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 alyA 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 Rabs, the lysosome and/or regulating its integrity by modulating fusion and fission events. Among these proteins are Rabs, 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 in vivo. Interference with Rabs (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 N-acetylmuramic acid and N-acetylgalactosamine (16). Despite the fact that Dictyostelium 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 Dictyostelium 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 for 4 min, and washed 2 times in Sörensen’s 17 mM sodium/potassium phosphate, pH 6.0. Freshly harvested and washed amoebae (2.5 × 10^9 cells) were kept by three cycles of freezing and thawing in 5 volumes of 10 mM sodium acetate, pH 4.5, supplemented with complete proteinase inhibitor mixture (Roche Applied Science). The lysate 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 column 2.5 × 20 ml; Bio-Rad) manually filled with a 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 Biocisent) 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 microbichinonic acid reagent assay (Fischer) and hen egg lysozyme as standard (18). Tricine SDS-PAGE was performed according to Schagger et al. (18). Protein concentration was determined using the microbichinonic acid reagent assay (Pierce) and hen egg lysozyme as external standards.

**Enzyme Assays**—Lysozyme activity was measured as described previously (26-28). Enzyme activity was assayed as a control. To monitor lysozyme activity during purification, column fractions were analyzed by using the lysozynetest method (21). For quantitative measurements of lysozymic activity, cell wall degradation of lysophilized Micrococcus luteus (Sigma) was determined turbidimetrically essentially according to Shugar (22). Degradation of cell walls of Staphylococcus aureus (Sigma) was tested in the assay. To determine the effect of pH and ionic strength on lysozyme activity, the turbimetric assay was adjusted according to Dobson et al. (23). Various buffers were prepared as specified by Miller and Goldar (24). They covered a pH range from 3.5 to 7.5, their ionic strength varying between 0.005 and 0.2. The occurrence of reducing N-acetyl amino sugars was tested as described by Reissig et al. (25). Chitinolytic activity was assayed using chitin glycol (Sigma) as a substrate (26). Activity against viable bacteria was determined in a microdilution susceptibility assay as published previously (27).

**Gene Disruption**—The sequence encoding AlyA was amplified from AX2 genomic DNA using primers bearing an EcoRI recognition sequence in front of the start codon (5′-CGCGGATCTATCAATGGAGATGGTTGTCTTCTTACTGTTAGGCGG-3′) and a BglII site after the last codon specifying an amino acid (5′-GGAGATCTTCGACATGAAACCCATTAAGCTGTTTACGCACG-3′), cleaved with the corresponding enzymes, and ligated into the same sites of pIC19H. After deleting a SalI site in the polylinker of this plasmid, which is also recognized by HincII, the lysozyme sequence was cut at its remaining HincII site, and a blunted cassette conferring bacterial resistance, isolated via BamHI and HindIII digestion from plasmid pUCBsr-Bam (28), was inserted. The resulting vector was cleaved with SmaI and NruI situated in the polylinker region to release the plasmid backbone required for replication and selection in Escherichia coli. The remaining linear 2.5-kilobase fragment containing 560 bp of lysozyme homologous sequences upstream and 550 bp downstream of the resistance cassette was transfected into Dictyostelium AX2 cells by electroporation, and the resulting clones were isolated by limited dilution into multiwell plates. To identify clones that carry a disrupted lysozyme gene, we performed PCR on preparations of genomic DNA (29) using primers (5′-GGGCGGTTCTCTTTATTTTCAAC-3′ and 5′-CTTCGCGAACCATGCTTGGC-3′) that flank 85 bp of the coding sequence containing two introns of different lengths which distinguish between the genes encoding lysozyme isoforms.

**Expression of Lysozyme and Tagged Variants**—To rescue possible defects in a lysozyme mutant, EcoRI and HindIII enzymes were used to cut out lysozyme from pCl19H, and the gene was inserted into the expression vector pDEX-RH (30) cut with the same restriction enzymes. To allow for detection of the transgene, a Myc epitope sequence cloned in pC20R (31) was cut out using Smal and AccI enzymes, blunted by filling in the two-base overhang, and ligated into the HincII site within the lysozyme sequence of the pDEX-RH expression construct. To monitor the distribution of lysozyme, a GFP fusion was constructed. The lysozyme coding sequence from the ATG up to the cleavage site of the prodomain was amplified by DPCR using a dPCR primer with a 5′-nuclease tag (courtesy of Peter Devreotes) as a template. The product was digested at the EcoRI and BglII cleavage sites that flank the PCR primers 5′-CGGATCCAAAAAGTTGGCTCTTTTTTTTATTTTTTCCAG-G3′ and 5′-GGAGAAGCTTTGACATGCTTTTATTTTATTTTTTTCCAG-G3′ and inserted into pDd-A15-gfp (32) as modified by (33), so that GFP replaces the C-terminal domain of lysozyme.

**Chitinolytic Activity**—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 test strains. These bacterial species were grown on blood agar 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 specific and unique determinant residing on many lysozymic enzymes (38). mAb AD 7.5 binds to an N-acetylgalactosamine 1-phosphate modification of another class of lysozymic enzymes (39). The antigen corresponding to mAb 130-80-2, an esterase gp70, accumulated in crystal structures of E. coli (38). mAb AD 7.5 binds to an N-acetylgalactosamine 1-phosphate modification of another class of lysozymic enzymes (39). The antigen corresponding to mAb 130-80-2, an esterase gp70, accumulated in crystal structures of E. coli (38). mAb AD 7.5 binds to an N-acetylgalactosamine 1-phosphate modification of another class of lysozymic enzymes (39). The antigen corresponding to mAb 130-80-2, an esterase gp70, accumulated in crystal structures of E. coli (38).

**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 Dicyostelium protein data base (dicybase/dbj/cgi-bin/blast.pl), resulting in sequence information for alyA (AA008434), alyB (AA008432), alyC (AA0051440), alyD (DDB0217279 and DDB016810), DdLsyc1, DdLsyc2, and DdLsyc3 (D. discoideum similar to c-type lysozymes; U66323, DDB0189256, DDB0205537, respectively). Dicyostelium genes coding for DlsyT41 and DdLsyc4 (lysozymes of D. discoideum similar to T4 phage-type lysozymes; DDB0217501 and DDB0167824, respectively) and DdLsycE1 and DdLsycE2 (lysozymes of D. discoideum similar to Entamoeba histolytica; DDB0167552 and DDB0198864, respectively) were also identified in the Dicyostelium genome. The amino acid sequence of lysozymes of diverse organisms used in the phylogenetic analysis retrieved from the protein Entrez data base of NCBI and listed below with their respective abbreviations in Fig. 7 (source organism; accession numbers): LysC (Gallus 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 (AA162083), Bathymodiolus thermophilus (AA162082). (Caenorhabditis elegans 1–3 (AAC10179, AAC19181, AAA83197), Caelytoga sp. 1 and 2

1 The abbreviations used are: Tricine, N-[2-hydroxy-1,1-bis(hydoxy-methyl)ethyl]glycine; Aly, ameba lysozyme; mAb, monoclonal antibody; GFP, green fluorescent protein; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; TRITC, tetramethylrhoda-
We purified a protein with lysozyme activity from axenically cultured Dictyostelium discoideum. The purified protein was subjected to N-terminal sequencing, yielding a single amino acid sequence up to residue 25 (YSCPKPCYGNMCCSTSPQNYLTD). This sequence was back-translated into nucleic acid sequence and used to search a cDNA database, identifying the corresponding gene on chromosome 2 in the database from the Dictyostelium genome project. The information on genomic structure and amino acid sequence is summarized in Fig. 2.

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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 \textit{alyA} and \textit{alyB} genes (see below) revealed both genes in genomic DNA from wild-type cells, whereas only a product corresponding to \textit{alyB} was detected in the mutant (Fig. 3A). The mutant carried the blasticidin resistance cassette precisely within the \textit{alyA} locus (Fig. 3B). As seen previously in strain 74, the knock-out mutant \textit{alyA138} had a total lysozyme activity corresponding to about 40% of wild-type cells and maintained this level over more than 2 months (Fig. 3C). When the plaque diameters of the \textit{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. 3D). Despite these changes, repeated PCR analyses did not reveal any alteration at the \textit{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 \textit{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 \textit{alyA138} mutant performed as efficiently as wild-type cells (Fig. 4A), 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 \textit{alyA138} mutant cells was almost 2-fold higher than the rate of wild-type phagocytosis (Fig. 4B), providing a reasonable explanation for the increased

\textbf{FIG. 2. Sequence features of the \textit{alyA} gene.} Nucleotide sequence of the cDNA clone SSF469 and derived amino acid sequence. The residues identified by peptide sequencing are shown in \textit{boldface}. \textit{CS} denotes the cleavage site positions between the signal peptide (residues 1–19), the mature protein, and the prodomain (residues 139–181). Above the nucleotide sequence, \textit{H2} denotes the single HincII site used for insertion of the resistance cassette and the Myc epitope, respectively (see Figs. 3 and 5). The positions of the six introns from the genomic clone are numbered I1–I6. The \textit{underlined} sequences indicate the position of primers used for PCR in Fig. 3.
Compensation of Lysozyme Deficiency

AlyA Endocytic Performance of the alyA Mutant and Rescue of the Phenotype—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.

The decrease of intracellular fluorescence was measured over time. B, 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. r.u., relative units. C, fluid-phase uptake is unaltered in the alyA138 mutant. Cells were allowed to internalize nutrient medium containing fluorescently labeled dextran, and intracellular fluorescence (relative units) originating from phagocytosed particles was measured at 15-min intervals. r.u., relative units. D, re-expression of AlyA reverts the plaque size to wild-type-like dimensions. Conditions were as for Fig. 3D. Filled bars are from wild-type cells, open bars represent the alyA138 mutant, and hatched bars denote the rescued strain. Values (in mm) are the mean of 6–7 measurements in at least three independent experiments (n as indicated). Error bars are S.E. r.u., relative units. E, re-expression of alyA increases total enzyme activity about 2-fold. Enzymatic activity in cell extracts is measured as the decrease in absorbance of a bacterial cell wall preparation over time.

![Graphs](image)

**Fig. 4.** Endocytic performance of the alyA mutant and rescue of the phenotype. A, degradation of bacteria by alyA138 cells (open circles) in vivo occurs with the same efficiency as in the wild-type strain (filled circles). Cells were challenged with GFP-expressing bacteria, and
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.
The only lysozymes of protozoan origin characterized at the molecular level to date are from *Entamoeba 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 database 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 serine and glycine. 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 other only 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 mannose-6-SO\textsubscript{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 isoform, 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 *alyA* mutant cells grow on lawns of *E. coli* and *K. aerogenes*.

However, when the *alyA*-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, *alyA* 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* *alyA* 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.
Fig. 7. Multiple sequence alignment and phylogenetic tree of lysozymes. A, multiple sequence alignment using ClustalX. The amino acid sequences of the ALY isoforms A–D are shown as proenzymes. The other amino acid sequences of putative lysozymes of D. discoideum, namely c-type (DdLysC1–3), phage-type (DdLysT41–2), and Entamoeba-type lysozymes (DdLysEh1–2), were found in databases and are aligned with representative homologues of the respective class of lysozymes from other organisms. B, phylogenetic tree of lysozymes from different species drawn and analyzed with PAUP 4.0. Data on sequences are given under “Experimental Procedures.” An unrooted distance tree (neighbor joining) is presented. Numbers at the nodes represent bootstrap proportions on 1000 replicates; values greater than 70% are depicted only. The ALY family members are marked by bold letters. The invertebrate lysozymes are considered a monophyletic family, and therefore, the entire branch is shaded.
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