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Original Article

Abnormalities of Endocytosis, Phagocytosis, and Development Process in *Dictyostelium* Cells That Over-Express *Acanthamoeba castellanii* Metacaspase Protein

***Entsar SAHEB [1], Wendy TRZYNA [2], Katherine MARINGER [3], John BUSH [3]**

1. *Dept. of Biology, College of Sciences, University of Baghdad, Baghdad, Iraq*
2. *Dept. of Biological Sciences, Marshall University, Huntington, West Virginia, USA*
3. *Dept. of Biology, College of Science and Mathematics, University of Arkansas at Little Rock, Arkansas, USA*

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***Correspondence**
Email:
ejsaheb@ualr.edu

Abstract

Background: *Acanthamoeba castellanii* forms a resistant cyst that protects the parasite against the host's immune response. *Acanthamoeba* Type-I metacaspase (Acmcp) is a caspase-like protein that has been found to be expressed during the encystations. *Dictyostelium discoideum* is an organism closely related to *Acanthamoeba* useful for studying the molecular function of this protozoan caspase-like protein.

Methods: The full length of Acmcp and a mutated version of the same gene, which lacks the proline rich N-terminal region (Acmcp-dpr), were cloned into the pDneo2a-GFP vector separately. The pDneo2a-GFP-Acmcp and pDneo2a-GFP-Acmcp-dpr were electro-transfected into wild type *D. discoideum* cells to create cell lines that over-expressed Acmcp or Acmcp-dpr.

Results: Both cell lines that over-expressed Acmcp and Acmcp-dpr showed a significant increase in the fluid phase internalization and phagocytosis rate compared to the control cells. Additionally, the cells expressing the Acmcp-dpr mutant were unable to initiate early development and failed to aggregate or form fruiting bodies under starvation conditions, whereas Acmcp over-expressing cells showed the opposite phenomena. Quantitative cell death analysis provided additional support for these findings.

Conclusion: Acmcp is involved in the processes of endocytosis and phagocytosis. In addition, the proline rich region in Acmcp is important for cellular development in *Dictyostelium*. Given its important role in the development process, metacaspase protein is proposed as a candidate drug target against infections caused by *A. castellanii.*

Introduction

Acanthamoeba is an opportunistic pathogen that can cause diseases in humans, such as granulomatous amoebic encephalitis and amebic keratitis (1-3). Metacaspases have been recently identified (4) and have been found in organisms lacking conventional caspases, including fungi, plants, and parasitic protozoa (5). *A. castellanii* has a metacaspase type-1 (Acmcp) that is highly expressed during the encystation process (6). Thus, this protein could be a possible drug target against dangerous protozoan including *Acanthamoeba* (7) and *Naegleria* (8). The sequence of metacaspases contains the His/Cys catalytic dyad (9, 10). Type-1 metacaspases have a prodomain with a proline-rich region, which is located at the N-terminus (4, 11). Interestingly, *A. castellanii* metacaspase has the most proline-rich region. This region has over 40 prolines in the first 150 residues. Additionally, Ala–Pro–Pro sequence is present 11 times in this region (6).

In several protozoan parasites that infect humans, genes that express metacaspases have been studied. Some of these studies have shown that metacaspases function in programmed cell death pathways. In *Plasmodium falciparum,* metacaspase (PfMCA1) has been shown to induce apoptosis, which is characterized by DNA fragmentation and disruption of transmembrane mitochondrial potential (12). Furthermore, it had been determined that *Leishmania major* metacaspase active catalytic domain has an important role in disrupting mitochondria functions in infected cells under oxidative stress (13). Although caspases generally function in apoptosis, not all members of this family follow the trend. The over-expression of PfMCA1 in yeast induces cellular growth inhibition (14). Interestingly, it has been shown that the *T. brucei* MCA4 protein is important for parasite virulence during mammalian infection (15). Another study provided evidence that *L. mexicana* metacaspase does not have a role in cell death and that it is a negative regulator of amastigote growth (16). Thus, these studies suggest metacaspase proteins have an alternative function involving instead cellular viability and/or stress signaling pathways.

Dictyostelium discoideum, a free-living amoeba, is a genetically tractable amoeba via developed genetic tools (17- 21). *Dictyostelium* shares some similarity with *A. castellanii* that is both organisms are members of Amoebozoa (22). In current research work, *D. discoideum* is considered an attractive model organism for functional studies of metacaspase since this organism possesses only a single paracaspase (23), and, as a result, there should be few overlapping functions with the other caspases. This study will help to gather insight into the novel roles that metacaspases may play outside of PCD.

D. discoideum has a well-known endocytic pathway and a highly skilled phagocytic nature (24). The extracellular fluids, such as the liquid medium used to grow laboratory strains, is endocytosed via the pinocytosis process. In *Dictyostelium,* the endocytic pathway is important as a primary source of nutrition (25, 26) and for the maintenance of plasma membrane lipids (27). Fluid is transported through endosomes into the lysosome vesicles in *D. discoideum* (28, 29). Particles, such as bacteria, are usually engulfed via the phagocytosis process. *D. discoideum* is highly skilled phagocyte cells that are capable of ingesting bacteria, yeast, and other small particles (30, 31). Additionally, *Dictyostelium* develops multi-cellular forms through a series of physiological and morphological stages by altering the properties of the cells (32). Under starvation condition, the amoebic cells aggregate and release cyclic adenylyl cyclase (cAMP). These molecules bind to specific surface receptors to activate the signaling pathways (33, 34). The multicellular organism next develops into a slug-shaped structure that migrates toward light

and heat after 6-8 hours (35, 36) in order to communicate with each other and to collect information from their surroundings (37). The slug then differentiates into a stalk leading up to a fruiting body that contains spores (38, 39, 19). Endocytosis, phagocytosis, development, and cell movement are all achieved through chemical and physical signals that the cells receive from their environment (40).

Several reported metacaspases are thought to function in the process of programmed cell death (41- 43), while other studies have shown that this protein can be activated in other cellular processes (44- 45). We have found that the metacaspase localize with the CV bladder and suggest that it has a role in regulating the function of the CV complex in *D. discoideum.* Supporting this idea, we have also found that Acmcp partially interacts with four associated CV complex marker proteins: RabD, Rab11, calmodulin, and vacuolar ATPase (V- (+) ATPase (46). In *D. discoideum,* the membrane transport system connects the endo-lysosomal and CV system (47). RabD regulates the function of the CV complex and localizes with two associated CV complex marker proteins, calmodulin and vacuolar ATPase (V-H+ ATPase) (47). This protein plays a role in the regulation of the early endocytic pathway in *D. discoideum* (48). In this report, we present experiments in order to determine the molecular function of Acmcp while using *Dictyostelium* as a model organism.

Materials and Methods

Construction of GFP tagged Acmcp and Acmcp-dpr expressing cell lines

A full length of *A. castellanii* type-I metacaspase (Acmcp) was cloned into the pDneo2a-GFP vector. In addition, a mutant version with the deletion proline region of metacaspase (Acmcp-dpr) was also cloned into the pDneo2a-GFP vector as previously described by (46). Briefly, Acmcp and Acmcp-dpr DNA were subjected to PCR cycles using a set of Acmcp primers that had a *Sal*I restriction enzyme recognition site sequence added to the sense primer (GTCGACA ATG GCA TAC CCC TAC GGA G) and *XhoI* restriction enzyme recognition site sequence added to the anti-sense primer (CTCGAG TTA CAT GAT GAA CTG GGC G). Both of the Acmcp constructs and the pDneo2a-GFP plasmid were digested with *Sal*I and *Xho*I restriction enzymes separately. These vectors' construct (pDneo2a-GFP-Acmcp and pDneo2a-GFP-Acmcp-dpr) were sequenced for errors and reading frame confirmation. This resulting construct, pDneo2a-GFP–Acmcp and pDneo2a-GFP–Acmcp, were electro-transfected into wild type *Dictyostelium* **(**AX4) cells, which were subsequently selected with G418 (Invitrogen). Western blots analysis was performed as previously described by (46).

Cells and cultural conditions

For all experiments, *D. discoideum*, WT-AX4, pDneo2a-GFP, GFP- Acmcp, and GFP-Acmcp-dpr over-expressing cell lines were grown axenically at 21^{0}C and were shaken at 150 rpm in HL5 medium. This medium was supplemented with 300 mg ml^{-1} of streptomycin sulfate and 100 mg ml^{-1} of penicillin. For the transformed cells, HL5 medium was supplemented with 10 mg ml^{-1} of G418.

Phagocytosis, endocytosis, exocytosis, and recycling assays

Phagocytosis was measured using fluorescent rhodamine isothiocynate latex beads (RITC- latex beads, Sigma Aldrich). Fluid phase endocytosis, exocytosis, and recycling rates were measured using rhodamine isothiocynate-dextran (RITC-dextran, Sigma Aldrich) as described by Rivero and Maniak, 2006 (49). Briefly, for phagocytosis assay, cells were harvested and re-suspended at 5 × 10^6 cells ml^{-1} in 10 ml of fresh nutrient medium. Cell suspension (100µL) was centrifuged and the pellet was used to determine protein content. Protein concentration was determined in

μg/mL using the BCA™ Protein Assay Kit (Pierce). Next, the rest of the cell suspension was incubated for 15 minutes on a rotary shaker and 100 μL of RITC-labeled latex bead suspension (molecular probes) at a relative ratio of 200:1 to *Dictyostelium* cells was added to the suspension. At time point 0, 1 mL samples were withdrawn into conical tubes containing 2 mL of ice-cold soerensen buffer (2 mM Na_2HPO_4, 14.6 mM KH_2PO_4, pH 6.0) to rapidly stop phagocytosis. This continued at selected time points (15, 30, 45, 60, 90, 120, and 180 minutes). Samples were collected on ice until the end of the experiment. After all samples had been collected, they were centrifuged at 800 g for 10 minutes at 4°C through an ice cold 10 mL cushion of PEG 8000 (20% w/v). By centrifuging, the suspension through a cushion of highly viscous solution of PEG particles with a diameter below 2μm will remain on top of the cushion, whereas amoebae are collected in the pellet. Cells then were washed twice with cold soerensen buffer and the pellets resuspended in 1 mL of lysis buffer (50 mM Na_2HPO_4, pH 9.3, 0.2% Triton X-100) and measured in the Horiba Jobin Yvon analytical grade FluorMax3 Spectrofluorimeter with an excitation of 470 nm and an emission of 515 nm. Relative fluorescence was plotted against time after subtraction of fluorescence at time 0 and correction for differences in protein content. Data are presented as relative fluorescence to WT, which is considered 100% at 60 minutes time point of the phagocytosis assay.

Endocytosis was measured over 180 minutes. Protein concentration was also determined. The cell suspension then was incubated for 15 minutes on a rotary shaker and 100μL of 100 mg/mL RITC-dextran solution was added to the suspension. At selected time points (0, 15, 30, 45, 60, 90, 120, and 180 min), 500μL was withdrawn and added to Eppendorf tubes containing 50μL Trypan Blue solution to quench the *Dictyostelium* cells of any particles that may stick to the cell surface. The tubes were then inverted and immediately pelleted. The cells were washed once with 1mL of soerensen buffer and the pellet was re-suspended in the same buffer. Fluorescence was immediately measured with excitation set to 544nm and emission set to 574 nm. Relative fluorescence was plotted against time after subtraction of fluorescence at time 0 and correction for differences in protein content. Data are presented as relative fluorescence to WT, which is considered 100% at 120 minutes time point of the endocytosis assay.

To find out the rate in which fluids leave the cell, exocytosis was measured over 180 minutes. The exocytosis assay was performed as a continuation of the endocytosis assay. After 180 minutes of loading with RITC-dextran (endocytosis) the cell suspension was centrifuged for 3 minutes at 500g and the cell pellet was washed and re-suspended in nutrient medium. The cell suspension was then dispensed into a 30-mL Erlenmeyer flask and incubated at room temperature on a rotary shaker. At selected time points (0, 15, 30, 45, 60, 90, 120, and 180 minutes), 500μL samples were drawn and immediately pelleted. The cells were washed once with 1mL of soerensen buffer, and re-suspended in the same buffer. Fluorescence was immediately measured. Relative fluorescence was plotted against time after subtraction of background fluorescence. For each sample, the time point 0 minutes was assigned 100% relative fluorescence and the rest of the time points were calculated relative to the corresponding 0 minutes time point.

Next, Recycling of the molecules to the plasma membrane was tested. Cultures were grown as previously described. Approximately 10^8 cells were harvested and re-suspended in 5 mL of fluorescent medium containing 20 mg TRITC-dextran. The cell suspension was collected and centrifuged. The cells were then re-suspended in non-fluorescent nutrient medium, and incubated on a rotary shaker. A 500 μL sample was immediately withdrawn (time point 0) and added to an Eppindorf tube containing 50 μL Trypan Blue solution. The cells suspension was pelleted and re-suspended in 1

mL soerensen buffer and fluorescence of TRITC was immediately measured. Samples were then collected and processed as described above for each of the remaining time points (15, 30, 45, 60, 90, 120 minutes). Relative fluorescence was plotted against time after subtraction of fluorescence at time 0. Data are presented as relative fluorescence to WT, which is considered 100% at 0 minutes time point of the recycling assay.

Development assay

For developmental studies, cells were grown axenically in HL5 medium on a rotary shaker (160 rpm) to 1 x 10^9 cells ml^{-1} and harvested by centrifugation. After repeated washing with developing buffer (5mMNa_2HPO_4, 5mMNa_2HPO_4, 5mMKH_2PO_4, 1mM$CaCl_2$, 2mM$MgCl_2$), cells were suspended in the same buffer at a density of 2 x 10^8 cells ml^{-1}. Then, 200 ml of the cell suspension were spread evenly in a 100 mm KK2 plate using a sterile glass spreader. The plates were then wrapped with plastic wrap including a wet paper towel, inverted, and incubated at 22 °C for the desired time to monitor the developmental stages (50). Photographs of multicellular development were taken using a model M 4201, 25× microscope (Switzerland).

Chemotaxis to cAMP

D. discoideum cell lines (WT-AX4, pDneo2a-GFP, GFP-Acmcp, and GFP-Acmcp-dpr cells) were grown axenically in HL5 medium to a density of 1 x 10^7 cells ml^{-1} and harvested by centrifugation. Cells were then washed twice with developing buffer and suspended in the same buffer at a density of 5 x 10^6 cells ml^{-1}. After shaking for 8 hours, 100 µl of the cell suspension were added to an outer 2 mm trough in a 60 mm agarose plate. At the same time, 100 µl of 5 µM cAMP were added to the center trough (49). Images were taken every hour using a Nikon 2000SE microscope with NIB, Image software with 10 × magnification. The edge of the trough was included in the image as a reference point.

Flow cytometry assay

To induce cell death in all cell lines, vegetative cells in late exponential growth phase were harvested and washed twice with SB (Soerensen Phosphate buffer: 14.5 mM KH_2PO_4, 2.5 mM Na_2HPO_4, and pH 6). Approximately 5 × 10^5 cell ml^{-1} was suspended in 1 ml of SB containing 3 mM cAMP (Sigma) for 8 hours at 22 °C. Cells were then washed with 1 ml of SB which was replaced afterward with either 1 ml of SB or 1 ml of SB containing 0.1 mM differentiation inducing factor DIF (1-(3-Chloro-2, 6-dihydroxy-4-methoxyphenyl)-1-hexanone, Sigma) and incubated for 30 hours at 22 °C. The cells were then suspended with a titer of 1× 10^6 cell ml^{-1} in SB and 300 µl aliquots. Test tubes for 1 µg ml^{-1} Propidium Iodide (PI) staining were used and incubated for 10 min at 22 °C with no wash. Cytometry analysis was then conducted using a FACSCalibur cytometer from Becton Dickinson using CellQuest software (51- 53).

Regrowth assay for cell death quantification

Vegetative cells in the late exponential growth phase were harvested and washed twice with SB. Approximately 5 × 10^5 cells ml^{-1} were added in 1 ml of SB containing 3 mM cAMP in a two Lab-Tek chamber and incubated for 8 hours at 22 °C. The cells were then washed with 1 ml of SB that was later replaced with either 1 ml of SB or 1 ml of SB containing 0.1 mM DIF and incubated for 24 hours at 22 °C. From each chamber, SB was removed and replaced with 1 ml of HL-5 medium and incubated for 72 hours at 22 °C. Cells were detached and counted using hemocytometer and phase contrast optics. Finally, the ratio of the number of cells re-growing in the DIF chamber to the number of cells in the control chamber was calculated (49).

Determining cell viability due to ATP levels

The number of viable cells in all cell lines was determined based on measuring the quantity of ATP (Adenosine-5'-triphosphate), which is a signal of the presence of active cells

(54). The CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega) was used for this test. Approximately 5×10^5 vegetative cells were harvested and suspended in 100 µl of 10 mM MES (Sigma). Next, the cells were transferred into opaque-walled 96-well plates (23). Cells were then lysed by adding 100 µl of the CellTiter-Glo reagent and mixing on a shaker for 2 min. The plates were then incubated at room temperature for 10 min. ATP levels present in the cellular extracts were measured using Microtiter® Plate Illuminometer B36580 with Revelation MLX software.

Results

Over-expression of Acmcp and Acmcp-dpr mutation alters phagocytosis rates of latex beads

In *D. discoideum,* the membrane transport system connects the endo-lysosomal and CV system (48). We previously showed that Acmcp and Acmcp-dpr localize in the CV bladder and have a role in regulating the function of the CV complex in *D. discoideum* (46). To determine if the over-expression of Acmcp would alter the phagocytosis rates within the cells, Acmcp and Acmcp-dpr over-expressing cell lines and the control cells were subjected to phagocytosis assay using RITC-latex beads for over 180 minutes. The rate of solid particle uptake into the cells was measured at sample time points (Fig.1A). Over the course of 60 minutes, cells expressing Acmcp had an increased rate of bead uptake (the fluorescence intensity of intracellular RITC-latex bead was 750 nm) which was about 3.5 times higher than the WT, pDneo2a-GFP, and Acmcp-dpr cells (their uptake levels were near 200 nm). Interestingly, after 60 minutes, the control cells (WT and pDneo2a-GFP) showed a decrease in the rate of phagocytosis of RITC-beads, whereas Acmcp cells showed a significant increase in phagocytosis rate over the course of 90 minutes (around 1861 nm), after which the rate began to decrease.

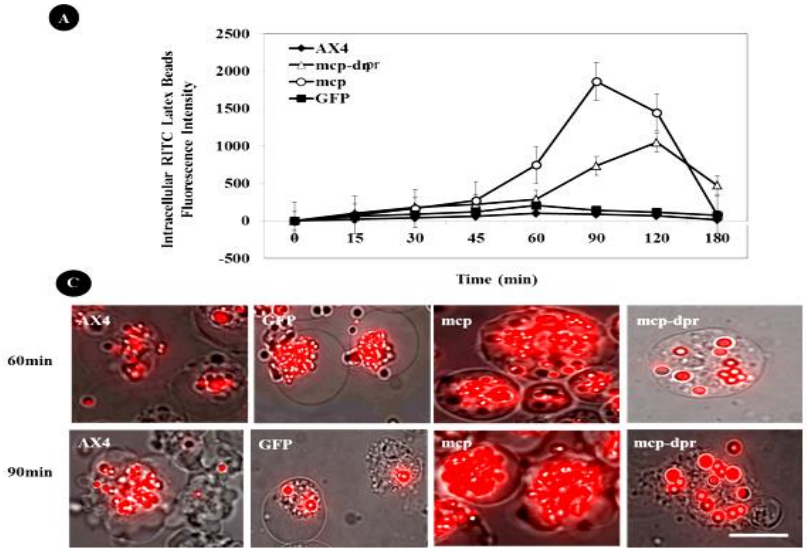

Fig. 1: Cells Over-expressing Acmcp in *Dictyostelium* has higher rates of phagocytosis during a period of 180 minutes as compared to control cell line. (A) Graphical representation with standard error showing differences in RITC latex bead phagocytosis rates in Acmcp (○) and Acmcp-dpr (Δ) cell lines over the WT (♦) and pDneo2a-GFP cells (■). Data are presented as relative fluorescence to AX4, which is considered 100%. After 180 minutes, cells over-expressing Acmcp-dpr had a high rate of phagocytosis compared to the other cell lines. (B) Overlaid images of bright field with visualization of red bead in Acmcp and Acmcp-dpr mutant cell lines show an increase in bead uptake compared to the WT-AX4 and pDneo2a-GFP, confirming the increased rates of phagocytosis observed in the graphical representation

In Acmcp-dpr cell line, phagocytosis reached its highest rate (1046 nm) at 120 minutes—which was still lower than the highest level reached by the Acmcp cell line. Surprisingly, after 3 hours, it seemed that the phagocytosis rate in the Acmcp-dpr cell line was still high, while all other cell lines had almost no latex beads (Fig.1A). This dramatic difference in the rates of phagocytosis among the cell lines was also visualized in photomicrographs of representative cell fields as shown in Fig.1B. This figure documents representative photomicrographs of RITC-bead uptake at 60 and 90 minutes of feeding. At 60 minutes, overlaid images of bright field with visualization of red beads in all cell lines revealed that the Acmcp-dpr mutant cells had more phagocytes beads inside individual cells when compared to the bead numbers in control cells. The Acmcp cell line showed a very high uptake of red beads (estimated to be 3 times) as compared to other cell lines after 90 minutes. Thus, over-expressing Acmcp or Acmcp-dpr altered the phagocytosis rates

Acmcp over-expression significantly increased the rate of fluid phase endocytosis

It was shown that RITC-dextran is good fluid phase marker as it is internalized and does not degrade in *Dictyostelium* (55). The rates of fluid phase uptake (Fig.2) were higher compared to that of the control cells. Wild type and mutant cells were fed RITC-dextran in HL5 growth media for over 120 minutes. Measurements of fluorescence intensity of RITC-dextran inside sampled cells were obtained and the data plotted as shown in Figures 2A and B. The level of total fluorescence intensity of the wild type cells was assigned a value of 100% for these assays. The results show that there was a significant difference in the endocytic rates between wild type and mutant cells. Cells over-expressing Acmcp or the Acmcp-dpr mutant had a significantly higher rate of RITC-dextran uptake at times past 15 minutes of feeding (36% and 33%, respectively, vs. the wild type value of 15%), and this trend was magnified until the 2 hour sample point (197% and 170% vs.70 and 100% for AX4 cells). Compared to the levels of the GFP vector only cell line uptake, the mutants still had a higher level of endocytosis, as well.

Based on the graphical results shown in Fig.2A and B, we wanted to visualize the endocytic pathway to confirm the data's significance. The wild type and mutant cells were again loaded with RITC-dextran for 120 minutes and subjected to fluorescence visualization of the RITC-dextran containing vesicles of the endosomal systems at 60 minutes. Representative photomicrographs of these cell lines are shown in Fig.2C. In these photographs, wild type and mutant cells contain a varied distribution of RITC-dextran endosomes that differ in size and shape. The arrows show wild type AX4 and pDneo2a-GFP cells containing diverse-sized endosomes. In Acmcp and Acmcp-dpr cells, the arrows point to the enlarged endosomes that were present in many of these cells. The overall amount of RITC-dextran contained in Acmcp and Acmcp-dpr cells was also much higher as compared to the other cell lines after 60 minutes. These data confirm that enhanced endosomal fusion and uptake activity resulted from the over-expression of the Acmcp and Acmcp-dpr proteins. In Fig.2D, overlaid images of bright field with visualization of red RITC-dextran confirm the increased uptake of RITC-dextran compared to the wild type and pDneo2a-GFP.

No significant differences in the rates of exocytosis or recycling were seen in mutant cell lines

Functional analysis was used to determine the exocytosis rates (Fig.3A and B). Cells were preloaded with RITC-dextran for 180 minutes. Samples were then taken from between 0 and 180 minutes of chase, and the amount of released (exocytose) RITC-dextran in the growth medium was determined. In the wild type and mutant cells, nearly all of the preloaded RITC-dextran had released by 180 minutes of tracking.

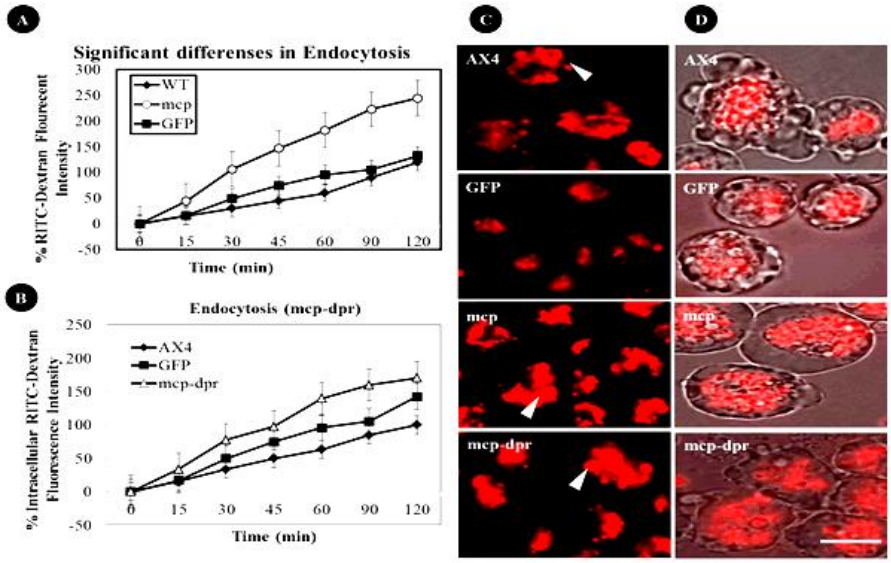

Fig. 2: The rate of endocytosis increased in cells over-expressing Acmcp or Acmcp-dpr versus control cell lines. Data are presented as relative fluorescence to WT, which is considered 100%. (A and B) Significant differences are shown in endocytosis rates. Cells expressing Acmcp and Acmcp–dpr over 120 minutes loaded with RITC- dextran showed significant differences in the rate of endocytic uptake. (C) The vesicles of the early endocytic system from endosome to lysosome were shown in red by visualization of RITC-dextran after 60 minutes of treatment. There does appear to be a difference for RITC-dextran in cell lines that express Acmcp and Acmcp–dpr mutant (indicated by arrows) compared to the controls. (D) Overlaid images of bright field with visualization of red RITC-dextran in Acmcp and Acmcp-dpr mutant cell lines show an increase in uptake of RITC-dextran compared to the WT and pDneo2a-GFP

The data revealed that the exocytosis rates in wild type and mutant cells were not different even though there were significant differences in the rates of endocytosis between the wild type and mutant cells. This suggests that the over-expression of Acmcp or Acmcp-dpr proteins did not alter exocytosis levels. Recycling of endocytosed RITC-dextran was also measured in the wild type and mutant cells (Fig.3C). Cells were pulsed with RITC-dextran for 10 minutes and tracked in growth media. At each sample point, the amount of intracellular RITC-dextran relative to the total amount in the sample was plotted on the graph. The flux time was nearly the same in each cell line. These data suggest that Acmcp does not have an effect on the rate of recycling through the cell.

Over-expressing Acmcp-dpr impairs Dictyostelium's development process

The unexpected effect of the over-expressing Acmcp on phagosomes allowed investigation into the possibility that this mutation may ultimately affect the developmental program of *Dictyostelium*. This program was triggered by starvation conditions. To address whether the Acmcp may have a role in mediating the developmental pathways, we set up a developmental assay scheme using the wild type and the mutant cell lines. First, to determine the mutation effects on streaming, cells were washed with developmental buffer and spread evenly in a KK2 plate. The cells were then monitored microscopically and photographed after 8 hours. Fig.4A illustrates that there was normal streaming among the control cells (AX4 and pDneo2a-GFP) and Acmcp cell lines. In the Acmcp-dpr mutant cells, there were little to no streaming cells. This observation suggests that deletion of the proline rich region inhibits the signal transduction and cells fail to stream toward a specific center. Next, to investigate the mutation effects on development behavior, cell aggregation was monitored.

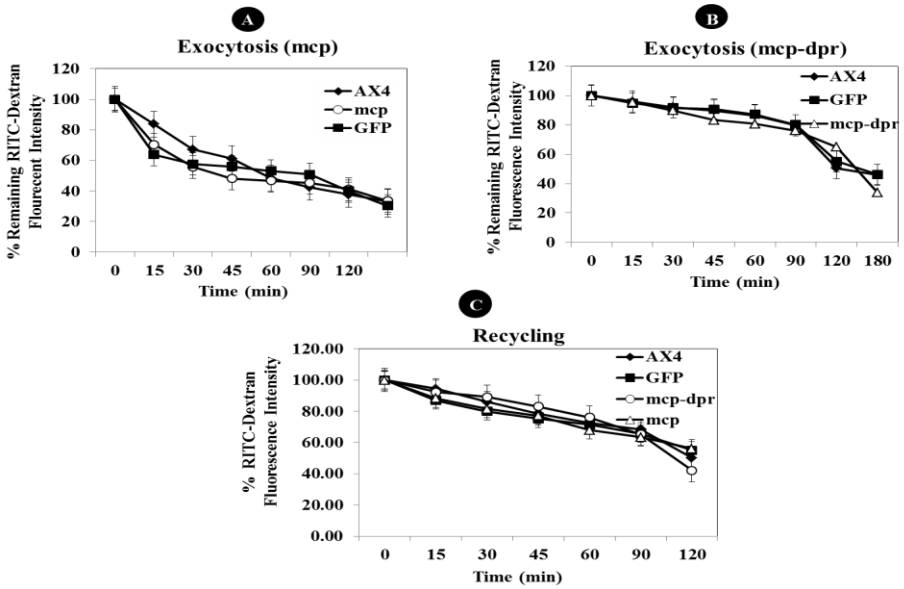

Fig. 3: No significant difference detected in the rates of exocytosis or fluid marker recycling in the mutant cell lines as compared to control cells. (A and B) After 180 minutes of accumulation, cells were washed and re-suspended in a growth medium. At the indicated times, the remaining intracellular RITC- dextran was measured. Acmcp and Acmcp-dpr cells did not show a significant change in the exocytosis process. (C) Graphical representation with standard error of RITC- Dextran recycling over 120 minutes showed no significant variation in recycling amongst all cell lines

Cells from all cell lines in mid-log phase were washed with developmental buffer and then plated as above and photographed after 16 hours of development. As seen in Fig.4B, representative photomicrographs of the wild type and pDneo2a-GFP cell lines show a normal aggregation event of *Dictyostelium* as the cells move to a central location before slug formation. However, Acmcp-expressing cells showed a wider pattern of aggregation. As seen previously, the Acmcp-dpr over-expressing cell line under these same conditions failed to aggregate, suggesting a defect in cell-cell communication.

This developmental assay was then extended to 24 hours in order to see the final formation of the stalk and spore-containing fruiting bodies. Fig.4C shows representative photomicrographs of the pDneo2a-GFP and Acmcp cells that had developed at the same rate and structure as parental wild type cells. Interestingly, the Acmcp-dpr cells exhibited a marked defect; they did not develop, and they lacked the fruiting bodies. These results show that mutant cells over-expressing Acmcp-dpr did not have the capacity to develop.

Acmcp-dpr over-expressing cell-line has less chemotactic sensitivity for cAMP due to its inability to initiate early development

The inability of Acmcp-dpr over-expressing cells to communicate with each other suggested a test to determine the chemotactic sensitivity for cAMP of these mutant cells. This experiment would provide preliminary information about how the deletion of the proline region affects the development pathway. To begin, the vegetative cells of all cell lines were incubated in the developing buffer for 8 hours to allow expression of the cAMP receptor and then added to an agarose plate containing 5μM cAMP.

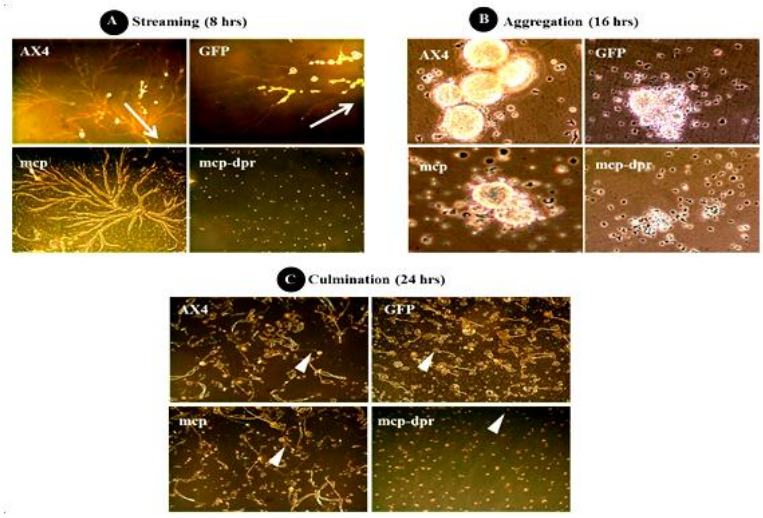

Fig. 4: Over-expression of the Acmcp-dpr protein in *Dictyostelium* causes severe defect in developmental process of these transformed cells. (A) Representative photomicrographs of streaming after 8 hours incubation at 22 °C on KK2 plates. WT and pDneo2a-GFP cells show the normal streaming event of *Dictyostelium* as the cells move to a central location before slug formation. The Acmcp cells showed fewer streaming events compared to the control cells. Acmcp-dpr over-expressing cells failed to stream under the same conditions. (B) Representative series photomicrographs of aggregation territories in absence of nutrition. After incubation for 16 hours, WT cells and pDneo2a-GFP show the normal aggregation event of *Dictyostelium* as the cells move to a central location before slug formation. Acmcp cells showed wider aggregation compared to the controls. A large quantity of non-aggregating cells was observed in Acmcp-dpr mutants. (C) Developmental representative series after 24 hours at 22°C. WT and pDneo2a-GFP cell lines developed into fruiting bodies in the absence of nutrition. The fruiting bodies of Acmcp expressing cells appear the same as those of the wild type and pDneo2a-GFP. Acmcp-dpr cells failed to develop or form fruiting bodies.

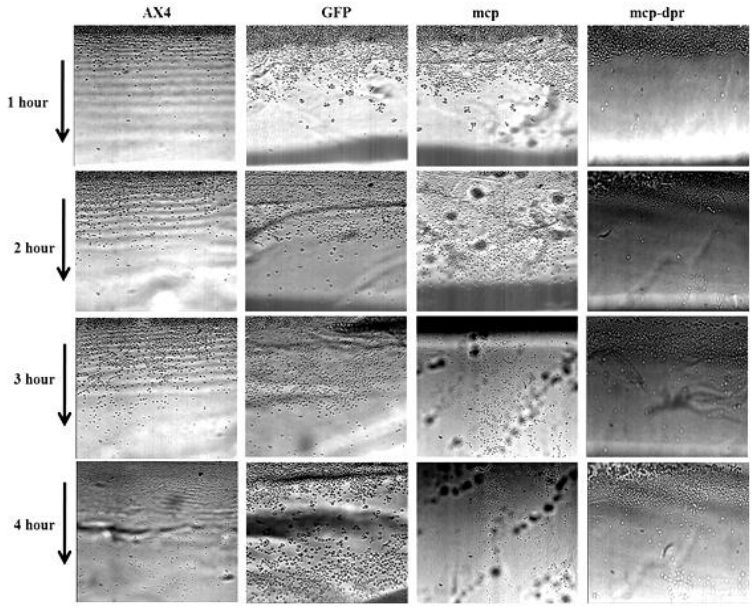

Fig. 5: Measurement of the chemotaxis activity in all cell lines using 5µM cAMP as chemo-attractant. After 4 hours, the *Dictyostelium* cells moved beyond one field of view on the agarose in a 60 × 15 mm Petri dish. The amoeboid control cells (WT and pDneo2a-GFP) clearly show trails toward the source of cAMP. Cells with Acmcp over-expression showed faster movement, while cells with Acmcp-dpr mutant scarcely moved towards the source of cAMP.

Wild type, pDneo2a-GFP, Acmcp, and Acmcp-dpr cells were then sampled at 1, 2, 3, and 4 hours (Fig.5), and representative photomicrographs were taken at each sample time point. Analysis of the data indicated that wild type AX4, pDneo2a-GFP, and Acmcp cells have normal chemotactic sensitivity to cAMP, which was characterized by their movement toward the source of cAMP. Cells that expressed Acmcp-dpr showed almost no movement towards the source of cAMP even after 4 hours of the experiment.

Under starvation conditions, cell death levels are reduced in Acmcp-dpr cell line

The severe defect in Acmcp-dpr cell line development and its inability to respond to the cAMP signal could be due the fact that deletion of the proline rich region from metacaspase protein may affect signal transduction pathway. Starving wild-type *Dictyostelium* cells and treating them with the differentiation factor (DIF) will cause autophagic cell death (51). To determine if the Acmcp-dpr over-expression altered the cell death process, all cell lines were starved, subjected to DIF treatment and PI staining. Cell death was then quantified. Results of a representative experiment showed that the Acmcp-dpr cell line had less dead cells in response to DIF compared to other cell lines (Fig.6A).

The percentage of dead cells in the control cell lines (wild type AX4 and pDneo2a-GFP) was approximately 29%, 25% respectively. Cells over-expressing the Acmcp-dpr mutant had approximately 5% dead cells, while cells over-expressing the Acmcp had approximately 18% dead cells (Fig.6B). These, together with previous results, demonstrate that *Acanthamoeba* metacaspase is playing a role in developmental phenomena in *Dictyostelium* cells.

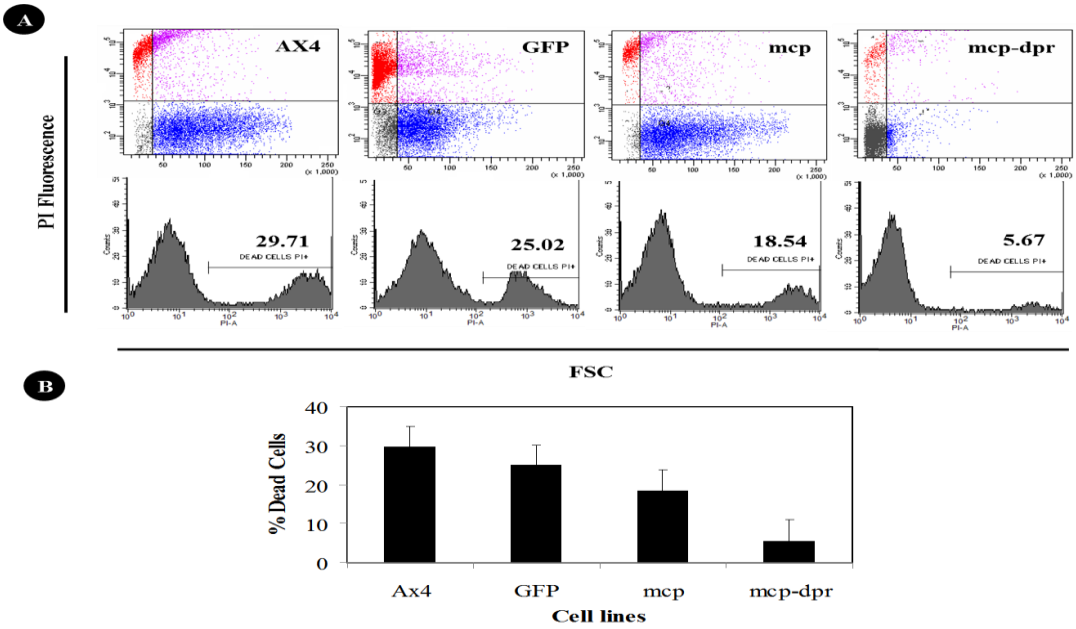

Fig. 6: Cell death quantification in *Dictyostelium* using flow cytometry analysis. WT, pDneo2a-GFP, Acmcp, and Acmcp-dpr cells were subjected to starvation and cAMP, then incubated with or without DIF for 6 hours. Cells were next stained with 1µg ml^{-1} Propidium Iodide for 10 minutes. Fluorescent PI-positive cells were quantified using flow cytometry. (A) Dot plot data with side scatter and forward scatter shows the dead cells distinct from living cells. (B) Quantification for DIF treatment cells undergoing apoptosis. Cells over-expressing Acmcp-dpr showed a lower rate of apoptosis compared to the other cell lines.

ATP levels, an indicator of cellular metabolic activity, increase in Acmcp-dpr over-expressing cells

ATP is a nucleotide correlates to the intracellular energy. When the ATP levels decrease, the cell performs basic apoptotic functions and dies. Our data showed that Acmcp-dpr cell line had less dead cells compare to the other cell lines. To test the metabolic activity of these cells, ATP levels were measured using CellTiter-Glo® Luminescent Cell Viability Assay. Fig.7A shows that the ATP levels in cells over-expressing Acmcp-dpr were two times greater than the ATP levels in the other cell lines (wild type, pDneo2a-GFP and Acmcp).

Cell survival ratio increase in Acmcp-dpr over-expressing cells

Our observation that the metabolic activity increased in cells over-expressing Acmcp-dpr gave us a reason to test the survival ability of the mutant cells. All cell lines were subjected to re-growth assay. After treatment with DIF for 72 hours, cells were counted, and the survival ratio was calculated. Wild type and pDneo2a-GFP had almost the same survival ratio (54% and 61%, respectively) while, in Acmcp-expressing cells, the ratio was 26%. In Acmcp-dpr over-expressing cells, the survival ratio was higher than in the other cell lines (around 105%) (Fig. 7B), indicating the high ability of these cells to survive.

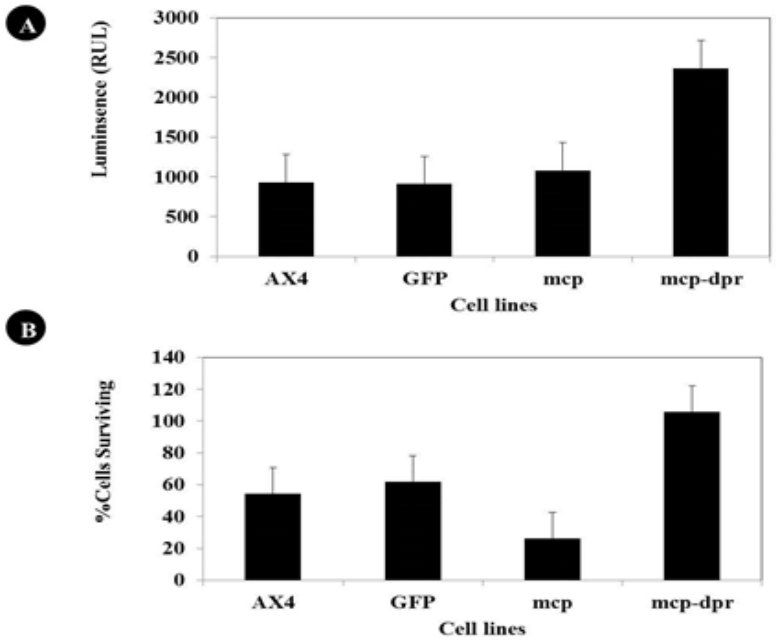

Fig. 7: Acmcp-dpr over-expressing cells have increased survival ratios and metabolic activity as compared to controls. (A) Survival ratios of the mutant cells after 72 hours of treatment with DIF. Cells over-expressing Acmcp showed lower survival rates while cells over-expressing Acmcp-dpr had a higher survival rate compared to the other cell lines. (B) To measure the metabolic activity, luminescence was recorded 10 minutes after the CellTiter-Glo® reagent was added to cells in a 96-well plate. The luminescent signal from Acmcp-dpr cells was two times greater than the signal from the other cell lines (WT, pDneo2a-GFP, and Acmcp).

Discussion

The process of cell signaling and signal transduction are important for cellular development and for parasitic infections. In terms of parasitic infections, the cysteine proteases have proven to be important key proteins for excystment, encystment, and tissue invasion (56). Metacaspases have been identified as cysteine proteases, and their potential as useful

drug targets against parasites is currently being investigated. This paper details a series of investigations into an important metacaspase from the parasitic *A. castellanii* that has been expressed into *D. discoideum* cells, which serves as a safer alternative model organism to gather insights into the novel roles of this protein. Especially important would be results that point to proteins or processes, which could targeted for the treatment of parasitic infections.

An analysis of *Dictyostelium* cells over-expressing Acmcp and Acmcp-dpr mutant was performed to identify Acmcp's possible function. When the mutant cell lines as well as the control cells were subjected to a phagocytes assay, the results showed an increase in the rate of this process in both Acmcp and Acmcp-dpr cell lines compared to the control cells. In addition, the over-expression of Acmcp resulted in an increased rate of endocytosis and abnormal fusion of early endosomes. This suggests that the Acmcp protein is involved in regulating phagocytosis and endocytosis processes. It was hypothesized that Rab11 in *Dictyostelium* regulates phagocytosis via recycling endosomal and contractile vacuole membranes to form a phagocytic cup (57). This protein associates with the endosomal membranes in *Dictyostelium* (58) and is delivered to the plasma membrane by fusion with endosomes (59). V-ATPase is an enzyme that transports protons across the plasma membranes. Reducing levels of v-ATPase has been shown to lead to a defect in the rate of phagocytosis and endocytic function (60). In addition, v-ATPase is retrieved from the phagosome membrane before exocytosis for rapid recycling of the v-ATPase by an actin tail (59). The increase of both endocytosis and phagocytosis in the mutant cells could be due to the presence of shared effectors protein among the vacuoles that led to a change in the phagocytosis rate when the Acmcp protein was over-expressed in the cell. Our previous observations have shown that Acmcp protein associates with the contractile vacuole and partially interacts with Rab11and the vacuolar v-ATPase (46). The over-expression of Acmcp protein caused increased endocytosis and phagocytosis, thus leading us to conclude that Acmcp function is possibly overlapped with Rab11 and v-ATPase function resulting in significant increases in the endocytosis and phagocytosis processes and thus could be involved in signaling and cellular development as well.

Under stressful conditions, *D. discoideum* undergoes a developmental program as a survival mechanism (18). This development phenomenon is comparable to the encystations process in *A. castellanii,* which is also triggered by environmental stress. Interestingly, the mutant cells over-expressing Acmcp-dpr were unable to initiate early development of cells streaming when we placed these cells under starvation conditions in a layer of development buffer. On the other hand, we saw normal development phenomena in Acmcp over-expressing cells compared to the controls. Furthermore, Acmcp-dpr cells failed to form fruiting bodies when cells were starved, indicating that the effect of the deletion of the Acmcp proline rich region on the developmental phenotype is severe. It was reported that, when *Dictyostelium* is starved, two main factors, cAMP, and differentiation-inducing factor (DIF), cause the differentiation of *Dictyostelium* cells into stalk cells, which are dead cells. Usually DIF acts on starved cAMP-subjected cells to promote their differentiation (61-63, 51). In the present study, after cells were starved, subjected to cAMP, and treated with DIF, Acmcp-dpr over-expressing cells showed lower levels of dead cells and a higher survival ratio compared to other cell lines. These data demonstrate that Acmcp-dpr cells tend to survive rather than developing into dead stalk cells. To assess if this effect is related to the cellular signal transduction, Acmcp-dpr over-expressing cells were subjected to the chemoattractant, cAMP, and they showed a defect in their chemo-tactic sensitivity for cAMP compared to the control cells. These

cells had not differentiated to the early development of cell aggregation; thus, they would not carry out cAMP chemotaxis because of a failure to reach the appropriate developmental stage.

Acmcp is associated with the contractile vacuole and interacts partially with the other contractile vacuole proteins, Calmoduline and vacuolar V-ATPase (46). It was found that treated trophozoites of an *Acanthamoeba* with Acanthamoebicidal fractions cause the formation of large contractile vacuoles that lead to cell lysis under hypotonic conditions within 1 hour. The study also reported that the treated cells had an increased intracellular acidity due to the elevation of the proton pumping or V-ATPase stimulation (64). The contractile vacuole and endosomal compartments in *D. discoideum* are involved in intracellular calcium storage that plays a critical role in cAMP-induced calcium release. The contractile vacuole system is involved in this process due to the presence of calmodulin and a vacuolar proton pump protein that provides the proton gradient for calcium transport. In addition, the vacuolar proton pump is present in endosomal membranes in *Dictyostelium* (65-67, 58). According to these facts, deletion of the proline rich region from Acmcp protein affects contractile vacuole function which in turn possibly affects the intracellular calcium storage that plays a critical role in cAMP-induced calcium release. Supporting this idea are data that reveal that Acmcp-dpr over-expressing cells have defects in their chemotactic activity to cAMP source. Thus, over-expressing Acmcp-dpr affects cell-to-cell signaling such that cells fail to complete the development process even under starvation conditions. On the other hand, Acmcp-dpr over-expressing cells have a highly active CV, which causes a higher growth rate compared to the control cells (46). A study in freshwater algae showed that an increase the intracellular osmolarity leads to an increase of the energy input rate for the cell wall and the contractile vacuole mechanism (68). The increases of ATP levels in Acmcp-dpr mutant cells confirm the increases in the energy input within the cells. These data indicate that over-expression the deletion proline region mutant metacaspase has altered ATP homeostasis and increased ATP levels per cell, which ultimately increased the intracellular energy within the cell. Moreover, the *Dictyostelium* vegetative cells with Acmcp-dpr mutant possess an active phagocytosis process compared to the other cell lines even after three hours of treatment. Thus, this action might have led to the down-regulation of cell-cell signaling and transition from the unicellular to the multicellular stage of development. It has been shown that phagocytosis leads to the suppression of developmentally regulated genes (69). Future studies will be required to determine if Acmcp has a role in Ca^{2+} homeostasis, potentially via its role in interacting with the CV localized Calmoduline and v-ATPase.

In this study, we found that Acmcp protein have a critical role in the process of fluid intake internalization, and phagocytosis regulation in *D. discoideum* which ultimately effect the cellular signaling pathway. The overexpression of Acmcp and Acmcp-dpr caused a severe defect in development phenomena due to the inhibition of cAMP production in these cell lines. Additionally, the results have shown that the proline rich region is important to regulate cAMP production. These results proposed that Acmcp have an effect on cell-cell signaling, which in turn effect on cell differentiation and development cycle in *Dictyostelium.*

Conclusion

Functional analysis revealed that Acmcp is involved in endocytosis and phagocytosis regulation and that the proline rich region in Acmcp is important in signal transduction and the development process in *Dictyostlium*. Taken together, processes involving the caspases-like proteins (metacaspase) in cell-cell signaling pathway in unicellular organisms provide new insight into the metacaspase potential as a

novel drug target against parasitic protozoan infection.

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