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Novel zinc protease gene isolated from *Dictyostelium discoideum* is structurally related to mammalian leukotriene A4 hydrolase

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ABSTRACT. The allantoicase (allC) gene of *Dictyostelium discoideum* allC RNAi mutant strain was silenced using the RNA interference technique. The mutant strain is motile, aggregated, and could not undergo further morphological development. The growth rate is high and the cells show a shortened cell cycle comparing with wild-type *D. discoideum*. However, the mechanisms regarding these actions remain unclear. mRNA differential display was used in this study to identify genetic differences. A novel *D. discoideum* gene (GenBank accession number: KC759140) encoding a new zinc protease was cloned. The amino acid sequence of the novel gene exhibited a conserved zinc-binding domain (HEX2HX18E) that allowed its classification into the M1 family of metallopeptidases. The gene encoded a 345-amino acid protein with a theoretical molecular mass of 39.69 kDa and a theoretical pI of 6.05. This protein showed strong homology with leukotriene A4 (LTA4) hydrolase of *Homo sapiens* (41% identity and 60% similarity at the amino acid level). By analyzing quantitative reverse transcription-

polymerase chain reaction data, this zinc protease gene was more highly expressed in *D. discoideum* allC RNAi mutant type than in wild-type KAx-3 cells during the trophophase. The novel zinc protease gene may function as an LTA4 hydrolase and contribute to the shortening of the allC RNAi mutant cell cycle.

Key words: *Dictyostelium discoideum*; Leukotriene A4 hydrolase; M1 family; Zinc-binding site

INTRODUCTION

During the proliferation of *Dictyostelium discoideum*, the cells feed on bacteria. After 24 h when food sources are exhausted, *D. discoideum* interacts and assembles with other cells in response to extracellular cyclic adenosine monophosphate (Parent and Devreotes, 1996), and then undergoes multicellular developmental stages, including aggregation, slug formation, and eventual formation of a fruiting body (Konijn et al., 1969; Ginsburg et al., 1995; Williams, 2010). The allantoicase (allC) gene of the allC RNAi mutant strain was silenced by RNA interference in a previous study. These cells showed a shorter cell cycle, a reduction of cellular size and cellular allC concentration, and interruption of development (Xue and Hou, 2012).

Using the mRNA differential display method, a novel zinc protease gene (1424 bp) was isolated from allC RNAi mutant cells. This zinc protease gene was overexpressed in allC RNAi mutant cells during trophophase. The amino acid sequence included a C-terminal leukotriene A4 and a conserved catalytic domain zinc-binding motif HEX2HX18E specific to metallopeptidases in the M1 family (Medina et al., 1991a; Vazeux et al., 1998; Lausten et al., 2001; Pham et al., 2007). This zinc-binding catalytic region (HEX2HX18E) can bind a single Zn ion for Zn-driven hydrolysis of the substrate. Its proximal glutamic acid is required for the hydrolysis of peptide bonds and subsequent release of the substrate. The distal glutamic acid and 2 histidines in this region can coordinate the Zn ion (Thompson et al., 2003). Additionally, the protein encoded by the zinc protease gene shows 39 and 41% identity to LTA4 hydrolase of *D. discoideum* and *Homo sapiens,* respectively. LTA4 hydrolase is classified as a member of the M1 family of zinc metalloproteases, which also includes enzymes such as aminopeptidases A, B, and N. Humans have 9 M1s, including 6 transmembrane proteins (aminopeptidase A, aminopeptidase N, insulin-regulated aminopeptidase, endoplasmic hormone-degrading ectoenzyme) and 3 soluble proteins [puromycin-sensitive aminopeptidase (PSA), aminopeptidase B and LTA4 hydrolase] (Tanioka et al., 2003; Albiston et al., 2004; Wendy, 2011). To date, only 3 proteins in the M1 family have been identified in *D. discoideum*: 2 homologs of mammalian PSA known as PsaA and PsaB, and LTA4 hydrolase. Furthermore, evidence suggests that PSA regulates the cell cycle and differentiation in *D. discoideum* (Catalano et al., 2011; Huber and O'Day, 2011). LTA4 hydrolase was found not only in mammalian species but also in several lower vertebrates, including fish and frogs and in some lower species (Pettitt et al., 1991; Strömberg-Kull and Haeggström, 1998). An LTA4 hydrolase showing 39% identity (53% similarity) to the human enzyme was cloned and characterized from yeast, *Saccharomyces cerevisiae* (Kull et al., 1999). The data showed that LTA4 hydrolase is an important specific zinc metallohydrolase involved in the 5-lipoxygenase pathway and a gatekeeper of chemotactic leukotriene B4 biosynthesis, which is an important proinflammatory mediator (Haeggström, 2004).

Little data is available regarding the specific mechanism of allC RNAi in mutant cells.

Here, we used the differential display method established in *Dictyostelium* cells (Chae and Maeda, 1998) to determine the genetic differences between wild-type and allC RNAi mutant type cells. We identified a novel gene in *D. discoideum* encoding a putative zinc protease with structural similarity to LTA4 hydrolase, which may provide insight into the development and evolution of *D. discoideum*.

MATERIAL AND METHODS

Cell culture and RNA preparation

Both KAx-3 cells and allC RNAi mutant cells were cultured in axenic medium HL5 at 22°C (Sussman, 1987). When the growth of KAx-3 and allC RNAi mutant cells was stable, the number of cells was approximately 2.65 x 10^6 and 5.42 x 10^8 cells/mL, respectively. Cells were harvested by centrifugation (3000 rpm) in 10 min, washed twice with ice-cold phosphate buffer (1.2 mM NaH_2PO_4; 0.4 mM Na_2HPO_4, pH 6.4) and once with ice-cold PDF buffer (9.2 mM K_2HPO_4; 11.8 mM KH_2PO_4; 20.1 mM KCl; 5.3 mM $MgCl_2$, pH 6.4). Total RNAs from the 2 strains were extracted using RNAiso Plus reagent (TaKaRa, Shiga, Japan).

Polymerase chain reaction (PCR)-based mRNA differential display

Using 1 mg total RNA was extracted from each strain, and reverse transcription was performed with PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa). Next, reverse transcript cDNA templates were diluted into 1000 mg/mL and amplified using the primers using the oligo d(T) primer (H-T11A 5'-AAGCTTTTTTTTTTTA-3'; H-T11C 5'-AAGCTTTTTTTTTTC-3'; H-T11G 5'-AAGCTTTTTTTTTTG-3') with one arbitrary primer (H-AP25 5'-AAGCTTTCCTGGA-3'; H-AP26, 5'-AAGCTTGCCATGG-3'; H-AP27 5'-AAGCTTCTGCTGG-3'; H-AP28 5'-AAGCTTACGATGC-3'; H-AP29 5'-AAGCTTAGCA GCA-3'; H-AP30 5'-AAGCTTCGATCGT-3'). PCR amplification reactions were carried out in a final volume of 25 µL containing 12.5 mL 2X Premix Taq™ (TaKaRa), 1 µL template, including 1000 ng cDNA, 0.5 µL of each primer, and 10.5 µL PCR-grade water. The thermal profile for PCR was as follows: predenaturaion at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 40°C for 2 min, elongation at 72°C for 2.5 min, and a step at 72°C for 15 min. The PCR products were visualized on a 1.2% agarose gel.

Plasmid constructs and cloning

Several different cDNA fragments expressing either in KAx-3 or allC were randomly selected. These cDNAs were collected and purified using the SanPrep Column DNA Gel Extraction Kit (Sangon Biotech, Shanghai, China), and then cloned into the pUCm-T vector (Sangon Biotech), and transformed into DH-5α cells. Clones were generated on SM solid medium. Recombinants were identified through blue/white screening. Plasmids were extracted using the SanPrep Column Plasmid Extraction Kit (Sangon Biotech) and evaluated by single digestion with *Pst*I. Positive clones were sequenced by Invitrogen (Carlsbad, CA, USA).

Quantitative reverse transcription-PCR (qRT-PCR)

SYBR Green qRT-PCR technology was used to analyze differences in the expression levels of targeted cDNA. qRT-PCR was performed using a C1000TM Thermal Cycler (Bio-Rad CFX 96™ Real-Time System, Hercules, CA, USA) according to manufacturer instructions. The sense primer and anti-sense primer were designed based on the targeted gene. Samples were run in triplicate with glyceraldehyde 3-phosphate dehydrogenase as a control. The specificity of the amplified products was evaluated using the $2^{-\Delta\Delta Ct}$ comparative Ct method (Livak and Schmittgen, 2001). qRT-PCR amplification reactions were carried out in a final volume of 25 μL, which contained 12.5 μL 2X SYBR Premix Ex Taq™ (TaKaRa), 1 μL diluted template, 200 ng cDNA, 10.5 μL PCR-grade water, and 0.5 μL of each primer. PCR reactions were as follows: predenaturation at 95 for 30 s, 35 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 20 s, and elongation at 72°C for 20 s. PCR data were analyzed using the CFX Manager™ software (version 1.0).

Sequence alignments and sequence homology analysis

The acquired full-length cDNA sequence was analyzed using BLAST in the NCBI blastx with other species. cDNA sequences with relatively high similarity from various species were retrieved from the NCBI GenBank database. Aligned sequences were analyzed using the ORF Finder and ClustalW Mutiple Alignmet programs (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The sequence homology was analyzed using NCBI blastp.

Phylogenetic analysis

A related neighbor-joining phylogenetic tree was constructed using the MEGA version 5.2.2 app (Tamura et al., 2007). The reliability of the branching tested using bootstrap re-sampling with 1000 replicates.

RESULTS AND DISCUSSION

Characterization of the acquired gene sequence and its deduced amino acid sequence

Using the PCR-based mRNA differential display method, a full-length cDNA fragment was amplified by the single primer H-AP28. This full-length cDNA was 1424 bp with an open reading frame of 1023 bp, a short 47-bp-long 5'-untranslated region and a 354-bp-long 3'-untranslated region. The first triplet (starting at nucleotide 48 in Figure 1) in the sequence was considered to be the initiation codon for protein translation because the first and only ATG triplet appeared before the zinc-binding motif HEX2HX18E. This cDNA sequence had no poly(A+) tail and ended at the TAA termination codon. The sequence was isolated from allC RNAi mutant cells and was not identified in KAx-3 cells using the same method. This complete sequence has been deposited in GenBank (accession No.: KC759140); its deduced amino acid sequence is shown in Figure 1. The deduced protein was composed of 340 amino acids, had a theoretical molecular mass of 39.69 kDa and a predicted isoelectric point of 6.0. In the open reading frame of this sequence, several typical figures were observed: a zinc-binding motif HEX2HX18E in the core region (residues 26-49), 5 potential N-linked glycosylation

sites, 1 potential N-myristoylation site, 6 potential casein kinase II phosphorylation sites, and 3 protein kinase C phosphorylation sites. The BLASTP search revealed that the putative amino acid sequence was highly similar at the LTA4 hydrolase C-terminus and partially similar in the core region of the M1 family (data not shown).

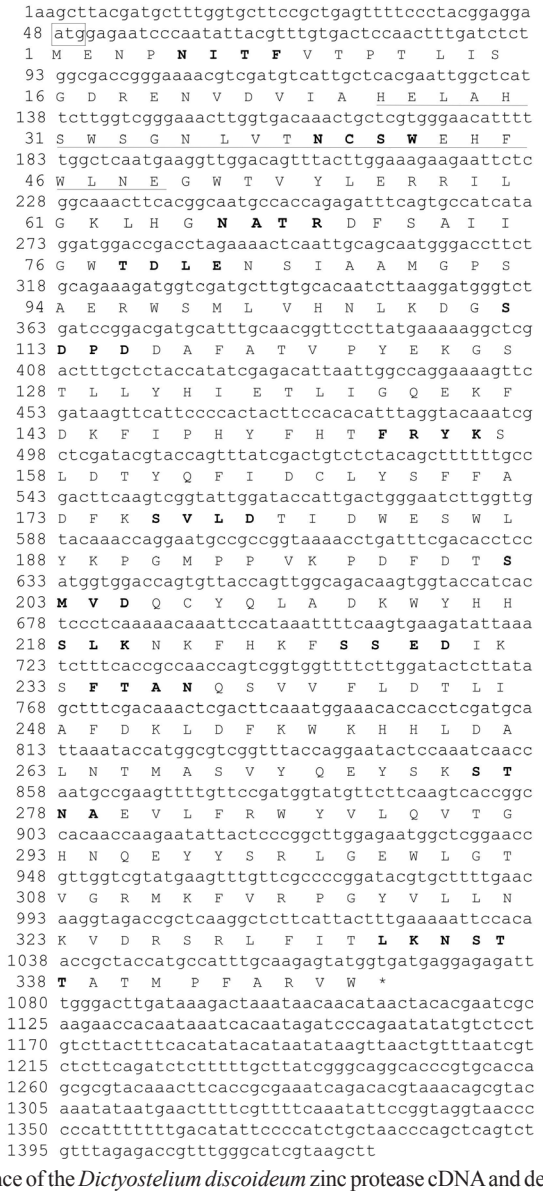

Figure 1. Nucleotide sequence of the *Dictyostelium discoideum* zinc protease cDNA and deduced amino acid sequence. The nucleotides from number 1-47 and 1111-1395 are the 5'untranslated regionand 3'untranslated region, respectively. The initiation codon and the stop codon are indicated with a square and an asterisk, respectively. The deduced amino acid sequence is shown in upper case letters. The zinc binding motif (HEX2HX18E) is underlined. The casein kinase II phosphorylation site, N-myristoylation site, and protein kinase C phosphorylation site are shown in bold.

Multiple alignment and sequence homology analysis of the deduced *D. discoideum* zinc protease with other species

Multiple alignment of the deduced amino acid sequence of the allC zinc protease with the *H. sapiens*, the *Mus musculus*, the *D. discoideum* LTA4 hydrolase, and rat aminopeptidase B (Ap-B) analysis are shown in Figure 2. Sequences were aligned using ClustalW2 Multiple Sequence Alignment program (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

In Figure 2, the residues His295, His299, and Glu318 both in *H. sapiens* and *M. musculus* LTA4 hydrolase sequences were conserved and together form the catalytic zinc site of zinc metallopeptidase and are involved in the epoxide hydrolase and the aminopeptidase activity of the mammalian LTA4 hydrolase (Vallee and Auld, 1990; Medina et al., 1991b; Baset et al., 1998). The His295, His299, and Glu318 match with the His26, His30, and Glu49 in the *D. discoideum* zinc protease. Except for the typical consensus zinc binding motif HEX2HX18E, the tryrosine residue Tyr383 in both of the *H. sapiens* and the *M. musculus* LTA4 hysrolase and conserved as Try380 in the *D. discoideum* LTA4 hydrolase are essential for the peptidase activity of mammalian aminopeptidase and acts as a proton donor in the general base mechanism (Blomster et al., 1995). The key residue of LTA4 hydrolase (Tyr383 in the *H. sapiens* and the *M. musculu*, Try379 in the *D. discoideum*) is also conserved and matched the Tyr116 in the *D. discoideum* zinc protease sequence. Interestingly, the Tyr378 in both the *M. musculus* and *H. sapiens* LTA4 hydrolase sequences, which is thought to be an essential amino acid involved in the covalent binding of LTA4 (Mueller et al., 1996), was replaced with Phe111 in the zinc protease sequence, Phe 375 in the *D. discoideum* LTA4 hydrolase, and Phe 491 in *Meyerozyma guilliermondii* (*M. guilliermondii*) LTA4 hydrolase homolog 2. There have been few studies examining this phenomenon.

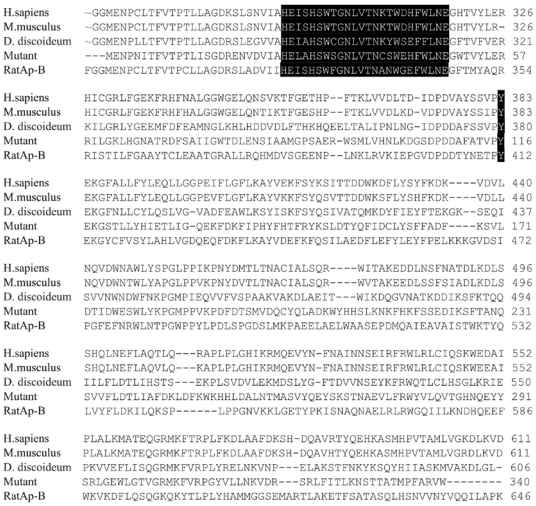

Figure 2. Multiple alignment of amino acid sequences of the *Homo sapiens* leukotriene A4 hydrolase, *Mus musculus* leukotriene A4 hydrolase, *Dictyostelium discoideum* leukotriene A4 hydrolase, rat Ap-B, and *D. discoideum* zinc protease. The last amino acid of each row is numbered according to the location in its own sequence. Both the zinc-binding motif, common among members of the M1 family of metallopeptidases (HEX2HX18E), and the tyrosine residue (number 383 in both the human and the mouse sequences), which is essential for the peptidase activity of the human and mouse LTA4 hydrolase/aminopeptidase, are indicated on a black background.

To verify the homology of the deduced zinc protease, we analyzed the homology of the proteins described by multiple alignment and included the *Dictyostelium* puromycin-sensitive aminopeptidase A and B (2 zinc-binding aminopeptidases of the M1 family have been identified in *Dictyostelium*) using the NCBI BLASTP method. The resulting data is shown in Table 1.

Table 1. Amino acid sequence identity (%) between *Dictyostelium discoideum* zinc protease and other aminopeptidases.

	Dd zinc protease	hLTA4	mLTA4	dLTA4	*Mg*LTA4	dPA-A	dPA-B	rAP-B
Dd zinc protease	100	41	41	39	47	30	29	32
hLTA4	41	100	93	44	40	26	26	39
mLTA4	41	93	100	43	40	27	27	39
dLTA4	39	44	43	100	39	27	28	38
*Mg*LTA4	47	40	40	39	100	26	26	32
dAP-A	30	26	27	27	26	100	65	27
dAP-B	29	26	27	28	26	65	100	27
rAP-B	32	39	39	38	32	27	27	100

A BLASTP search of NCBI identified LTA4 hydrolases followed by aminopeptidases as the most closely related proteins to the *D. discoideum* zinc protease. Dd zinc protease, *D. discoideum* zinc protease; hLTA4, *Homo sapiens* LTA4 hydrolase; mLTA4, *Mus musculus* LTA4 hydrolase; dLTA4, *D. discoideum* LTA4 hydrolase; MgLTA4, *Meyerozyma guilliermondii* LTA4 hydrolase homolog 2; dPA-A, *D. discoideum* puromycin-sensitive aminopeptidase A; dPA-B, *D. discoideum* puromycin-sensitive aminopeptidase B; rAP-B, Rat aminopeptidase-B.

The *D. discoideum* zinc protease showed relatively high identity with the *M. guilliermondii* LTA4 hydrolase homolog 2 (47%), *H. sapiens*, and *M. musculus* LTA4 hydrolase (41%) at the amino acid level, with lower identity to the *D. discoideum* LTA4 hydrolase (39%) and puromycin-sensitive aminopeptidase A and B (30 and 29%, respectively). In contrast, the *M. guilliermondii* LTA4 hydrolase homolog 2 showed 40% identity with *H. sapiens* and *M. musculus* LTA4 hydrolase. The *D. discoideum* LTA4 hydrolase was 44 and 43%, respectively. The phenylalanine (F111 of zinc protease and F375 of LTA4 hydrolase) in *D. discoideum* may have evolved into tyrosine in mammalian LTA4 hydrolase. This zinc protease gene may be more advanced than *M. guilliermondii* LTA4 hydrolase homolog 2, but less advanced than mammalian LTA4 hydrolase, suggesting an evolutionary relationship.

Furthermore, the identity of the primary protein structure between *D. discoideum* zinc protease and mammalian LTA4 hydrolases extends over nearly the entire sequence, but some divergence was observed in the N-terminus. Although the *D. discoideum* zinc protease sequence was approximately 271 amino acid shorter than *H. sapiens* LTA4 hydrolase, a region of approximately 300 amino acids overlapped, functioning as an aminopeptidase of the M1 family. This region contained the canonical zinc-binding motif HEX2HX18E. Thus, the zinc protease may function as an LTA4 hydrolase.

Interestingly, members of the M1 family generally contain a GXMEN motif and HEX2HX18E motif. GXMXN is another conserved exopeptidase motif (Iturrioz et al., 2001) present in the M1 family. However, the deduced *D. discoideum* zinc protease identified in this study only contained the HEX2HX18E motif and not the GXMXN motif. There are also some exceptions, such as a bifunctional aminopeptidase B (Ap-B) from the rat testis (Sandrine et al., 1997). Ap-B exhibits 33% identity and 48% similarity with LTA4 hydrolase and has the capacity to hydrolyze leukotriene A4 (Table 1). However, this requires further analysis.

Phytogenetic analysis

To further explore the structural and evolutionary relationships between the *D. discoideum* zinc protease and other members of the M1 metalloproteases family, phylogenetic analysis was conducted using the neighbor-joining method revealed that the zinc protease was within the LTA4 hydrolase cluster but segregated from the *D. discoideum* LTA4 hydrolase clade (Figure 3). This suggests that the *D. discoideum* LTA4 hydrolase and the zinc protease belong to a subfamily of M1 metalloproteases.

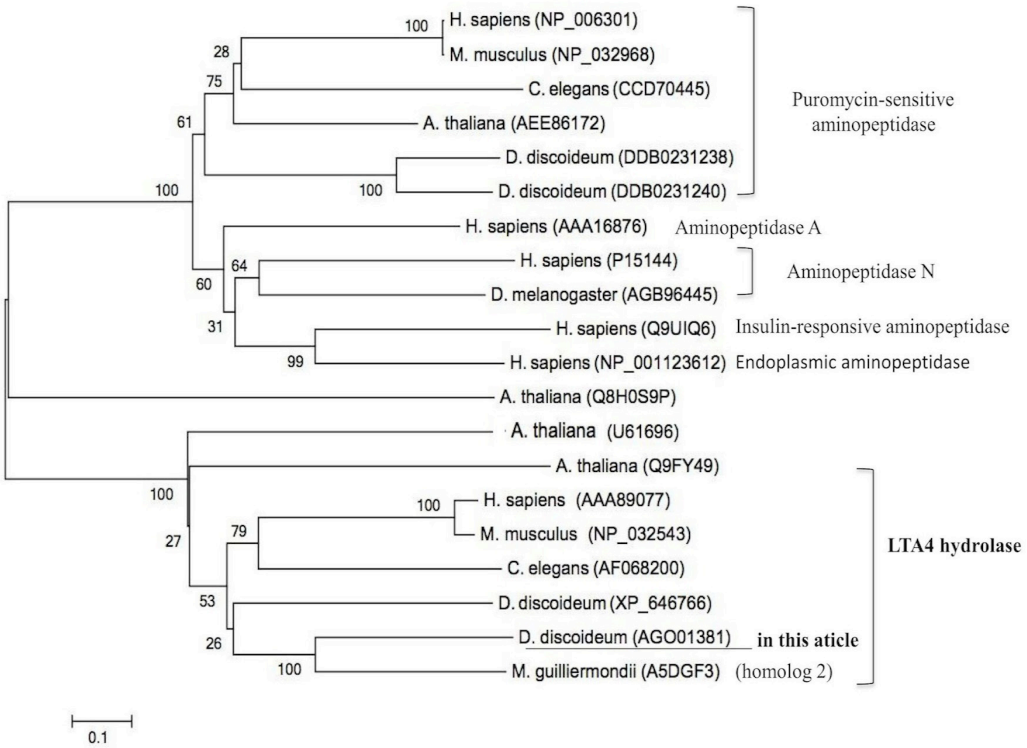

Figure 3. Neighbor-joining phylogenetic tree of the *Dictyostelium discoideum* zinc protease and similar proteins of M1 metallopeptidases. The numbers indicate the percentage frequencies with which the phylogram topology represented here replicate for every 1000 bootstrap interactions.

qRT-PCR analysis

To further verify the results of mRNA differential display and determine the relative expression level of the wild-type and mutant, the zinc protease cDNA transcript was detected both in KAx-3 cells and allC RNAi mutant cells in the trophophase using qRT-PCR. As shown in Figure 4, zinc protease gene expression in KAx-3 cells was extremely low; however, the expression level was high in allC RNAi mutant cells and was significantly different from KAx-3, according to previous mRNA differential display results showing that the LTA4 hydrolase transcript was not detected in KAx-3 cells or in allC cells.

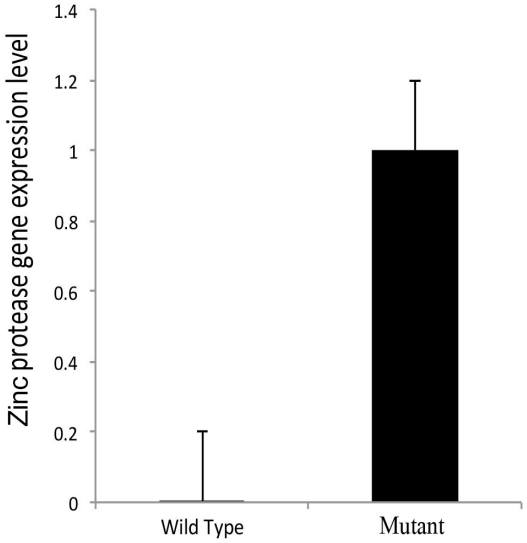

Figure 4. Relative expression level of *Dictyostelium discoideum* zinc protease gene expression in KAx-3 and allC RNAi mutant cells. The zinc protease gene represents significant differences in wild-type and allC mutant type, respectively.

The *D. discoideum* zinc protease identified in this study was found to be homologous and structurally related to LTA4 hydrolase, so that we deduced the zinc protease has a similar biochemical function as LTA4 hydrolase. Furthermore, LTA4 hydrolase is a key enzyme in the 5-lipoxygenase pathway and the gatekeeper of chemotactic leukotriene B4 (LTB4) biosynthesis. It is reported that LTB4, as one of the important bioactive compounds in the 5-lipoxygenase pathway, can enhance cell proliferation, increase cell survival, and suppress apoptosis (Hoque et al., 2005).

Because the zinc protease gene identified in this study was overexpressed in mutant cells and was significantly different from wild-type, combined with the mutant cells phenotype of a shortened cell cycle, smaller size, and fast cell proliferation, the abnormal behavior of mutant cells may have been influenced by overexpression of the *D. discoideum* zinc protease gene. The products of LTB4 and gene sequence have not been reported in *D. discoideum*. Thus, we could not determine LTB4 gene expression directly in *D. discoideum*. *Dictyostelium discoideum* is an amoeba that assembles, via cyclic adenosine monophosphate-dependent chemotaxis, into a multicellular organism in case of starvation. Exogenously added LTB4 can induce chemotaxis of leukocytes and cell aggregation *in vitro*, and this slime mold ancestral putative zinc protease together with LTB4 is thought to participate in a primitive intercellular communication system. Thus, this study provides a foundation for a multicellular development-related study.

CONCLUSIONS

In this study, a zinc protease gene encoding a 36.69-kDa aminopeptidase has been cloned from allC RNAi mutant cells of *D. discoideum*. The primary structure of the encoded protein shares relative homology with the mammalian LTA4 hydrolase, suggesting that this cDNA encoding zinc protease has an evolutionary relationship with mammalian aminopep-

tidase as well as similar function to LTA4 hydrolase. Additionally, this novel gene may be a key factor contributing to the faster growth and shortened cell cycle of the allC RNAi mutant.

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