A Dictyostelium Mutant with Reduced Lysozyme Levels Compensates by Increased Phagocytic Activity*

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Lysozymes are bacteria-degrading enzymes and play a major role in the immune defense of animals. In free-living protozoa, lysozyme-like proteins are involved in the digestion of phagocytosed bacteria. Here, we purified a protein with lysozyme activity from Dictyostelium amoebae, which constitutes the founding member, a novel class of lysozymes. By tagging the protein with green fluorescent protein or the Myc epitope, a new type of lysozyme-containing vesicle was identified that was devoid of other known lysosomal enzymes. The most highly expressed isoform, encoded by the <i>alyA</i> gene, was knocked out by homologous recombination. The mutant cells had greatly reduced enzymatic activity and grew inefficiently when bacteria were the sole food source. Over time the mutant gained the ability to internalize bacteria more efficiently, so that the defect in digestion was compensated by increased uptake of food particles.

The lysosome is the most potent degradative organelle within the eukaryotic cell. It contains hydrolytic enzymes that fulfill essential functions. In humans many mutations affecting its constituents lead to the diseased state, often with dramatic consequences. Mutations of this sort fall into two classes. The first class affects proteins involved in trafficking to and from the lysosome and/or regulating its integrity by modulating fusion and fission events. Among these proteins are Rab, proteins belonging to a family that act as molecular timers (1) controlling virtually every trafficking step in the cell (2) and the LYST proteins, which when mutated cause enlarged lysosomes manifested as Chediak-Higashi Syndrome (3). The second class of mutations affects lysosomal enzymes with metabolic roles. These enzymes degrade macromolecules that the cell wants to dispose of, producing small metabolites that are useful building blocks or provide energy if completely degraded in the cytosol and mitochondria. Tragically, many of the lysosomal enzymes are exoenzymes, i.e. they digest macromolecules from their ends. If one of these enzymes is lacking, degradation proceeds up to the residue that cannot be removed, and as a consequence, all steps that ensue will be blocked. This leads to the accumulation of partially degraded intermediates within the lysosomes culminating either in the formation of toxic compounds or blowing up the volume of the lysosome until it sterically interferes with vital cellular activities. Depending on the cell type that is most seriously harmed, be it neurons, muscle, or leukocytes, patients will suffer from mental retardation, cardiac failure, or immunodeficiency (4, 5).

In contrast, free-living amoebae are unlikely to develop lysosomal storage diseases because their endocytic pathway differs from that of mammalian cells. In mammalian cells, the lysosome is a dead-end of vesicular trafficking. In most cell types, it accumulates non-degradable material and retains it for the lifetime of the cell and organism. In amoebae, endocytosed cargo transits through the cell. Any material that cannot be degraded because specific enzymes are lacking is released from the cell by exocytosis after an hour. Despite these differences, amoebae such as Dictyostelium can serve as bona fide model systems to study lysosomal function <i>in vivo</i>. Interference with Rab (6, 7) and LYST proteins (8, 9) produces phenotypes in Dictyostelium that are comparable with those observed in mammalian cells, indicating that many lysosomal constituents are functionally conserved in evolution. On the other hand, a deficiency of cathepsin D leads to profound defects in mice (10) and sheep (11) but is tolerated well in Dictyostelium (12).

More recently mice have been generated that specifically lack the M isoform of lysozyme, which constitutes the most prominent bacteriolytic activity in the airways (13). Although homozygous mutants increase the level of expression of the lysozyme P isoform to compensate for their deficiency, they remain much more sensitive to infections of the lung (14). Lysozymes are not only found in animals but are also present in numerous phylogenetically diverse organisms such as plants, fungi, bacteria, and bacteriophages (15). Several different classes of these enzymes have been described revealing that structurally diverse proteins fulfill the function of degrading the peptidoglycan of bacteria by splitting a 1,4-linkage between <i>N</i>-acetylmuramic acid and <i>N</i>-acytlyglucosamine (16). Despite the fact that <i>Dictyostelium</i> has been regarded as a good model for elucidating molecular mechanisms underlying phagocytosis of cells from higher organisms, e.g. mammalian defensive cells, nothing is known about the molecular armament that it uses to efficiently eliminate the enormous number of bacteria phagocytosed for nutrition. We have isolated a protein with lysozyme activity from <i>Dictyostelium</i> amoebae. The protein is the first antimicrobial polypeptide characterized in this protozoan organism at the molecular level, and it appears to be a member of a previously unrecognized lysozyme family. The enzyme is stored in a unique organelle, and disruption of its gene had a dramatic effect on the phenotype.

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**EXPERIMENTAL PROCEDURES**

**Purification of Lysozyme**—The AX2 strain of Dictyostelium discoideum was cultured at 22 °C axenically (17). Amoebae in late-logarithmic phase were harvested, sedimented at 320 × g at 4 °C for 5 min, and washed 2 times in Sörensen's 17 mM sodium/potassium phosphate, pH 6.0, followed by centrifugation and washing in 200 mM Tricine (2.5 × 10⁶ cells) were kept at 4 °C for 3 days by three cycles of freezing and thawing in 5 volumes of 10 mM sodium acetate, pH 4.5, supplemented with complete protease inhibitor mixture (Roche Applied Science). The lysose was centrifuged at 150,000 × g at 4 °C for 1 h. Subsequent purification steps were carried out at 10 °C, and samples were kept on ice. The 150,000 × g supernatant was applied to a chromatography column (Econo-PAQUE column 2.5 × 20 cm). Bio-Rad) manually filled with Bio-Gel matrix (Bio-Gel A-0.5m; Bio-Rad). The column was equilibrated with 10 mM sodium acetate, pH 4.5. Adsorbed protein was eluted by washing the column with the same buffer (350 ml) and with 100 mM sodium acetate, pH 4.5 (200 ml). Finally, the column was washed with 500 mM sodium acetate, pH 4.5 (200 ml). Fractions with lysozyme activity were pooled and loaded on a Resource S cation exchange column (1 ml; Amersham Biosciences) equilibrated with 10 mM sodium acetate, pH 4.5. Adsorbed protein was eluted by first washing the column with the same buffer (5 ml), then by use of a gradient from 0 to 300 mM NaCl (25 ml) and finally by a wash with 1 M NaCl. Each fraction was tested for lysozyme activity.

**Protein Analysis**—Protein concentration was determined using the microbicinchoninic acid reagent assay (Pierce) and hen egg lysozyme as standards. Enzyme activity was measured with a dPCR using a TRITC-coupled polyclonal antibody (courtesy of Peter Devreotes) as a template. The product was digested at the EcoRI and BglII cleavage sites that flank the PCR primers 5'-CGGAATTCTTTTATTACTGTGATCATGAGCTGTTTGAT-3' and 5'-GGAGAATCTTGAACGGTTGCTGATTGAAGATACTAC-3' and inserted into pDd-A1-gfp (32) as modified by (33), so that GFP replaces the C-terminal domain of lysozyme.

**Endocytosis and Cell Death**—The uptake of particles and fluid phase was measured as described previously (34, 35). To determine the capacity of cells to degrade bacteria in vivo, we used the assay established by Maselli et al. (36), except that we used E. coli expressing the green fluorescent protein (37) to measure the decrease in intracellular fluorescence after phagocytosis. To assay the growth properties of cells on bacterial lawns, we used M. luteus, Klebsiella aerogenes, and E. coli as substrates (26). These bacterial species were prepared on plates, harvested in Sörensen's phosphate buffer (as above), and mixed with about 80 cells of the appropriate Dictyostelium strain, and plaque diameter was measured after 5 days of growth on SM agar plates. In this assay no dramatic growth differences were observed between Gram-negative and Gram-positive bacteria.

**Immunofluorescence and Antibodies**—mAb 221-342-5 recognizes a strong and uniform determinant residing on many lysozymes (38). mAb AD 7.5 binds to an N-acetylglucosamine 1-phosphate modification of another class of lysozymal enzymes (39). The antigen corresponds to mAb 130-80-2, an esterase gp70, accumulated in crystalization of another class of lysosomal enzymes (39). The antigen corresponds to mAb 221-342-5 recognizing a strong conserved determinant residing on many lysozymes (38). mAb AD 7.5 binds to an N-acetylglucosamine 1-phosphate modification of another class of lysozymal enzymes (39). The antigen corresponds to mAb 130-80-2, an esterase gp70, accumulated in crystalization of another class of lysosomal enzymes (39). The antigen corresponds to mAb 130-80-2, an esterase gp70, accumulated in crystalization of another class of lysosomal enzymes (39). The antigen corresponds to mAb 130-80-2, an esterase gp70, accumulated in crystalization of another class of lysosomal enzymes (39).

**Data Base Searches and Accession Numbers**—A computer-assisted homology search was performed using the Internet BLAST and FASTA searches in the nucleic acid data base of the National Center for Biotechnology Information (NCBI) and the Dictyostelium protein data base (dbtydb.org/cgi-bin/blast.pl), resulting in sequence information for alyA (AA008434), alyB (AA008432), alyC (AA0051440), alyD (DDB021729 and DDB016810), DldLysC1, DldLysC2, and DldLysC3 (lysozymes of D. discoideum similar to type B lysozymes; U66323, DDB019256, DDB020557, respectively). Dictyostelium genes coding for DmdlyT41 and DmdlyT42 (lysozymes of D. discoideum similar to T4 phage lysozymes; DDB0217501 and DDB0167824, respectively) and DldLysE1 and DldLysE2 (lysozymes of D. discoideum similar to Entamoeba histolytica; DDB0167552 and DDB019884, respectively) were also identified in the Dictyostelium genome. The amino acid sequence of lysozymes of diverse organisms used in the phylogenetic analysis of the protein sequences retrieved from the protein database of the NCBI and listed below with their respective abbreviations in Fig. 7 (source organism; accession numbers): LysC (Galbus gallus c-type lysozyme; 2CDS_A), LysT4 (enterobacteria phage T4 type lysozyme; 1LYD), LysG (goose type lysozyme; LZGSG). The following lysozymes represent the i-type lysozymes of invertebrates: Bathymodiolus azoricus (AAN16208), Bathymodiolus thermophilus (AAN16209), Caenorhabditis elegans 1–3 (AAG11079, AAC19181, AAA83197), Caelytoma sp. 1 and 2.

1 The abbreviations used are: Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; A1, amoeba lysozyme; mAb, monoclonal antibody; GFP, green fluorescent protein; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; TRITC, tetramethylrhodamine isothiocyanate.
Fig. 1. Purification of AlyA and activity determination at various conditions. A, a protein preparation with lysozyme activity eluted isocratically from a Bio-Gel column was subjected to Resource S cation exchange chromatography. Bound protein was eluted with an increasing NaCl gradient (dotted line), and active fractions were pooled as indicated by the bar. The inset shows the different steps of purification of AlyA on a silver-stained SDS gel. Lane 1, amoebic acid extract; lane 2, Bio-Gel fraction; lane 3, Resource S fraction. Molecular masses are indicated at the left. B, cell walls of *M. luteus* were incubated with the purified protein at various pH and at ionic strength reaching from 0.005 to 0.2, and degradation as a measure of lysozyme activity was determined photometrically.

(AAN16211, AAN16212), *Chlamys islandica* (CAB63451), *Drosophila melanogaster* 1-3 (CAA21317, AAF57939, AAF57940), *Hirudo medicinalis* (AA96144), *Mytilus galloprovincialis* (AAN16219), *Mytilus edulis* (AAN16207), *Tapes japonica* (BAB3389), *Asterias rubens* (AAR29291), *E. histolytica* EhLys 1 and 2 (Q27650, AAC67235).

Protein alignments were performed using the software ClustalX (42), and phylogenetic relationships were analyzed using PAUP 4.0 (43). Bootstrap analysis (1000 replicates) was added to test tree robustness.

RESULTS

Purification of a Novel Lysozyme and Identification of the alyA Gene—We purified a protein with lysozyme activity from axenically cultured *Dictyostelium* amoeboae, exploiting its adsorption to a Bio-Gel matrix. The main part of active material found in acid extracts of amoebae was only weakly retained by the column. It was eluted as a single protein peak with the purified protein at various pH and at ionic strength reaching from 0.005 to 0.2, and degradation as a measure of lysozyme activity was determined photometrically.

The purified lysozyme was subjected to N-terminal sequencing, yielding a single amino acid sequence up to residue 25 (YSCPKPACYGMNCSTSPNQSYVLTD). This sequence was back-translated into nucleic acid sequence and used to search a cDNA database (44). A cDNA clone, SSF469, was retrieved, the deduced amino acid sequence of which perfectly matched the experimentally determined amino acid sequence of lysozyme. Using this clone we could identify the corresponding gene on chromosome 2 in the data base from the *Dictyostelium* genome project (45) and named it alyA (for amoeba lysozyme). The information on genomic structure and amino acid sequence is summarized in Fig. 2.

The N-terminal tyrosine residue of the mature enzyme is preceded by a stretch of 19 mostly hydrophobic amino acid residues that may serve as a signal peptide necessary for the transport of the protein into the endoplasmic reticulum membrane. MALDI-TOF analysis of the purified AlyA protein yielded a molecular mass of 12.652 kDa. This value confirms the molecular mass estimated from SDS-PAGE and is in good agreement with a mature peptide of 119 residues that would have a calculated molecular mass of 12.633 kDa provided that 12 cysteine residues participate in disulfide bonding. No glycosylation of the purified protein was experimentally evident as judged by a glycan detection assay. The only putative N-glycosylation site in the primary translation product is at Asn149, beyond the C terminus of the mature protein. Neither the mature protein nor its precursor molecule reveal significant similarity to any protein of other organisms reported so far, in particular not to other bacteriolytic enzymes.

**Phenotypic Instability of aly Knockouts**—To analyze the in vivo function of this novel protein, we disrupted the alyA gene. Therefore, we electroporated *Dictyostelium* cells in the presence of a linear DNA fragment in which genomic regions of alyA flanked a cassette conferring blasticidin resistance to transformed cells. As soon as clones appeared on the primary Petri dish, we re-cloned them by limited dilution in liquid growth medium. In the first attempt to isolate a mutant lacking alyA, we screened about 100 clones by PCR analysis of genomic DNA and obtained a single mutant cell line. Cell homogenates from this clone 74 had a lysozyme activity that was reduced to about 50% of wild-type levels. When plated on a bacterial lawn, the mutant initially formed plaques where the food bacteria were consumed that grew only to half the size measured for the wild-type strain. This phenotype, however, was not stable. Plating experiments repeated over the course of 2 weeks revealed that the diameter of plaques increased in the alyA mutant until no difference was seen to wild-type cells. In contrast, the enzyme activity remained low.
Because of this phenotypic instability, we repeated the transformation to obtain another mutant. In the second attempt, one mutant was found among about 150 clones analyzed. The following analysis focuses on this clone, 138. PCR analysis using a primer pair that binds to both alyA and alyB genes (see below) revealed both genes in genomic DNA from wild-type cells, whereas only a product corresponding to alyB was detected in the mutant (Fig. 3 A). The mutant carried the blastidine resistance cassette precisely within the alyA locus (Fig. 3 B). As seen previously in strain 74, the knock-out mutant alyA138 had a total lysozyme activity corresponding to about 40% of wild-type cells and maintained this level over more than 2 months (Fig. 3 C). When the plaque diameters of the alyA138 strain were measured and compared with wild-type values, they were found to increase from 60% to 90% within about 10 days, steadily rising to 150% after 40 days and finally stabilizing at a constant value corresponding to 180% of wild-type cells (Fig. 3 D). Despite these changes, repeated PCR analyses did not reveal any alteration at the alyA locus (data not shown). All of the subsequent experiments were performed using cells from this stage.

To test whether the increased plaque size was due to an AlyA-independent increase in the capacity to degrade bacteria, we fed the cells with GFP-expressing bacteria and measured the disappearance of their fluorescence signal. Unexpectedly, the alyA138 mutant performed as efficiently as wild-type cells (Fig. 4 A), although it was still reduced in lysozyme activity. To find out whether the increased plaque size was caused by an increased efficiency of phagocytosis, we measured the internalization of an indigestible particle, fluorescently labeled yeast. Indeed, the rate of particle uptake by alyA138 mutant cells was almost 2-fold higher than the rate of wild-type phagocytosis (Fig. 4 B), providing a reasonable explanation for the increased
Compensation of Lysozyme Deficiency

The phenotype of the alyA mutant was rescued by re-expression of AlyA. The rate of phagocytosis was increased in the alyA138 mutant and can be rescued to wild-type-like levels upon re-expression of alyA(open squares). Cells were incubated together with fluorescent yeast particles, and the intracellular fluorescence signal (relative units) originating from phagocytosed particles was measured at 15-min intervals. The decrease of intracellular fluorescence was measured over time. The rate of phagocytosis is increased in the alyA138 mutant and can be rescued to wild-type-like levels upon re-expression of alyA(open squares). Cells were incubated together with fluorescent yeast particles, and the intracellular fluorescence signal (relative units) originating from phagocytosed particles was measured at 15-min intervals. The decrease of intracellular fluorescence was measured over time. The rate of phagocytosis is increased in the alyA138 mutant and can be rescued to wild-type-like levels upon re-expression of alyA(open squares). Cells were incubated together with fluorescent yeast particles, and the intracellular fluorescence signal (relative units) originating from phagocytosed particles was measured at 15-min intervals. The decrease of intracellular fluorescence was measured over time.

**Rescue of Phenotypic Defects**—In the case of unstable phenotypes, it is particularly important to check whether the re-expression of the product corresponding to the disrupted gene restores the phenotype to the wild-type-like character. To this end we inserted the entire coding region of alyA including the introns into an expression vector and produced stably transformed cell lines in the alyA138 mutant background. RT-PCR analysis of various clones generally revealed a more intense band corresponding to the alyA gene (data not shown), which reflects the high copy number integration of the plasmid into the genome. In clone 2.4 phagocytosis was reduced to approximately wild-type levels (Fig. 4C), plaque size returned to normal (Fig. 4D), and the lysozyme activity in homogenates was also partially rescued (Fig. 4E).

Because we failed to produce a polyclonal antibody in chicken, which was suitable for the localization of the enzyme in cells, we resorted to analyzing the distribution of genetically tagged AlyA proteins bearing an Myc epitope in the middle of the mature enzyme or a GFP extension at its C terminus (Fig. 5A). In the former construct the enzyme prodomain was maintained, whereas the GFP tag eliminated the cleavage signal, replacing the C-terminal prodomain. These constructs were individually transformed into the alyA mutant background. The presence of the transgene was verified by PCR (Fig. 5B), and the protein was detected in Western blots (Fig. 5C). For all rescued clones we established that the endogenous copy of alyA was still disrupted (data not shown). Although expression of each of the tagged lysozymes increased the enzymatic activity of cell extracts only mildly (Fig. 5D), the efficiency of phagocytosis approached wild-type levels (Fig. 5E), indicating that the hybrid molecules were sorted to proper destination in the cells.

**Localization of the alyA Product**—Both tagged proteins localized to numerous small vesicles distributed throughout the cytoplasm. Because the GFP moiety replaced the prodomain in the hybrid construct, we compared the subcellular distributions of AlyA-GFP and Myc-tagged AlyA directly in cells transformed with a mixture of both plasmids. Fig. 6A documents that the majority of GFP-containing vesicles are also loaded with the Myc-tagged enzyme. The perinuclear ring and the peripheral structures labeled with the anti-Myc-antibody alone represent the endoplasmic reticulum (data not shown), suggesting that the Myc epitope in the middle of the AlyA protein reduces the efficiency of protein folding. To investigate the identity of the labeled vesicles, we checked the AlyA-GFP-expressing strain for the distribution of three classes of lysosomal enzymes known to reside in different types of lysosomes.
Surprisingly, neither enzymes bearing the common-antigen 1, a mannose-6-SO₄-containing oligosaccharide (Fig. 6B), nor enzymes characterized by the N-acetylglucosamine 1-phosphate modification co-distribute with AlyA (Fig. 6C). Vesicles containing crystal-forming esterases are also devoid of AlyA-GFP (Fig. 6D). We, therefore, conclude that the enzyme resides in a novel type of vesicle in Dictyostelium. To test whether these vesicles deliver their contents into phagosomes, AlyA-GFP-expressing cells were fed with synthetic particles. One of the rare events, where internalized beads are surrounded by a rim of fluorescence, is shown in Fig. 6E. More frequently we saw fluorescent granules accumulating in the vicinity of a bead as if docked to the phagosome membrane (also visible in Fig. 6E). We assume that the protein is rapidly degraded when it encounters the degradative enzymes of phagosome but is rather stable in the storage vesicles.

FIG. 6. Localization of Myc- and GFP-tagged AlyA. Shown are individual channels and overlay images of single confocal sections through AlyA-GFP expressing cells (green) stained with various antibodies in indirect immunofluorescence (red). A, a cell expressing additionally Myc-tagged AlyA as detected by mAb 9E10. B, a cell stained for common-antigen 1 using mAb 221–342-5. C, immunofluorescence using mAb AD7.5 directed against the N-acetylgalosamine-1-phosphate modification or the crystal protein, which is the target of mAb 130-80-2 binding (D). For E, cells were allowed to phagocytose latex beads as seen in the transmission image. Bar, 5 μm.
Compensation of Lysozyme Deficiency

The only lysozymes of protozoan origin characterized at the molecular level to date are from *E. histolytica* (20, 46), a parasitic amoeba colonizing the human small intestine. *Entamoeba* is thought to have diverged from the evolutionary path to higher organism virtually at the same time as *Dictyostelium* (47), and it was tempting to think that *Dictyostelium* lysozymes are more closely related to the *Entamoeba* lysozymes than to enzymes from other organisms. We purified the major protein with lysozyme activity from extracts of *Dictyostelium* amoebae. The protein was characterized and N-terminally sequenced, and a subsequent data base search identified a gene product with unknown function and allowed the elucidation of the entire primary structure of the protein. The enzyme described here is not related to any known lysozyme, and we suggest viewing it as the founding member of a new lysozyme class.

Several other sequences for putative gene products that may have lysozyme-like properties can be extracted from the *Dictyostelium* data base (Fig. 7A). A recent survey of the genome reveals the existence of 11 lysozyme genes that potentially code for lysozymes belonging to four classes; two are of the bacteriophage T4 type, and three are homologous to the C-type lysozymes. *Dictyostelium* also has two genes that may code for proteins closely related to the unconventional lysozymes first described from *E. histolytica* (20, 46). Searching with the sequence of the AlyA protein turns up three additional members of this so far unique lysozyme gene family. The predicted protein sequences of ALY B and ALY C are characterized by 12 and 22 exchanged residues, respectively. The fourth and most divergent member of the proteins encoded by the *aly* family, ALY D, carries a 77-residue insertion in the middle of the molecule, which consists almost exclusively of serines and glycines. The remaining sequence is only about 40% identical to AlyA. In summary, no other organism is as well equipped with lysozyme genes as *Dictyostelium*.

Many of the animals investigated so far appear to possess a single type of lysozyme only; multiple isoforms of a single lysozyme type can be found when the protein has also been recruited as a digestive enzyme (23). The only other organisms known to date bearing genes potentially coding for more than one type of lysozyme are *D. melanogaster* and *C. elegans*. They possess c-type and i-type lysozymes or *E. histolytica*-type and i-type lysozymes, respectively (16, 46). Phylogenetic analysis revealed that the newly recognized AlyA-D described here represent a novel type of lysozymes (Fig. 7B). It is reasonable to assume that the amoebic lysozymes, as in other phagocytic cells, are constituents of an intracellular armament that fulfill a digestive function to exploit bacteria for nutrition. In addition, these enzymes may prevent uncontrolled microbial growth within the digestive vacuoles.

Irrespective of whether AlyA is tagged with a Myc epitope in the middle of the molecule or with a GFP moiety replacing the C-terminal prodomain, the enzyme accumulates in the same type of small vesicle (Fig. 6A). This observation indicates that the prodomain is not required for proper sorting of AlyA, an observation that has been made with other lysosomal enzymes as well (48, 49). Because the N-terminal signal peptide is likely to be cleaved immediately after entry into the endoplasmic reticulum, the mature enzyme must encompass all the signals required for correct subcellular targeting.

Interestingly, AlyA is concentrated in a specific sort of vesicle devoid of other lysosomal enzymes (Fig. 6). This finding increases the number of primary lysosomes to four, because it has been convincingly shown that mannos-6-SO$_4$-modified enzymes and N-acetylglucosamine 1-phosphate-modified proteins do not coexist in the same vesicle (39), and the number and morphology of esterosomes (40, 50) identifies the AlyA-containing organelles as a novel class of lysosomes. A possible reason for sorting AlyA away from the majority of proteases lies in the sensitivity of the molecule; it is very short-lived in total cell extracts at acidic conditions and becomes increasingly stable during purification. Indeed, immunofluorescence experiments reveal that bead-bearing phagosomes only rarely contain detectable steady state levels of the enzyme (Fig. 6), but its acidic pH optimum is in good agreement with the pH values found within *Dictyostelium* endosomes.

When *aly*, the gene encoding the major lysozyme isomorph, is disrupted, the total lysozyme activity of cells extracts is reduced to 40% of wild-type cells as measured by the degradation of bacterial cell walls *in vitro* (Fig. 3C). In support of these findings, the initial ability of cells to form plaques on a lawn of bacteria is reduced to a similar degree (Fig. 3D). Taken together these two observations clearly support a role for AlyA in efficient degradation of phagocytosed bacteria, which is perfectly consistent with its well known activity to cleave the bacterial peptidoglycan, the major constituent of the cell wall of Gram-positives. As peptidoglycan is also a constituent of the cell wall of Gram-negative bacteria, it is not surprising to find the same initial results when *aly* mutant cells grow on lawns of *E. coli* and *K. aerogenes*.

However, when the *aly*-deficient cells are cultivated for prolonged times, a surprising observation can be made. The efficiency of plaque formation increases gradually until it reaches wild-type levels, finally exceeding these values by almost 2-fold (Fig. 3D). The reasons for these phenotypic changes are not trivial, because the enzymatic activity of lysozyme in both strains remains constantly at 40% of wild-type level *in vitro* (Fig. 3C). Most importantly, *aly* mutant cells attain wild-type properties after re-expression of wild-type and hybrid AlyA transgenes (Figs. 4 and 5), indicating that the changes observed in the mutant over time are not the consequence of an accumulation of a number of second site mutations. Instead, the compensatory events leading to increased phagocytosis in the mutant are more likely to involve metabolic changes, regulation within networks of gene expression, or epigenetic phenomena.

In principle, compensation that leads to the increased plaque size in the *Dictyostelium* *aly* mutants can be of two kinds. Either the ability to degrade bacteria is enhanced or the rate of phagocytosis is augmented. The first possibility can be ruled out because when the mutants were measured for their ability to attack GFP-expressing bacteria, they perform similar to wild-type cells (Fig. 4A). On the contrary, when the uptake of non-degradable yeast particles is measured, the rate of phagocytosis for all strains corresponds to the behavior observed in the plaque-forming assay (Figs. 4 and 5).

The rate of phagocytosis depends on the dynamics of the actin cytoskeleton, and many *Dictyostelium* mutants affected in actin-binding proteins show a reduced rate of particle uptake (51). Interestingly, a number of mutants exist that are characterized by an increased rate of phagocytosis (52–54). When the expression levels of a variety of these proteins were analyzed by Western blotting of mutant cell extracts, no differences to the wild type were detected (data not shown). On the other hand, it may be informative to look in more detail into signal-transducing cascades. In particular, overexpression of the small GTPases RacC or Rap1 is known to result in increased phagocytosis, whereas fluid-phase uptake remains unaffected (55, 56). Although these proteins could possibly signal to the cytoskeleton, it remains to be investigated how they would perceive the level of AlyA within the compartments of the secretory and/or endocytic pathway.
补偿溶菌酶缺陷

图7. 多序列比对和溶菌酶的系统发育树。A，使用ClustalX进行的多序列比对。图中显示了ALY同源物A–D的氨基酸序列。图中还展示了D. discoideum的其他多肽类（DdLysC1–3）、噬菌体类（DdLysT41–2）、Entamoeba类溶菌酶（DdLysEh1–2）的氨基酸序列，并与来自其他物种的代表性同源物进行了比对。B，使用PAUP 4.0绘制的溶菌酶系统发育树。数据来源见“实验方法”部分。使用了邻近加入法构建的未根化距离树。节点处的数字表示1000次重复的Bootstrap比例；大于70%的值只在图中显示。ALY家族成员用粗体字标示。无脊椎动物溶菌酶被认为是一个单系群，因此整个分支被着色。
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