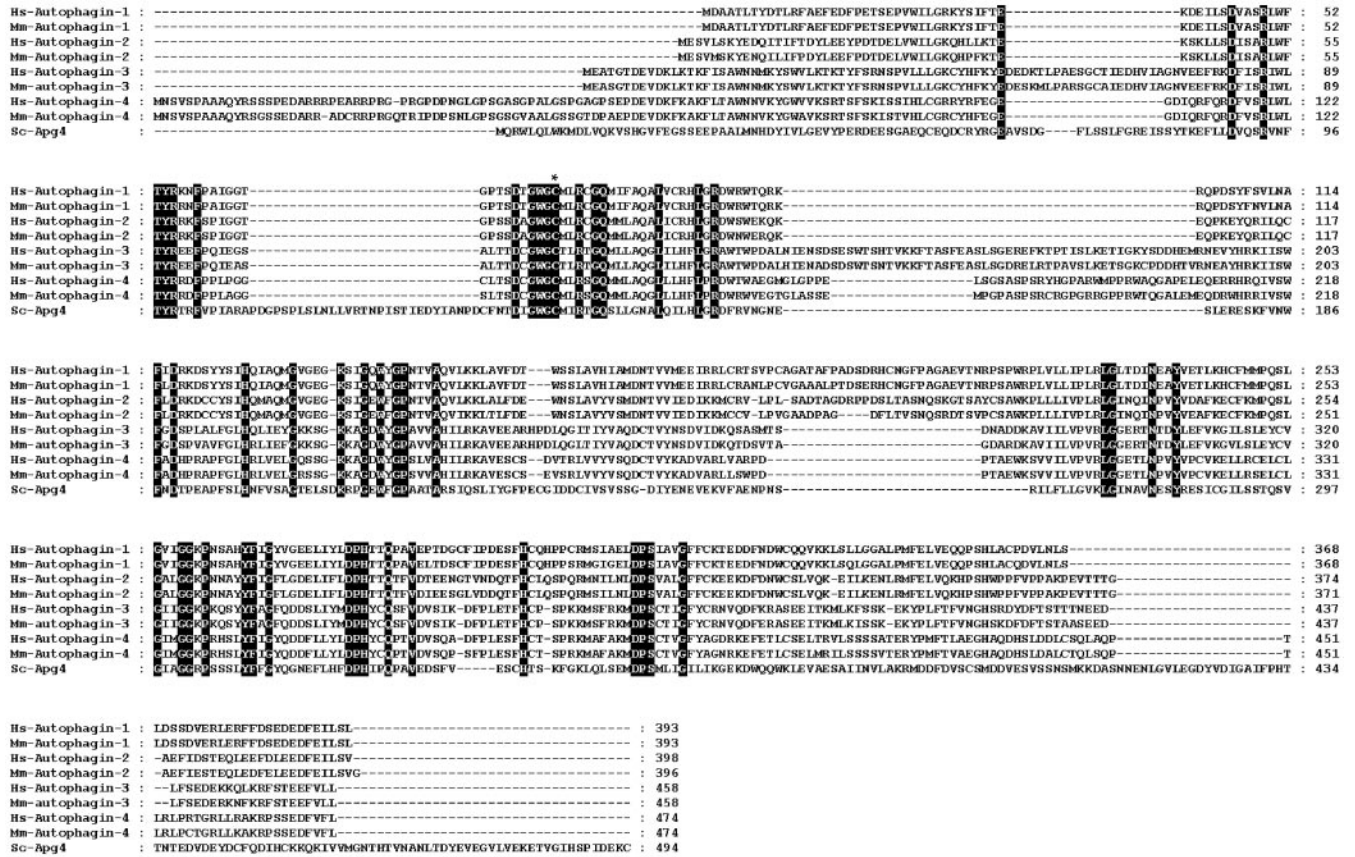


FIG. 1. Amino acid sequence of human and mouse autophagins and comparison with yeast Apg4/Aut2. The multiple alignment was performed with the ClustalX program. Gaps are indicated by *hyphens*. Common residues to all sequences are shaded. The Cys residues characteristic of cysteine proteinases are shown with an asterisk.

namics). Protein purity was confirmed by SDS-PAGE followed by silver staining.

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 AEBSEF. The fluorometric measurements were made in a LS 50-B PerkinElmer spectrofluorometer (λ_{ex} = 328 nm and λ_{em} = 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 μM 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 *k_{cat}/K_m* 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 proteases related to yeast Apg4/Aut2.

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 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 auto-

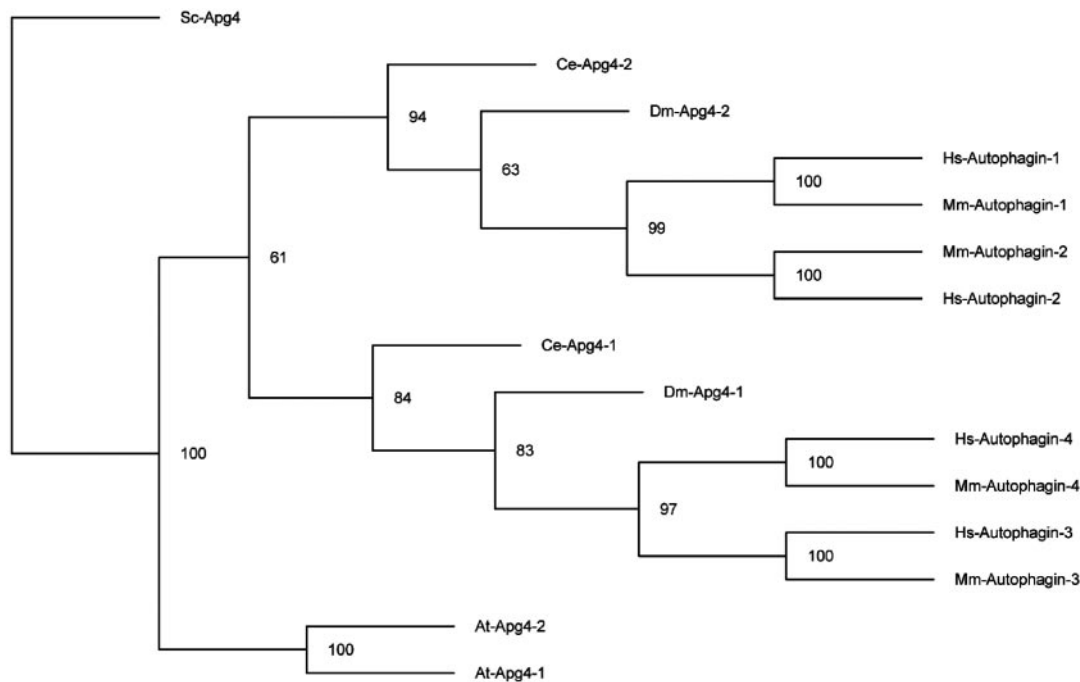


FIG. 2. **Phylogenetic tree of the autophagin family.** Amino acid sequences of the different human and mouse autophagins and of related proteins identified in *Arabidopsis thaliana*, *C. elegans*, and *D. melanogaster* were aligned using the Phylip program package (version 3.6). Numbers represent reliability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

phagin-3; and GenBank™ accession numbers NM_032885 and AK056210 for autophagin-4). These proteins have also been annotated in the protease data base MEROPS (www.merops.ac.uk) as members of the C-54 family of cysteine proteases (C54.003, C54.002, C54.004, and C54.005, respectively). However, 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 that they maintain a significant sequence similarity with yeast Apg4/Aut2 along the entire protein sequence with the exception of notable divergences in both the N- and C-terminal ends of the diverse proteins (Fig. 1). Human autophagins also exhibit the 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 oxanion 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-*

norhabditis elegans (2, 31). To further explore the evolutionary and structural relationships between human autophagins and related proteins, we next performed a computational phylogenetic tree analysis (Fig. 2). This analysis revealed that the autophagins can be classified into two subfamilies of closely related members. Autophagins-1 and -2 should be grouped together with *CeApg4-2* and *DmApg4-2*, whereas autophagins-3 and -4 should be closer to *CeApg4-1* and *DmApg4-1*.

In summary and according to this structural analysis, we can conclude that the four identified and cloned human cDNAs encode members of a novel family of intracellular proteins are closely related to the founding member of this family, the yeast cysteine protease Apg4/Aut2.

Expression Analysis of Autophagins in Human Tissues and Cancer Cell Lines—As a preliminary step to study the physiological role of autophagins in human tissues, we examined by Northern blot analysis the expression pattern of these genes in a variety of tissues including colon, small intestine, ovary, testis, prostate, thymus, spleen, pancreas, kidney, skeletal muscle, liver, lung, placenta, brain, and heart. The filters containing poly(A)⁺ RNA from these tissues were sequentially hybridized with radiolabeled full-length cDNA probes for autophagins-1–4, and the results obtained are shown in Fig. 3. A single transcript of ~4.5 kb was mainly detected in skeletal muscle after hybridization with a probe for autophagin-1 with some expression being also apparent in heart, liver, and pancreas. Autophagin-2 transcripts of ~3.2 kb were detected in several tissues, the strongest signal corresponding to skeletal muscle. A major 3.5-kb autophagin-3 transcript was detected in many tissues with the highest expression levels found in skeletal muscle, heart, liver, and testis. Additional autophagin-3 transcripts of 3.0 kb could be also observed in the same tissues. Finally, a unique autophagin-4 transcript of ~2.4 kb was detected in skeletal muscle and at lower levels in the testis.

We also evaluated the expression of autophagins in human fetal tissues with the finding that autophagin-2 is detected in fetal liver. No significant levels of autophagins-1, -3, and -4

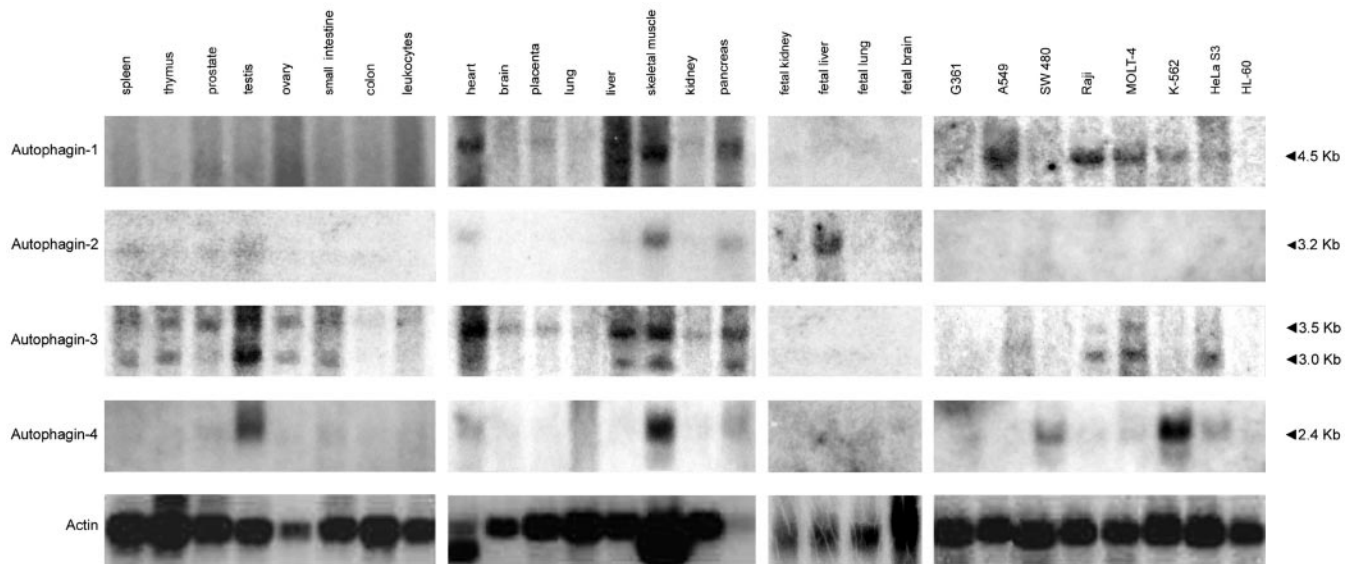


FIG. 3. **Analysis of expression of autophagins in human tissues and cell lines.** Filters containing $\sim 2 \mu\text{g}$ of polyadenylated RNAs from the indicated adult and fetal tissues, and cancer cell lines were hybridized with the full-length cDNAs isolated for human autophagins. RNA sizes are indicated. Filters were subsequently hybridized with a human actin probe to ascertain the differences in RNA loading among the different samples.

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

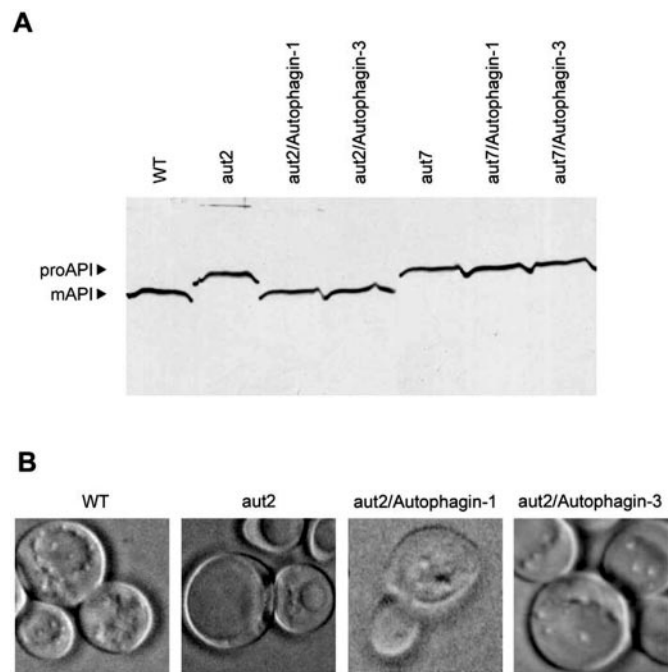


FIG. 4. **Complementation studies with human autophagins in autophagy-defective yeast strains.** A, Western blot analysis of aminopeptidase I (API) in *Aut2* and *Aut7* cells transformed with pGAD-Autophagin1 or pGAD-Autophagin3 or left nontransformed. Indicated cells were lysed as described under "Experimental Procedures," and API processing was analyzed using a rabbit serum anti-API. proAPI and mature aminopeptidase I (mAPI) are indicated by arrowheads. Wild-type (WT) cells were included as a positive control. B, *Aut4* yeast mutants were transformed with pGAD-Autophagin1 (*aut2/Autophagin-1*) or pGAD-Autophagin3 (*aut2/Autophagin-3*) or left nontransformed (*aut2*). Micrographs of cells were taken after 4 h of starvation in the presence of 1 mM PMSF to examine the accumulation of autophagic bodies. WT cells were included as a positive control.

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

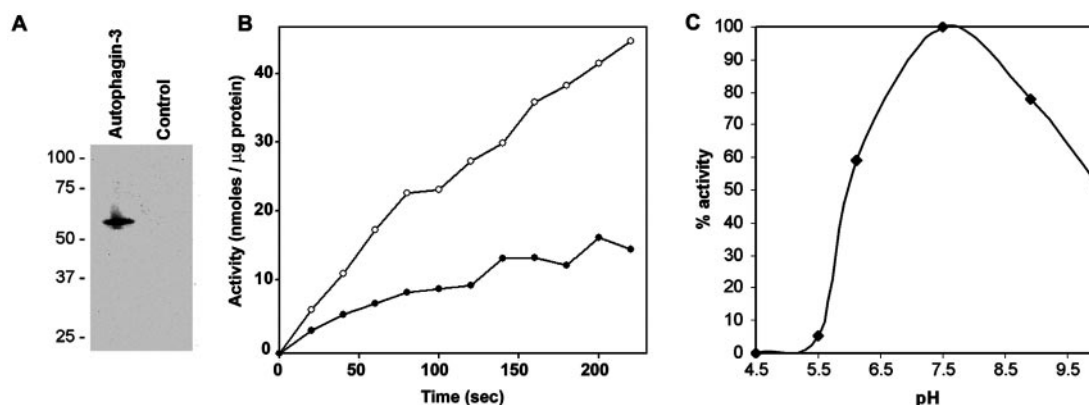


FIG. 5. **Production of recombinant autophagin-3 in mammalian cells and enzymatic analysis.** A, 50 μ l of conditioned medium from 293EBNA cells transfected with pCEP-Autophagin3-His (lane 1) and with pCEP-Pu (lane 2) were analyzed by Western blot using the penta-His monoclonal antibody (Qiagen). The sizes of the molecular mass markers are shown on the left. B, fluorogenic peptide Mca-Thr-Phe-Gly-Met-Dpa-NH₂ (5 μ M) 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 AEBSEF. The fluorometric measurements were made at $\lambda_{\text{ex}} = 328$ nm and at $\lambda_{\text{em}} = 393$ 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").

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, *aut2*, and *aut7* 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 protease inhibitor blocks the activity of vacuolar proteases, thereby hampering the autophagosome degradation and leading to the accumulation of these structures inside the vacuole. After 4 h of incubation, cells were washed, fixed, and photographed. In agreement with the results observed in the maturation of proAPI, only *aut2* mutant cells carrying autophagin-1 or -3 cDNA were able to restore the process of autophagy 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, the most widely distributed family member in human tissues, we produced recombinant autophagin-3 in an eukaryotic expression system. For that 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 culture medium as well as a His₆ tag at the C-terminal end to facilitate detection and purification (27). The resulting plasmid, pCEP-Autophagin3-His, was transfected into 293EBNA cells, and the secreted protein was detected by Western blot using a monoclonal antibody against the His₆ epitope (Fig. 5A). A single band of the expected molecular mass (54 kDa) was detected, confirming that the protein was expressed and correctly secreted to the culture medium. Recombinant autophagin-3 was purified from the conditioned medium using a nitrilotriacetic acid-agarose column. The purified protein was recovered at very low levels, and it was necessary to use Western blot analysis to clearly detect its presence. Nevertheless, no evidence of additional proteins co-purifying with autophagin-3 was observed. This recombinant autophagin-3 was used to assay its proteolytic activity using as a substrate the fluorescent peptide Mca-Thr-Phe-Gly-Met-Dpa-NH₂. To design this substrate, we first took into account the amino acid sequence of Apg8/Aut7 in the region that is cleaved by Apg4/Aut2: Thr-Phe-

Gly[↓]Arg. This sequence is conserved in the equivalent region of two human proteins MAP-LC3 (microtubule-associated protein light chain 3) and GATE-16 (Golgi-associated ATPase enhancer of 16 kDa) proposed to be functional homologues of yeast Apg8/Aut7 (22, 24–26). The sequences for MAP-LC3 and GATE-16 are Thr-Phe-Gly[↓]Met and Thr-Phe-Gly[↓]Phe, respectively. The sequence of GABARAP (γ -aminobutyric acid receptor-associated protein), another Apg8/Aut7 homologue present in mammalian tissues, is only partially conserved in the putative cleavage region (Val-Tyr-Gly[↓]Leu), and it was not considered for the purpose of synthesis of a consensus peptide substrate for Apg4/Aut2-related proteases. On the other hand and despite the fact that residue located after the cleaved Gly residue does not appear to have influence in the proteolytic cleavage of the susceptible bond (2), we introduced a Met residue at that position of the synthetic peptide since this is the residue present in human MAP-LC3 (22). In addition, a Apg8/Aut7 mutant with a Met at that position instead of the naturally occurring Arg residue is perfectly cleaved by Apg4/Aut2 (2).

After synthesizing the fluorogenic substrate Mca-Thr-Phe-Gly-Met-Dpa-NH₂, it was incubated with 10 ng of purified autophagin-3 and the hydrolyzing activity of the protease was followed by fluorometric measurements. As shown in Fig. 5B, autophagin-3 exhibited significant proteolytic activity against the synthetic substrate. This proteolytic activity was diminished by *N*-ethylmaleimide, an inhibitor of cysteine proteases including yeast Apg4/Aut2, providing additional evidence that the human autophagin-3 is a functional homologue of this yeast cysteine protease. The presence in the enzyme assay of inhibitors against serine proteinase or metalloproteinases did not have any effect on the autophagin-3 proteolytic activity against the fluorogenic substrate. As an initial attempt to determine the catalytic activity of this protease, a kinetic study was performed using the fluorogenic substrate described above. For this study, autophagin-3 (34 nM in 2 ml of reaction buffer) was incubated with different concentrations of substrate, and the k_{cat}/K_m was deduced as described by Northrop (28). The k_{cat}/K_m was determined to be $3.06 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. To further characterize 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 proteases produced by 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 mammalian expression system revealed that it is a catalytically active cysteine proteinase. In fact, the recombinant human protein exhibits a significant proteolytic activity against a fluorogenic substrate designed in this work to specifically analyze the activity of Apg4/Aut2-related proteases. This fluorogenic peptide contains the sequence around the Apg4/Aut2-cleavage site of Apg8/Aut7, the natural substrate of this yeast protease. Furthermore, this sequence is absolutely conserved in two human proteins, MAP-LC3 and GATE-16, proposed to play equivalent roles to yeast Apg8/Aut7 in the conjugation cascade associated with autophagy (22, 24–26). The finding that autophagin-3 hydrolyzes the peptide containing the sequence present in these two human proteins, is consistent with the possibility that MAP-LC3 and GATE-16 are *bona fide* substrates for human autophagins. It is also remarkable that this degrading activity was diminished by *N*-ethylmaleimide, an inhibitor of Apg4/Aut2p that also blocks the process of autophagy in yeast.

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 data reporting the association of autophagy abnormalities in pathological conditions involving skeletal muscle including some forms of muscular dystrophy (8, 34, 35). These putative associations between autophagins and skeletal muscle diseases may also imply the possibility that inherited alterations in these genes could be linked to familial forms of these pathologies. Chromosomal location analysis of autophagin genes indicate that they are not clustered in the human genome mapping to chromosomes 1p31.3 (autophagin-3), 2q37 (autophagin-1), 19p13.2 (autophagin-4), and Xq22 (autophagin-2). Genetic lesions in these regions have been linked to several diseases including muscular disorders whose responsible genes remain to be characterized. Of special interest is the finding of an autosomal dominant vacuolar neuromyopathy, which exhibits a muscle pathology with features of autophagic diseases and which is linked to 19p13 (36), the region where the autophagin-4 gene is located. The X-linked vacuolar myopathies distinct from Danon disease caused by mutations in LAMP-2 (lysosome-associated membrane protein-2) at Xq24 (8) have also been reported (35). It will be of future interest to examine the possibility that the autophagin genes could be a target of some of these genetic abnormalities. Likewise, the identification in this work of the putative murine orthologues of human autophagins opens the possibility to generate mice deficient in these genes that could contribute to clarifying the role of this proteolytic 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 may 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 or activity has been 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), sentrin/sumo-specific processing proteases or SUSPs, and now autophagins. According to our most recent estimations derived from human genome sequence analysis, at least 5 ubiquitin C-terminal hydrolases, 50 USPs, 7 sentrin/sumo-specific processing proteases, and 4 autophagins are produced by human tissues. Interestingly, a novel family of metalloproteases with deubiquitinating properties has also been identified recently (49, 50). The large and growing number of human proteases belonging to these different families underscores the relevance of conjugation/deconjugation systems for the regulation of multiple biological processes (51–53). Further studies directed to clarify the functional roles of autophagins will be very useful in establishing their relative importance in the context of the diverse ubiquitin-related modification systems occurring in human tissues.

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