Macroautophagy Is Required for Multicellular Development of the Social Amoeba Dictyostelium discoideum*

Received for publication, December 6, 2002, and in revised form, January 22, 2003
Published, JBC Papers in Press, March 7, 2003, DOI 10.1074/jbc.M212467200

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Macroautophagy is a mechanism employed by eukaryotic cells to recycle non-essential cellular components during starvation, differentiation, and development. Two conjugation reactions related to ubiquitination are essential for autophagy: Apg12p conjugation to Apg5p, and Apg8p conjugation to the lipid phosphatidylethanolamine. These reactions require the action of the E1-like enzyme, Apg7p, and the E2-like enzymes, Apg3p and Apg10p. In Dictyostelium, development is induced by starvation, conditions under which autophagy is required for survival in yeast and plants. We have identified Dictyostelium homologues of 10 budding yeast autophagy genes. We have generated mutations in apg5 and apg7 that produce defects typically associated with an abrogation of autophagy. Mutants are not grossly affected in growth, but survival during nitrogen starvation is severely reduced. Starved mutant cells show little turnover of cellular constituents by electron microscopy, whereas wild-type cells show significant cytoplasmic degradation and reduced organelle number. Bulk protein degradation during starvation-induced development is reduced in the autophagy mutants. Development is aberrant; the autophagy mutants do not aggregate in plaques on bacterial lawns, but they do proceed further in development on nitrocellulose filters, forming defective fruiting bodies. The autophagy mutations are cell autonomous, because wild-type cells in a chimera do not rescue development of the autophagy mutants. We have complemented the mutant phenotypes by expression of the cognate gene fused to green fluorescent protein. A green fluorescent protein fusion of the autophagosome marker Apg8 mislocalizes in the two autophagy mutants. We show that the Apg5-Apg12 conjugation system is conserved in Dictyostelium.

Protein turnover in eukaryotes is accomplished by two major mechanisms, autophagy or proteasomal degradation. Three modes of autophagy have been identified: chaperone-mediated autophagy, microautophagy, and macroautophagy. In chaperone-mediated autophagy, specific proteins containing targeting sequences are bound by chaperones that mediate direct transport across the lysosomal membrane. A lysosomal receptor, lysosomal-associated membrane protein type 2a, interacts with the substrate to facilitate transport into the lysosome. Microautophagy is required for basal protein degradation in rat liver (4) or glucose-induced peroxisome degradation in the methylotrophic yeast Pichia pastoris (5) and involves vacuolar membrane invagination to capture cargo directly. Macroautophagy is a non-selective mechanism used to deliver cytoplasmic components, including entire organelles, to the lysosome or vacuole during starvation (reviewed in Ref. 6). Initially, a membrane distinct from the vacuole/lysosome encloses a portion of cytoplasm to form a double-membraned vesicle called an autophagosome or autophagic vacuole. The autophagosome docks at and fuses with the vacuole/lysosome, releasing a single-membrane-enclosed vesicle called an autophagic body, which is degraded by resident hydrolyses. Molecular genetic analysis in the budding yeast, Saccharomyces cerevisiae, has identified many of the genes that are required for autophagy (7–9). These genes are required for phosphorylation reactions (10), a phosphatidylinositol 3-kinase complex (11, 12), and two novel ubiquitin-like conjugation reactions (13, 14). Starvation releases the repression of autophagy by the target of rapamycin (Tor) protein kinase, leading to the activation of another serine/threonine kinase, Apg1p, which activates autophagy through unknown downstream targets (15). Apg1p kinase activity is regulated by the phosphorylation status of a binding partner, Apg13; hyperphosphorylated Apg13 associates weakly with Apg1p. Upon Tor inactivation, Apg13p is dephosphorylated and binds more tightly to and activates Apg1p.

Autophagosome formation also requires the activity of two protein conjugation systems mechanistically related to ubiquitination. In the first, the carboxyl-terminal glycine of Apg12p is conjugated to an internal lysine of Apg5p, through the action of E1-like and E2-like enzymes, Apg7p and Apg10p, respectively (16–18). Apg16p binds to Apg5p and then oligomerizes to produce Apg12p-Apg5p-Apg16p oligomers (19, 20). A second conjugation system involves the conjugation of Apg8p/Aut7p to the membrane lipid phosphatidylethanolamine (14, 21), through the action of the E1-like and E2-like enzymes, Apg7p and Apg3p/Aut1p, respectively (22). Apg8p/Aut7p is the only component of the autophagy machinery in yeast that is transcriptionally up-regulated upon starvation (23).

Dictyostelium discoideum is a soil amoeba that feeds on bacteria but upon starvation completes a complex developmental cycle to produce a multicellular organism (reviewed in Ref. 24). Starving amoebae aggregate using a cyclic AMP signaling relay to form mounds of about 100,000 cells. The mound undergoes morphogenesis to produce a mature fruiting body composed of a sphere of spores held aloft a cellular stalk. To complete development and construct a fruiting body, Dictyo-
stelium needs to mobilize resources allotted previously to growth. We reasoned therefore that autophagy would be important for Dictyostelium development. Additionally, given its genetic tractability, we believe that Dictyostelium is a good organism to extend the observations on autophagy made in budding yeast, plants, and mammalian cells and to ask questions about the control of autophagy during development. We find that apg5 and apg7 are essential for Dictyostelium development. Mutants disrupted in these genes do not aggregate on agar plates cleared of Klebsiella pneumoniae, as the wild-type parents do, but will aggregate if starved on nitrocellulose filters or non-nutrient agar. The autophagy mutants have no evident growth defects but die rapidly when starved of amino acids. Protein turnover, which is normally induced by starvation, is reduced in apg5 and apg7. GFP fusions of Apg5 and Apg12 are present in two forms in growing and starving cells, corresponding to free and conjugated forms, implying similar conjugation reactions to those discovered in budding yeast. We also examine the subcellular localization of a GFP fusion of Apg8 in Dictyostelium and show the effects of autophagy on the ultrastructural organization of cells during starvation.

**EXPERIMENTAL PROCEDURES**

**Strains**—All mutations were created in the strain DH1, which is a uracil auxotroph. All strains were grown in HL5 medium or on lawns of K. pneumoniae (25).

**Development**—For multicellular development, axenically grown cells in mid-log phase (2–4 × 106 cells/ml) were washed twice in cold Soensen C (SorC) buffer (16.7 mM NaH2PO4, 50 mM CaCl2, pH 6.0). The cells were resuspended in SorC buffer and plated on 25-mm, 0.45 µm nitrocellulose filters (Millipore Corp.), which rested on SorC-soaked Whatman grade 17 filter pads, at a density of 3.3 × 106 cells/cm2 or 1.5 × 106 cells/filter (25). For development on non-nutrient agar, cells were resuspended at 105 cells/ml, and 10 µl of these cells were plated on 35-mm SorC-1% Phytigel (Sigma) agar plates and allowed to dry.

**Identification of D. discoideum Autophagy Genes—Dictyostelium orthologues of budding yeast autophagy genes were identified by searching the partial Dictyostelium genome sequence at dicty.sdsu.edu/ with the S. cerevisiae amino acid sequence. This super computer at the University of San Diego, La Jolla, CA contains sequence data from all of the members of the Dictyostelium Genome sequencing consortium. These include the Institute of Biochemistry I, Cologne, Germany, the Genome Sequencing Centre, Jena, Germany, the Baylor College of Medicine Dictyostelium Genome Center, and the Sanger Centre Dictyostelium Project. Sequence data obtained from the Genome Sequencing Centre, Jena are available at genome.imb-jena.de/dictyostelium. The sequence data produced by the D. discoideum Genome Project at the Sanger Centre can be obtained from ftp.sanger.ac.uk/pub/databases/D.discoideum_sequences/. Where contigs were not available, we assembled individual reads into contiguous sequences. The locations of intron/exon boundaries were inferred from sequence homology to the consensus splice donor and acceptor sites in Dictyostelium (26), by the AT richness of introns in D. discoideum, and by identifying fusions that maintained a single open reading frame.

**Gene Disruption**—The complete apg5 gene was obtained by PCR reactions with primers 5–1A (ATGCTATGCTTGAAGAGATA) and 5–1B (ATGCTATGCTTGAAGAGATA) to generate the 5′ region, and 5–2A (ATGCTATGCTTGAAGAGATA) and 5–2B (ATGCTATGCTTGAAGAGATA) to generate the 3′ region. A ClaI site was generated in the PCR product by inclusion of appropriate sequence in the primers. The PCR products were ligated into pCR2.1-TOPO (Invitrogen). The basicidicin resistance cassette was removed from pCR2.1-TOPO and cloned into the generated vector for filters. The complete apg7 gene was obtained by PCR reactions with primers 7–1A (ATGCAAAATGATCAGTCTCT) and 7–1B (GTCGTTGACTATGCAACATG) to generate the 5′ region, and 7–2A (GTCGTTGACTATGCAACATG) and 7–2B (GTCGTTGACTATGCAACATG) to generate the 3′ region. The PCR products were cloned into pCR2.1-TOPO, transformed into DH1 cells, selected on 35-mm glass bottom microwell dishes (MatTek Corporation), and the following day the medium was replaced with SorC 4 ml of these cells were plated on SM+G418 (40 µg/ml) plates with G418-resistant K. pneumoniae to obtain clones. GFP expression in transformants was confirmed by Western blotting with rabbit polyclonal serum against GFP (Molecular Probes). Protein Turnover Assays—Protein turnover assays were performed on filters obtained following two oligonucleotides: CATGGTGCTTGAAGAGATA and CTGAGGCTTGAAGAGATA. This linker provides compatible cohesive ends for XbaI and NcoI sites. The linkers nucleotides were annealed, and the ends were phosphorylated with T4 polynucleotide kinase and then purified with a Sephadex G50 column (Amersham Biosciences). The correct orientation of the insert was confirmed by sequencing of the junctions with the primers pTX-SacI5′ (TTAACC CACCGG TCTCTT) and pTX-KpnI3′ (ATCGATCAA GCTTAAA), and pTX-KpnI5′ (TTAACC CACCGG TCTCTT) and pTX-KpnI3′ (ATCGATCAA GCTTAAA). GFP fusion constructs were electroporated into DH1 cells and autophagy mutants, and transformants were selected with the drug G418 (5 µg/ml) in HL5 medium for 1 week. Transformants were harvested from Petri dishes and plated onto SM plates, and clones of mutant or wild-type phenotype were selected for further analysis. Homologous recombination of the knockout construct with the endogenous locus was confirmed by PCR or by Southern blotting (Invitrogen). The expression analysis—Northern blot analysis was conducted as described previously (28), except that Nytran membrane (Schleicher & Schuell) was used to immobilize RNA. Cells were harvested on nitrocellulose filters for development, and a filter was harvested every 4 h for RNA extraction. 5–µg total RNA of wild-type or mutant cells was glyoxysomes, agarose gels, transmittance, and hybridized with random primer-labeled DNA probes. Probes were obtained by isolation of appropriate restriction fragments following separation on low melting temperature agarose gels.

**GFP Fusion Constructs**—GFP fusions were produced by cloning the full-length genomic sequence of each gene (generated by PCR) into the pTX-GFP vector (29), a plasmid in which the expression of the gene of interest is under the control of the constitutive actin15 promoter. Isolation of the apg7 sequence was described earlier (see “Gene Disruption”). The apg7 gene was obtained by PCR with the primers 5–5GFP (GTTCAGTATGCTTGAAGAGATA) and 3–5GFP (GTTCAGTATGCTTGAAGAGATA) to generate the 5′ region. The apg7 gene was obtained with the primers 3–5GFP and 5–5GFP (GTCGTTGACTATGCAACATG) to generate the 3′ region. A ClaI site of the apg12 gene was obtained by PCR with the primers 12–1 (TTCACC CACCGG TCTCTT) and 12–3 (ATCGGAGGAAGAAGAAAA). PCR products were cloned into pGEM-T Easy (Promega). The apg12 sequence was cut from pGEM-T Easy with NcoI and SacI and ligated into XbaI/SacI-digested, dephosphorylated pTX-GFP. The apg7 sequence was cut from pGEM-T Easy with SacI and ligated into SacI-digested, dephosphorylated pTX-GFP. The apg7 and apg12 sequences were cut from pGEM-T Easy with NcoI and SacI and ligated into XbaI/SacI-digested pTX-GFP, along with a linker composed of the following two oligonucleotides: CATGGTGCTTGAAGAGATA and CTGAGGCTTGAAGAGATA. This linker provides compatible cohesive ends for XbaI and NcoI sites. The linkers nucleotides were annealed, and the ends were phosphorylated with T4 polynucleotide kinase and then purified with a Sephadex G50 column (Amersham Biosciences). The correct orientation of the insert was confirmed by sequencing of the junctions with the primers pTX-SacI5′ (TTAACC CACCGG TCTCTT) and pTX-KpnI3′ (ATCGATCAA GCTTAAA), and pTX-KpnI5′ (TTAACC CACCGG TCTCTT) and pTX-KpnI3′ (ATCGATCAA GCTTAAA). GFP fusion constructs were electroporated into DH1 cells and autophagy mutants, and transformants were selected with the drug G418 (5 µg/ml) in HL5 medium for 1 week. Transformants were harvested from Petri dishes and plated onto SM+G418 (40 µg/ml) plates with G418-resistant K. pneumoniae to obtain clones. GFP expression in transformants was confirmed by Western blotting with rabbit polyclonal serum against GFP (Molecular Probes). Protein Turnover Assays—Protein turnover assays were based on those of White and Sussman (30). Developing cells were recovered from Millipore filters in SorC buffer, pelleted by centrifugation, and resuspended in 50 ml Tris containing protease inhibitors (Complete, Mini; Roche Molecular Biochemicals). Cells were lysed by freeze-thaw before determining protein levels using the Pierce Coomassie Plus protein assay reagent, following the manufacturer’s instructions. Protein turnover assays were also conducted in axenic medium. Cells were shaken in FM medium (31) lacking amino acids at a density of 5 × 106 cells/ml. Three 1-ml aliquots of cells were harvested every 8 h and prepared as described above to generate filters for filters.

**Electron Microscopy**—Electron microscopy procedures were as described (32). Briefly, cells were fixed with 2% phosphate-buffered glutaraldehyde, pH 7.2, followed by 2% osmium tetroxide fixation in the same buffer, supplemented by centrifugation and enrobbed in agar, dehydrated with an aqueous/aceton series, and embedded in TAAB epon resin embedded with 1 µm of filtered C6 at an inclination of 4°.

**Fluorescence Microscopy**—To examine the localization of GFP fusion proteins, axenic cells were incubated overnight in FM medium on 35-mm glass bottom microwell dishes (MatTek Corporation), and the following day the medium was replaced with SorC 4–6 h prior to visualization (representing starving cells) or visualized directly (representing growing cells). Samples were viewed on a Zeiss Axiovert 135 research microscope, and images were captured with a cooled CCD camera and processed using Metamorph 5 imaging software (Universal Imaging Corporation).

For chimeric development, cells were resuspended at 107 cells/ml
and 100 µl of these cells were plated on 35-mm SorC-1% Phytagel (Sigma) agar plates and allowed to dry. Structures were viewed after 32 h under a Zeiss Axioplan 2 microscope, and images were captured with a cooled CCD camera and processed using Openlab 3 imaging software (Improvision). We tapped the Petri dishes lightly against a hard surface to make the fruiting structures collapse onto the Phytagel. This treatment caused the sorus to burst in most cases, releasing the contained spores. This procedure made photography of these structures easier, because the number of Z-sections required to capture the full depth of the fruiting body was minimized.

**FIG. 1.** Amino acid alignment of *D. discoideum* (Dd) Apg5 and Apg7 with orthologues from *A. thaliana* (At), *H. sapiens* (Hs) and *S. cerevisiae* (Sc). A, alignment of Apg5. The asterisk indicates the conserved lysine residue that forms an isopeptide bond with the carboxy-terminal glycine of Apg12p. The accession numbers are as follows: *A. thaliana*, NP_197231 (RefSeq); *H. sapiens*, Q9H1Y0 (Swiss Prot); *S. cerevisiae*, S65160 (PIR). B, alignment of Apg7. The active site cysteine is indicated with an asterisk. The ATP binding motif is indicated by a dashed line. The accession numbers are as follows: *A. thaliana*, BAB88385 (GenBank™); *H. sapiens*, NP_006386 (RefSeq); *S. cerevisiae*, NP_012041 (RefSeq). Alignments were performed using the BCM Search Launcher at searchlauncher.bcm.tmc.edu/multi-align/multi-align.html. The ClustalW 1.8 algorithm was used for multiple sequence alignments, and the resulting alignment was shaded using the Boxshade server at www.ch.embnet.org/software/BOX_form.html. Identical residues are shaded black, and similar residues are shaded gray.

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The Role of Autophagy in Dictyostelium Development

Identification of Autophagy Genes in D. discoideum—To identify autophagy genes in Dictyostelium, we searched the near complete D. discoideum genome sequence data base with budding yeast protein sequences using the BLAST algorithm. We identified putative Dictyostelium orthologues for Tor (AY204354), Apg1 (AY191011), Apg2 (AAM45260), Apg3 (AAL93575), Apg4 (AY191018), Apg5 (AY191012), Apg6 (AY191013), Apg7 (AY191014), Apg8 (AY191015), Apg9 (AY191015), and Apg12 (AY191016) (Fig. 1, and data not shown). Orthologues for Apg10, Apg13, Apg14, Apg16, and Apg17 were not identified. Similar searches of the Arabidopsis thaliana genome by others (33, 34) identified orthologues for Apg10, Apg13, Apg14, Apg16, and Apg17 orthologues were not found, consistent with our search of the Dictyostelium genome. Whereas multiple isoforms of Apg1, Apg4, Apg8, Apg12, and Apg13 are present in Arabidopsis, we identified only a single homologue for each autophagy protein in Dictyostelium, with the exception of Apg4. We identified two possible orthologues of Apg4, although incomplete sequence is available for one (data not shown).

In this study, we focus on Apg5 and Apg7. The homology of the Dictyostelium genes to orthologues in S. cerevisiae, A. thaliana, and Homo sapiens is shown in Fig. 1. Apg5 has a 54-amino acid asparagine-rich stretch in the amino terminus of the protein that is absent from the orthologues of the other species. Although asparagine-rich stretches are common in Dictyostelium proteins, their function is unknown. The lysine residue that is the site of isopeptide bond formation with Apg12 is conserved in all four species and corresponds to Lys-128 of A. thaliana, Lys-130 of H. sapiens, Lys-149 of S. cerevisiae, and Lys-186 of D. discoideum. Apg5 shares highest homology with the Arabidopsis gene, 24% identity over 393 residues. The homology to the human gene is also high, 29% identity over 277 residues. The degree of sequence conservation between species is even higher for Apg7. The Dictyostelium protein shares highest homology with the human protein: 39% identity over 719 residues. The ATP binding motif, Gly-X-Gly-X-X-Gly (amino acids 368–373), and the active site cysteine (Cys-563), are conserved in the Dictyostelium Apg7 protein.

Regulation of Autophagy Genes—We examined the expression of autophagy genes during growth and throughout development of Dictyostelium. Transcription of Apg1, Apg8, and Apg9 appears to be induced by starvation (Fig. 2). The expression levels of Apg5, Apg7, and Apg12 were very low, which made it difficult to conclude whether starvation induces expression. We were unable to detect an Apg6 transcript by Northern blotting. In budding yeast, only Apg8 expression appears to be up-regulated by starvation (23).

We generated two autophagy mutants by insertional mutagenesis: Apg5<sup>−</sup> and Apg7<sup>−</sup>. The Apg5<sup>−</sup> strain does not produce a transcript, and therefore we consider the insertional mutation a null. The Apg7<sup>−</sup> strain expresses a truncated transcript of a size expected from the site of insertion of the resistance cassette and at similar levels to the uninterrupted transcript in parental DH1 cells. We believe that the Apg7<sup>−</sup> strain is a null mutant, because the insertion precedes the ATP binding motif and the active site cysteine. The complemented strains, Apg5<sup>−</sup> (act15/apg5-gfp) and Apg7<sup>−</sup> (act15/gfp-apg7), express a transcript of the appropriate size composed of the structural gene and the GFP open reading frame (Fig. 2, right panel). The transcript levels are high throughout growth and development, because expression is from the constitutive act15 promoter.
Autophagy Is Required for Development—We tested the growth of autophagy mutants and parental strain in both shaking axenic culture (by hemocytometer counts) and on bacterial lawns (by plaque size). The two autophagy mutants grow as well as the parent under both conditions (data not shown). Both mutants show defective development when developing within plaques on bacterial lawns and when developed on nitrocellulose filters (see Fig. 3) or non-nutrient agar (data not shown). However, the defect observed under the two sets of conditions is strikingly different. When developed on nitrocellulose filters, mutant cells produce larger aggregates than wild-type, from which multiple tips arise. These tips give rise to slugs that rarely migrate from the enlarged mounds. The slugs culminate to form small, abnormal fruiting bodies with thickened stalks and empty sori. 

Autophagy Mutations Are Cell Autonomous—To determine whether the developmental defect of autophagy mutant cells could be rescued by development in the presence of their wild-type counterparts, we performed mosaic development experiments. We thought that perhaps nutritionally stressed cells would be cross-fed by wild-type neighbors. Mutant and wild-type cells were mixed at a 1:3 ratio. We examined spores produced by chimeric fruiting bodies for the presence of autophagy mutant cells (autophagy mutants do not produce spores on their own). Terminal developmental structures were treated with Triton X-100, which kills amoebae but not mature spores, and surviving cells were plated on K. pneumoniae lawns on SM plates. Wild-type spores produce plaques that contain developmental structures, whereas apg5− or apg7− produce clear plaques, because mutant amoebae do not aggregate within plaques on bacterial lawns. We examined 273 spores from 1:3 mixtures of apg5− and DH1, and 243 spores from 1:3 mixtures of apg7− and DH1. We never recovered any non-aggregating plaques on SM plates (we expect 25% of recovered spores to be mutant). Thus, the autophagy mutations we have created are cell autonomous, because spore production cannot be rescued by mixing with wild-type cells.
To follow the fate of specific cells, we used marked strains expressing GFP from a constitutive actin15 promoter. We examined the developmental phenotype of 25% marked:75% unmarked cell mixtures on 1% Phytagel-SorC. We determined the localization of GFP-expressing cells in the mosaic fruiting structures by taking Z-sections under a fluorescence microscope. Marked wild-type cells distribute to all structures of the fruiting body (Fig. 4 B). The autophagy mutant cells appear to aggregate normally in mixtures with wild-type cells, although they accumulate predominantly at the periphery of mounds (data not shown). However, when slugs are formed, autophagy mutant cells accumulate at the rear of the slug and remain in the slime sheath after culmination commences, resulting in exclusion from the sorus (Fig. 4, D and F).

**Autophagy Mutants Are Hypersensitive to Amino Acid Starvation**—We tested the ability of the autophagy mutants and wild-type cells to survive when starved of amino acids. This treatment is commonly used in budding yeast to stimulate autophagy, and yeast autophagy mutants die more rapidly than wild-type yeast when starved in this way (16, 35–39). We determined viability by the ability to form plaques on bacterial lawns. Amino acid-free FM medium was inoculated with 5 × 10^5 cells/ml, the cells were incubated on a shaker at 22 °C, and aliquots were plated with K. pneumoniae on SM/5 plates every 2 days. The parental strain, DH1, survives well, and viability only starts dropping slightly after 8 days (Fig. 5). The autophagy mutants are highly susceptible to amino acid starvation; the number of viable cells drops rapidly after 4 days to ~10% of parental levels. The complemented strain, apg5^− (act15/apg5gfp) strain rescues viability completely, whereas the apg7^− (act15/gfpapg7) strain shows an intermediate phenotype.

**Bulk Protein Turnover Is Reduced in Autophagy Mutants**—Autophagy may have an important function in protein turnover when Dictyostelium cells starve. We determined total protein levels of cells starving on nitrocellulose filters. We find that protein turnover during starvation and development is reduced in autophagy mutants. Cells were placed on nitrocellulose filters for development and harvested at the indicated times. Total cellular protein was measured using the Bradford assay. Protein levels in the parental DH1 strain typically drop 30–40% after 24 h of development. The apg5^− mutant does not show an appreciable drop in protein levels. The apg5^− (act15/apg5gfp) strain shows wild-type levels of protein degradation.
Fig. 7. Localization of GFP-Apg8 in live cells. GFP-Apg8 shows cytoplasmic localization and labels punctate structures in wild-type cells growing in FM medium (A, phase image; B, GFP fluorescence). In wild-type cells starving in SorC (C, phase image; D, GFP fluorescence), a similar distribution of GFP-Apg8 fluorescence is seen. In the autophagy mutants, GFP-Apg8 again shows cytoplasmic localization, but in addition, a single large punctate structure is labeled, irrespective of nutrient conditions. E and F, apg5<sup>−</sup> in FM medium; G and H, apg5<sup>−</sup> in SorC; I and J, apg7<sup>−</sup> in FM medium; K and L, apg7<sup>−</sup> in SorC.

Fig. 8. Apg5-GFP and GFP-Apg12 fusion proteins are present in two forms. GFP was fused to the carboxyl terminus of Apg5 and the amino terminus of Apg12 and overexpressed from a constitutive act15 promoter in the parental strain, DH1, and the two autophagy mutants, apg5<sup>−</sup> and apg7<sup>−</sup>. Cells were grown in HL5 medium (G) or starved in SorC (S) for 6 h and immunoblotted with anti-GFP antibody. The non-conjugated Apg5-GFP and GFP-Apg12 are present in all strains regardless of growth conditions. However, the Apg5-GFP-Apg12 conjugate is only present in DH1 and apg5<sup>−</sup> but is absent in the strain lacking E1-like activity, apg7<sup>−</sup>. The band above free ApG5-GFP is a nonspecific band. The GFP-Apg12-Apg5 conjugate is present only in the wild-type strain and is absent in the strain lacking a target for conjugation (apg5<sup>−</sup>) or the E1-like activating enzyme (apg7<sup>−</sup>).

parental cells lose 33% of total protein over 24 h, whereas apg5<sup>−</sup> (and apg7<sup>−</sup>; data not shown) loses less than 15% of total protein over the same time period (Fig. 6). The complemented strain, apg5<sup>−</sup> (act15/apg5-gfp), displays protein degradation comparable with the parental strain (similar results are seen for the apg7<sup>−</sup> (act15/gfp-apg7) strain; data not shown). A Dictyostelium mutant that does not aggregate, yakA<sup>−</sup> (40), was also tested to control for the effects of multicellular development on protein turnover; protein levels drop by 26% during 24 h of starvation (Fig. 6), meaning that protein degradation also tested to control for the effects of multicellular development on protein turnover; protein levels drop by 26% during 24 h of starvation (Fig. 6), meaning that protein degradation is conserved in the Cytoplasm—To determine the localization of autophagy proteins in Dictyostelium cells and to ask whether this occurs normally in the apg5<sup>−</sup> and apg7<sup>−</sup> mutants, we created fusions of the autophagy proteins with GFP. We fused GFP to the amino termini of Apg7, Apg8, and Apg12 and to the carboxyl terminus of Apg5. We examined the distribution of GFP fusion proteins by fluorescence microscopy. Free GFP and GFP-Apg7 show diffuse cytoplasmic localization during both growth and starvation (data not shown). Diffuse cytoplasmic localization and, rarely, a single punctate spot are observed for ApG5-GFP and GFP-Apg12 in two forms. GFP was fused to the carboxyl terminus of Apg5 and the amino terminus of Apg12, and both forms are present in all strains regardless of growth conditions. However, the ApG5-GFP-Apg12 conjugate is only present in DH1 and apg5<sup>−</sup> but is absent in the strain lacking E1-like activity, apg7<sup>−</sup>. The band above free ApG5-GFP is a nonspecific band. The GFP-Apg12-Apg5 conjugate is present only in the wild-type strain and is absent in the strain lacking a target for conjugation (apg5<sup>−</sup>) or the E1-like activating enzyme (apg7<sup>−</sup>).
absent in the \( \text{apg5}^- \) and \( \text{apg7}^- \) mutants, where the target of conjugation and the E1 activating enzyme, respectively, are absent. This is the predicted result if the Dictyostelium system functions like that of budding yeast.

**Wild-type Cells Show Extensive Cytoplasmic Degradation When Starved of Amino Acids**—To study the defect in autophagy mutant cells, we examined ultrastructure by transmission electron microscopy. We looked at both growing cells and cells starved of amino acids for 36 h to stimulate autophagy. Growing mutant amoebae are indistinguishable from their growing wild-type counterparts (Fig. 9, A and C, and E). Many small vesicles concentrated at the cell periphery are observed (Fig. 9A); these are macropinosomes resulting from feeding on the FM medium. When cells are starved, we observe dramatic differences between wild-type and autophagy mutant strains. In the parent DH1, large vesicles containing predominantly membrane, but also a little granular material, are observed. Most organelles have been degraded, leaving an almost empty cytoplasm (Fig. 9B). However, in starved autophagy mutants, vesicles are observed that contain organelles and cytoplasmic components, but the contents have undergone little or no degradation. The cytoplasm of mutant amoebae contains many organelles and is densely packed with glycogen (Fig. 9, D and F). It appears that amoebae of the wild-type parental strain recycle cellular components to aid in survival during nitrogen starvation, whereas mutant amoebae do not. These studies reveal the presence of what appear to be autophagosomal structures in the autophagy mutants (see arrows in Fig. 9, D and F), although additional evidence besides morphology is required to substantiate this conclusion.

**DISCUSSION**

In this study, we show that the autophagy pathway characterized in yeast, plants, and mammalian cells is conserved in *D. discoideum*. We identified putative Dictyostelium orthologues of *S. cerevisiae* proteins for Apg1–9, Apg12, and Tor. We were unable to locate orthologues of Apg10, Apg13, Apg16, or Apg17, either because they do not exist in social amoebae, are poorly conserved in evolution, or because we searched an incompletely sequenced genome. Unlike *A. thaliana*, we find only a single isoform of each protein with the exception of Apg4. We examined the expression of some of the identified autophagy genes in Dictyostelium and observe up-regulated transcription of \( \text{apg1, apg8, and apg9} \) when cells are starved (Fig. 2). In yeast, the expression of only one autophagy gene, \( \text{APG8\textsuperscript{y}} \), is significantly induced by starvation (23).

To analyze the functional conservation of macroautophagy, we generated and characterized the first autophagy mutants described in *D. discoideum*, in the \( \text{apg5} \) and \( \text{apg7} \) genes. Apg5 is the target of Apg12 conjugation, requiring the activity of the E1-like enzyme, Apg7. This conjugation reaction is chemically similar to ubiquitination and is required for the formation of autophagosomes (13, 42). Both Dictyostelium genes show significant homology with their budding yeast, plant, and mammalian counterparts (Fig. 1), with all residues known to be essential for function conserved. In addition, the Apg12-Apg5 conjugation system appears to be present in Dictyostelium (Fig. 7). Two forms of the GFP fusion proteins are detected in wild-type cells, but the higher molecular mass form of Apg5-GFP is...
absent in apg7−, and the conjugated form of GFP-Apg12 is absent in both apg5− and apg7−. This conjugation pattern is consistent with the functions assigned to Apg5, Apg7, and Apg12 from studies in budding yeast and mammalian cells. Apg5-GFP and GFP-Apg12 show diffuse cytoplasmic localization and rarely a single dot in growing and starving cells (data not shown). The GFP-Apg8 fusion shows a change in localization pattern dependent on the nutritional status and genetic background of the cell. In the wild-type strain, GFP-Apg8 localizes to many punctate spots in the cytoplasm (Fig. 7, B and D), in addition to diffuse cytoplasmic localization. Interestingly, the frequency of the dots is lowest in rich growth medium, H1L5 (data not shown), and is higher in the defined medium, FM, and in phosphate buffer (SorC). These punctate structures may represent preautophagosomal complex(es) and/or forming/complete autophagosomes. In the apg5− and apg7− mutants, GFP-Apg8 labels only a single punctate structure that is much larger than the dots observed in the wild-type strain (Fig. 7, F, H, J, and L), and the localization/frequency of these structures does not change when cells are starved. It is unclear what these large structures represent, but immunoelectron microscopy with anti-GFP antibodies in GFP-Apg8-expressing strains may be informative.

We find that apg5− and apg7− are dispensable for growth in Dictyostelium but are required for normal development. When apg5− and apg7− mutants develop, they form large multi-tipped aggregates that fail to complete normal morphogenesis. Instead, aberrant fruiting bodies composed of thickened stalks and empty sori are formed (Fig. 3). These structures produce no viable, mature spores. Additionally, development and spore production cannot be rescued by co-developing autophagy mutant cells with wild-type cells in a chimeric organism (Fig. 4). Thus, these autophagy mutants are cell autonomous. It is not clear how these mutations prohibit proper development, but it may include interference with chemotaxis, motility, adhesion, intercellular signaling, or differentiation.

The apg5− and apg7− mutants exhibit additional attributes of autophagy mutants. Survival in medium lacking amino acids is severely reduced (Fig. 5), similar to that described for yeast and plants. Yeast autophagy mutants die rapidly when starved of amino acids, and plants carrying autophagy mutations are viable, mature spores. Yeast autophagy mutants die rapidly when starved of amino acids, and plants carrying autophagy mutations are viable, mature spores. Dictyostelium autophagy mutants die rapidly when starved of amino acids, and plants carrying autophagy mutations are viable, mature spores.

Acknowledgments—We thank all members of the Kessin laboratory and Howard Shuman and Hubert Hilbi for helpful discussions. We thank Thomas Huckaba, Edgar Gomes, and Chiann-Mun Chen for help with microscopy. We are grateful to the Dictyostelium Genome Sequencing Consortium for their efforts.

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