Alteration of cell aggregation and adhesion in *Dictyostelium* amoeba by an overexpression of paracaspase protein

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Abstract

Dictyostelium discoideum amoeba is a powerful system to study gene function through genetic and functional analysis. Paracaspase (Pcp) is a caspase-like protein that has recently identified in *D. discoideum.* Study paracaspase molecular function in *Dictyostelium* will provide insight into its cellular role in more complex organisms and the possibility to use it as a drug target against the parasitic amoeba. In this study, *pcp* was tagged with Green Fluorescent Protein (GFP) and over-expressed in the cell. The knockout version of *pcp* (*pcp*-) was also investigated. In the course of our examination, it was observed that cells overexpress Pcp was unable to complete the developmental process, leading us to examine its role in development. Those cells were unable to initiate early development and failed to aggregate under starvation conditions. Aggregation is severely defected as cells have decreased cell-cell cohesion. In particular, these cells demonstrated a reduction in adhesion. This data suggests that over-expression of Pcp causes a signaling defects crucial to normal development in *Dictyostelium.* On the other hand, cells with *pcp*- mutant demonstrated larger aggregation in normal media as well as under starvation conditions compared to the control cell lines. As a result, we propose that *Dictyostelium* Pcp is a regulated protein involved in cellular functions including signal relay pathways that are essential for cell aggregation and development. Thus, Pcp protein is a candidate drug target against the amoebic parasitic infection by prevent development to their infective stages.

Keywords: *Dictyostelium* amoeba, paracaspase protein, cell aggregation

زيادة تعبيربروتين البراكاسباس يغير تجمع والتصاق خلايا اميبا *Dictyostelium*

انتصار جبار صاحب

قسم علوم الحياة، كلية العلوم، جامعة بغداد، بغداد، العراق

الخلاصة:

خلايا الأميبا (الديكتستيليم) تمثل نظام قوي لدراسة وظيفة الجينات من خلال التحليل الجيني والوظيفي. بروتين البراكاسباس هو بروتين اكتشف مؤخرا في هذه الاميبا. دراسة الوظيفة الجينيه لهذا البروتين سيوفر معلومات وافيه حول دوره الوظيفي في الكائنات الاكثرتعقيدا وكذلك إمكانية استخدامه كعلاج ضد الأميبا الطفيلية. في هذه الدراسة تم زيادة تعبير جين البراكاسباس في خلايا الاميبا وايضا تم ازالة البراكاسباس في مجموعة اخرى من خلايا الاميبا

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وكنتيجه لذلك فقد لوحظ ان الخلايا التي تنتج كميات اكبر من البراكاسباس كانت غير قادرة على اكمال دورة حياتها حتى في الادوار الاولى . اضافة الى ذلك كانت هذه الخلايا غير قادره على الالتصاق او التجمع للانتقال الى الاطوار اللاحقة في حين تم مشاهدة العكس في الخلايا التي لا تحوي على البراكاسباس. من هذه النتائج يمكن الاستنتاج بان بروتين البراكاسباس له دور اساسي في اصدار الاشارة الجينيه للخلايا لتبدا بالتطور واكمال دورة حياتها مما يرشح هذا البروتين لان يكون علاج ضد الاصابات بالاميبا الطفيلية ومنع تطورها الى اطوارها المعدية.

Introduction

Protease paracaspases are cysteine-dependent, and their genes have recently been shown to be similar to those of caspases. The sequences of paracaspase have been discovered in metazoans and in *D. discoideum* [1]. Numerous studies have demonstrated the cellular function of paracaspase in animals. It has been demonstrated that paracaspase- expressing mucosa-associated lymphoid tissue 1 (MALT1) in humans has an alternative caspase function. Paracaspase MALT1 protease inhibition could be a novel drug target for aggressive B cell lymphoma [2, 3]. It's generally appreciated that paracaspase need not be involved in cell death [4] instead; it's associated with both localization and the function of the contractile vacuolar system [5]. It has been shown that there is a functional relation between the caspase and paracaspase families to facilitate non-apoptotic phenomena within these cells [6].

D. discoideum has proved very helpful model organism in studying the signaling pathways. It possesses a simple and well-defined life cycle includes two phases: the growth phase and the development phase [7]. During the development phase, *Dictyostelium* can develop into a multicellular form [8]. Stress conditions such as starvation induce the first transition from growth to development [9]. The amoebic stage initiates the second change by aggregating and communicating through the secretion of cyclic adenylyl cyclase (cAMP) [10]. DdCAD-1 is one of the first proteins that expressed at the cell surface during cell aggregation. Cells start interacts through cell-cell adhesions and stream to promote aggregation process [11, 12]. A protein termed gp80 involved in cells adhesion which is induced by cAMP as development proceeds [13]. When the cells release cAMP, some of the nucleotides activate protein kinase A within the cells. On the other hand, a significant amount of cAMP is produced outside the cells leading to changes in gene expression [14, 15]. Then, the cells start to move via chemotaxis to the extracellular cAMP which mediates the aggregation to groups of cells and differentiate into sorocorp that contain a stalk and fruiting body containing spores [16-20]. *Dictyostelium* cell death is mimic to apoptosis in that some cytoplasmic and chromatin condensation occurs however, differs from apoptosis because it involves vacuolization and, lacks DNA fragmentation [21, 22]. In this study, the overexpressing of Pcp in *Dictyostelium* cells has been caused a defect in cells aggregation, cell-cell adhesion while opposite phenomena was observed in cells with *pcp*- mutant. This result suggests that Pcp is a candidate for the regulation of cellular processes important at early stages in *Dictyostelium* development.

Materials and methods

Cells and culture conditions

For all experiments, *D. discoideum* wild-type (AX4) (kindly provided by dictybase.org), AX4 with pDneo2a-GFP, AX4 with GFP-Pcp over-expressing and AX4 with *pcp*- knockout cell lines were grown axenically at 21 ^{0}C and shaken at 150 rpm in HL5 medium (1% oxoid proteose peptone, 1% glucose, 0.5% yeast extract (Fisher Biotech), 2.4 mM Na_2HPO_4, and 8.8 mM KH_2PO_4, pH 6.5). This media was supplemented with 300 mg/ml of streptomycin sulfate and 100 mg/ml of ampicillin (Sigma). Additionally, for the transforming cells, HL5 medium was supplemented with 10 mg/ml of G418 (Invitrogen). *D. discoideum* cells with *pcp*- knockout mutant was kindly provided by Dr. Pierre Golstein (Centre d'Immunologie de Marseille-Luminy, Parc Scientifique de Luminy, Case 906, 13288 Marseille Cedex 9, France). The mutant *D. discoideum* with *pcp*- mutant cells were maintain in HL5 media supplemented with 10 mg/ml blasticidine.

Construction of GFP tagged pcp expressing cell lines

A full length of *D. discoideum* paracaspase (pcp) was cloned into the pDneo2a-GFP vector. These vectors' construct (pDneo2a-GFP -pcp) was sequenced for errors and reading frame confirmation. Primer design, PCR sequencing of the DNA, cloning, and Western blots analysis were performed as previously described [5].

SDS-polyacrylamide gel electrophoresis

Protein overexpression was analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). To prepare the protein sample, 1 x 10^6 cells was collected of each *Dictyostelium* strain. Freshly prepared Lysis Buffer (150 µL) was added to each sample. The mixture was then homogenized using 0.3 gm of glass beads and vortex each sample for 2 minutes at 4^{o}C. The samples were centrifuged at 4000 rpm for 10 min to remove un-dissolved debris. The supernatant (50 µL) from cell extract was transferred to new tube. Freshly prepared 2x SDS-loading dye (50 µL) were added. Next, samples were heat treated at 99^{o}C for 10 minutes. The samples were then centrifuged for 1 minute at 14000 rpm. The samples (20 µL) were loaded onto the polyacrylamide gel made of 10% resolving gel and 5% stacking gel and subjected to electrophoresis at a constant current of 100 volts per gel, using a Mini Protein II unit (BioRad Laboratories, Inc., Richmond, CA, USA). After separation, the gels were stained for 3 × 20 min with Coomassie Brilliant Blue R-250 in 45% (v/v) methanol and 10% (v/v) acetic acid, and de-stained with 45% methanol (v/v) and 10% (v/v) acetic acid. The gel was then exposed for autoradiography.

Cell size rate determination

All cell lines were grown in HL5 medium until a density of 3×10^6 cells/ml was obtained. Cells were washed with cold Sorensen phosphate buffer (14.5 mM KH_2PO_4, 2.5 mM Na_2HPO_4, pH 6), vortexes, and left on ice for 20 minutes. The cells were photographed, and the diameters of 50 cells were measured for each mutant using the BrightLine® on a Nikon 2000SE microscope with IPLab3.7 software × 1000 times' magnification.

Starvation buffer and HL5 media assays

Comparisons were made for cells in HL5 media and starvation buffer. To visualize the cells under normal environment, cells were harvested and allowed to settle on a glass cover slip, then viewed and photographed at 1000x magnification using the BrightLine® GFP filter set on a Nikon 2000SE microscope and IPLab 3.7. To prepare aggregation studies, cells were incubated for 3 hours in starvation buffer. Cells were harvested and allowed to settle on a glass cover slip, then viewed and photographed.

Developmental assay

For developmental studies, cells were grown axenically in HL5 medium on a rotary shaker (160 rpm) to 1 x 10^9 cells/ ml and harvested by centrifugation. After repeated washing with developing buffer (5 mM Na_2HPO_4, 5 mM Na_2HPO_4, 5 mM KH_2PO_4, 1 mM $CaCl_2$,and 2 mM $MgCl_2$), cells were re-suspended in the same buffer at a density of 2 x 10^8 cells/ml. Next, 200 ml of the cell suspension were spread evenly in 100 mm KK2 plate. The plates were wrapped with plastic wrap and a wet paper towel. The plates were then inverted and incubated at 22°C for the desired time period to monitor the developmental stages. This method was adapted from dictybase.org. Photographs of multicellular development were taken using a model M 420 1, 25× microscope (Switzerland).

Cell cohesion assays

Cell cohesion assays were performed as described by [23, 12, 24]. To determine cell cohesion, 2×10^7 cells/ml were collected in 1 ml KK2 buffer and shacked for 4 hours. Cells were then centrifuged at 700g for 5 min and re-suspend to a concentration of 2.5×10^6 cells/ml in 8 mL of KK2 buffer to determine cell cohesion during development and in HL-5 medium to determine cell cohesion during vegetative growth with or without 10 mM EDTA. Next, 200µl samples were collect for each time point (0, 20, 40, 60, and 80) in triplicate. Aggregates were dispersed by vortex vigorously for 15 s and then shacked at 180 rpm to

reform the aggregates. At the indicated times, the number of non-aggregated cells, including singles and doubles, were scored using a haemocytometer. The number of aggregating cells was determined by subtracting this number from the total number of cells and was expressed as a percentage of the total.
To assess the EDTA-resistant cell-cell adhesion, cells were harvested and re-suspended in KK2 and starved in a rotary shaker at 22^{o}C for 4 hours. Cells were then collected and re-suspended in KK2 + 10mM EDTA to inhibit EDTA-sensitive cohesion. Aggregates were dissociated by brief vortex, and 0.2 ml samples were placed in plastic tubes and rotated vertically at 180 rpm at room temperature for cell re-association. The percentage of cell aggregation was monitored at regular intervals for 60 minutes. Experiments were generally repeated three times.

Results and Discussion

SDS-PAGE for protein expression

SDS-PAGE of whole cell proteins was used for identification the overexpression of pcp. Protein overexpression patterns are depicted in figure -1. The overexpression of Pcp proteins was recorded by increasing Pcp band intensity in the gel (Lane 3) in comparison with that observed in control WT-AX4 band (Lane 2).

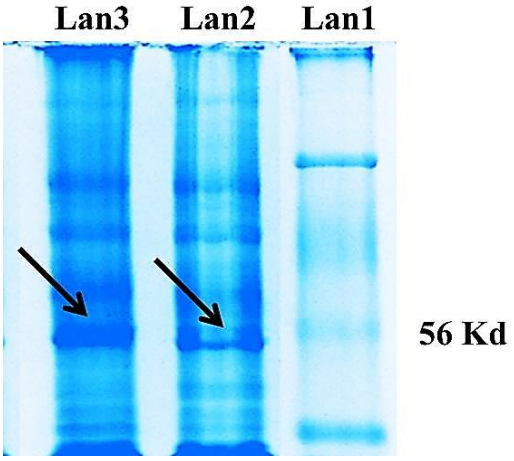

Figure 1- Coomassie blue-stained SDS-PAGE gel of whole cell proteins for three cell lines in *Dictyostelium* and visualized by autoradiography. The apparent molecular weights (in kD) of markers are indicated on the left. Band is visible on all cell lines in the following order: Lane 1: the apparent molecular weights (in kD) marker (Bio-Rad, Missisauga, ON, Canada); Lane2: WT-AX4; Lane 3: AX4 with Pcp over-expression.

Cell size representation

The diameters of 50 cells were measured for the vegetative amoebae in order to investigate the overall effects of *pcp* over-expression and knockout. Figure- 2 represents the graphical data (figure -2A) and photographs (figure - 2B) concerning cellular size as measured by average diameter. All cell lines were washed with Sorensen phosphate buffer and left on ice for 20 minutes. Then, cells were photographed, and diameters were measured. Graphical averages including the appropriate standard errors are shown. There is an apparent significant decrease in the average cellular size of the *pcp*- mutant cells compared to the AX4 (figure -2A, B). Analysis of the data suggests that the mutations affect overall cellular metabolism.

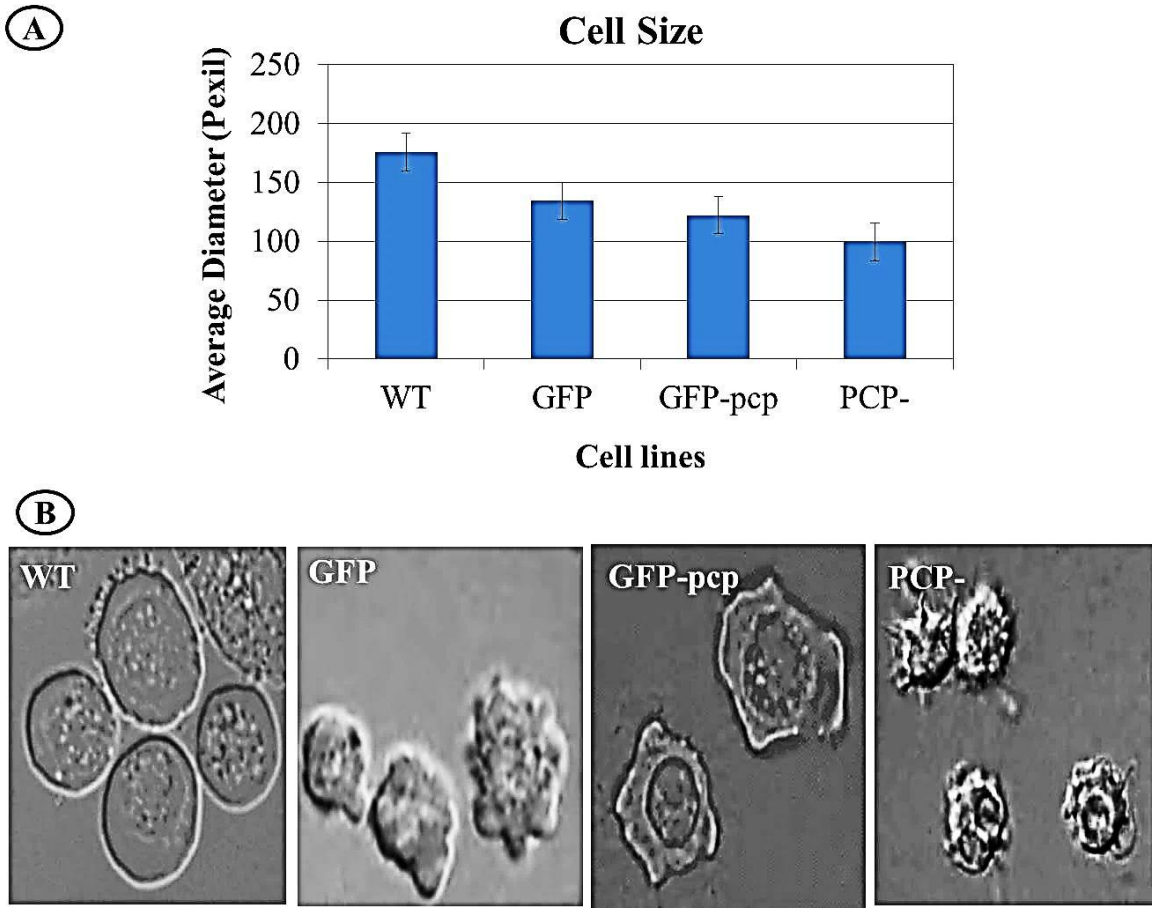

Figure 2- Cell size representation in wild type and mutant cell lines. A) Graphical representation with standard error and B) Photographs, showed decreasing in the cell size rate of the Ax4 with *pcp*- mutant cell line compared to the other cell lines (WT-AX4, AX4 with vector only (pDneo2a-GFP) and Ax4 with GFP-pcp overexpressing).

Controls and the mutant's cells aggregation pattern

To investigate the phenotype mutant cells in normal growth media, cells were collected by centrifugation and spotted on cover slips for 10 minutes and then processed for microscopy. Figure -3 presents both GFP-Pcp and *pcp*- mutant cell lines compared to the control cells. In normal growth medium (isotonic condition), the wild type AX4 and pDneo2a-GFP cell lines maintained an amoeboid shape. However, the GFP-Pcp cell line displayed an increased size of cells, apparently due to the effects of the over-expression of the Pcp protein. Interestingly, *pcp*- mutant cells were smaller and aggregated into groups (figure -3A). Cells with *pcp*- mutant were then watched through 1 hour. The aggregate formed in this cell line were comparatively larger than the respective controls. It is indeed interesting that the membranes of streaming cells make contact through filopodia structures (figure - 3B). Next, the aggregation pattern of cells with *pcp*- mutant was tested after incubation in starvation buffer. Large aggregate formation was seen in the presence of this buffer. In contrast to the control cell lines, *pcp*-expressing mutants formed aggregates that were larger and seemed to attract a greater number of cells into each aggregate mound (figure - 3C). On the other hand, there were no aggregation in the cells overexpress pcp.

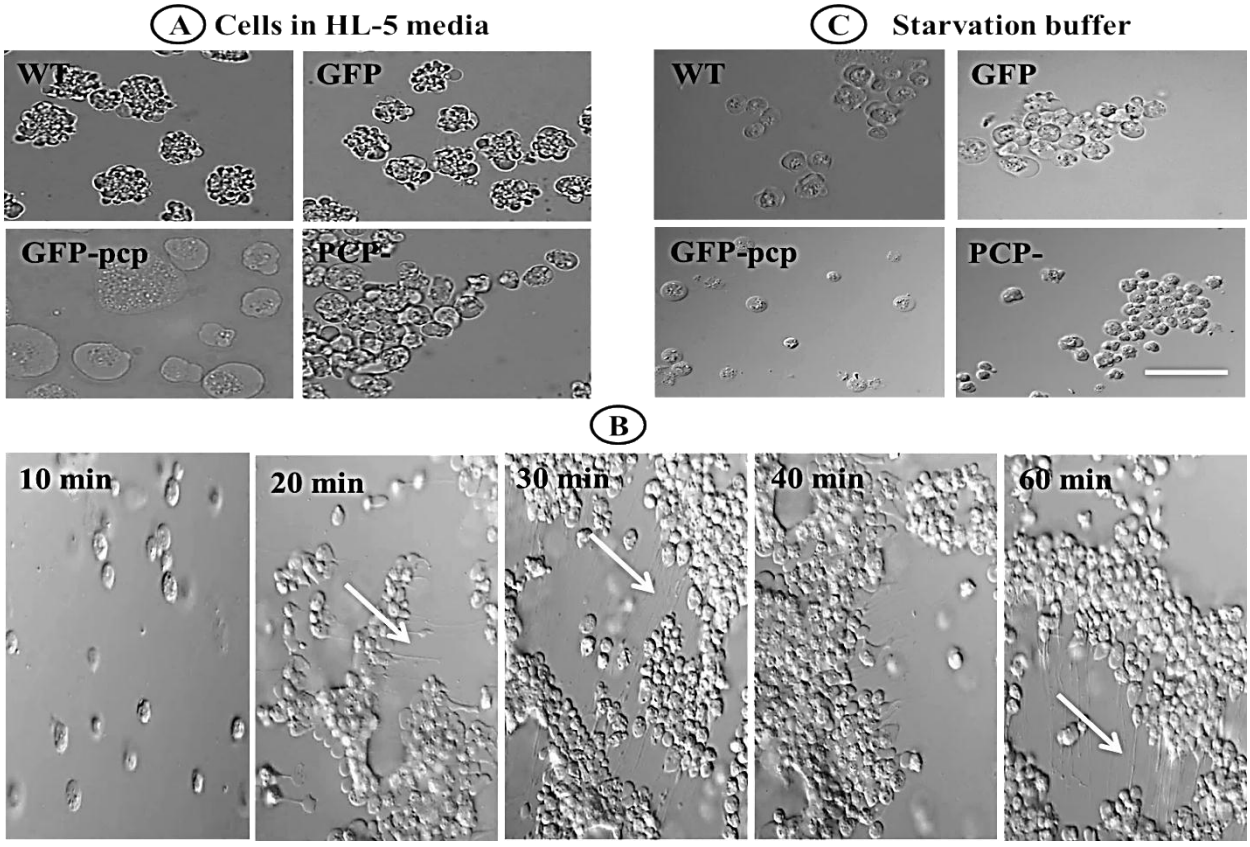

Figure 3- Effect of different environment on the controls and the mutant's cells aggregation pattern. (A) Cellular appearance in isotonic environment (HL5 medium). The GFP-Pcp cells appear different with enlarged cell size. Cells with *pcp*- mutant showed smaller size and they aggregate in groups. B) The aggregate formed in HL5 medium were comparatively larger than the respective controls. Cells with *pcp*- mutant were watched under microscope for an hour. The membranes of streaming cells make contact through filopodia. C) Aggregation pattern of the cells with *pcp*- mutant after incubation in starvation buffer. Large aggregate formation was seen in the presence of this buffer. There were no aggregation in the cells overexpress pcp. Scale bar = 200 μm.

Dictyostelium development severely fault due to Pcp over-expressing

The surprising effect of the *pcp* mutation on cellular aggregation suggested an investigation into the effects that these mutations with alter physiology may have on the *Dictyostelium* developmental process. To begin, the development process is triggered by starvation conditions, follow by cell streaming, slug formation and cellular differentiation into stalk and spore cell types in a mature development form (www.dictybase.org). This developmental assay was extended to 24 hours in order to observe the final formation of stalk and spore containing fruiting bodies. Figure- 4 shows representative photomicrographs of the pDneo2a-GFP and *pcp*- mutant cells that initially developed at the same rate and structure as parental AX4 cells. However, the *pcp*- cells had fruiting bodies with smaller, thinner stalks and developed fast within 16 hrs. Interestingly, the Pcp over-expressing cells exhibited a marked defect; they did not develop, and they lacked fruiting bodies. This experiment suggests that cells over-expressing Pcp did not have the capacity to form multi-cellular structures possibly due to the effects of altered phagocytosis and endocytosis on the developmental signaling process.

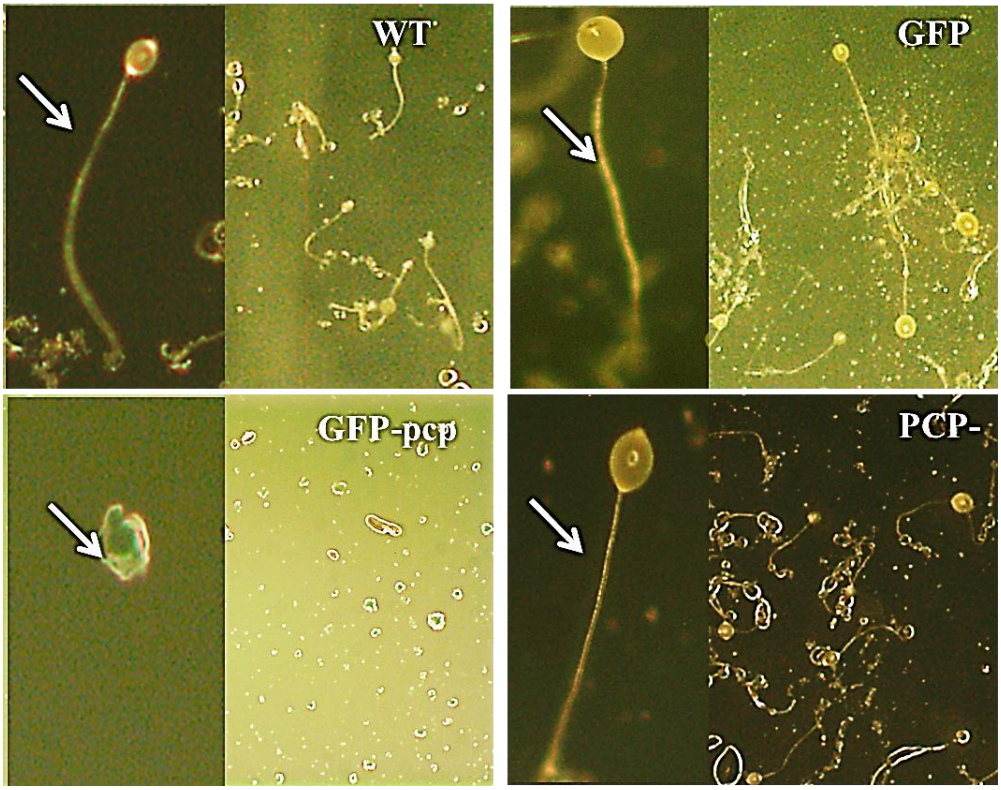

Figure 4- Representative photomicrographs of the streaming in control cells versus GFP-pcp and *pcp*- expressing cell lines. Developmental representative series photomicrographs on KK2 plates after 24 hours at 22^{o}C. Wild type AX4 cells and AX4 with pDneo2a-GFP developed normally and formed stalks with fruiting bodies. The fruiting bodies of *pcp*- mutant cells are shorter than the wild type and pDneo2a-GFP. AX4 with GFP-Pcp overexpressing cells had failed to develop or forming fruiting bodies.

Pcp overexpressing cells have decreased in cell aggregation and adhesion

Since Pcp overexpression cause severe defect in cellular aggregation while cells with *pcp*- mutant showed opposite phenomena, we tested the possibility that Pcp may have a role in cell–cell interactions. Control cells expressing GFP alone were identical to the parental cell line, Ax4. In contrast, the *pcp*- mutant cells initially formed aggregates that were larger (figure -4) than those of control cell lines, suggesting that these cells may be hyperadhesive. As development proceeded, these large aggregates increased. Interestingly, *Dictyostelium* mutant cells that overexpressing Pcp did not aggregate nor did they form fruiting bodies and were able to complete development.

Given the observation that Pcp overexpressing cells are not able to aggregate normally, the possibility that the lack of aggregation observed may be the result of reduced cell–cell adhesion. We measured adhesitivity in the Pcp over-expressing, and *pcp*- mutant cells during development as previously described [23, 13, 24]. Cells were first washed free of antibiotics and then starved for 4 hours. The cells were then dissociated by vigorous vortexing to break up any existing aggregates and then allowed to re-associate in rolling tubes. Cells with *pcp*- mutant were more adhesive than parental wild-type cells, reaching a higher level of adhesivity by 1 h of development. This supports the notion that this mutant is hyper-adhesive (figure - 5). GFP-pcp overexpressing cells were less adhesive than wild-type cells over the same time period which could be predicted by analysis of (figure - 5). This suggests that pcp may directly or indirectly regulate cell–cell adhesion.

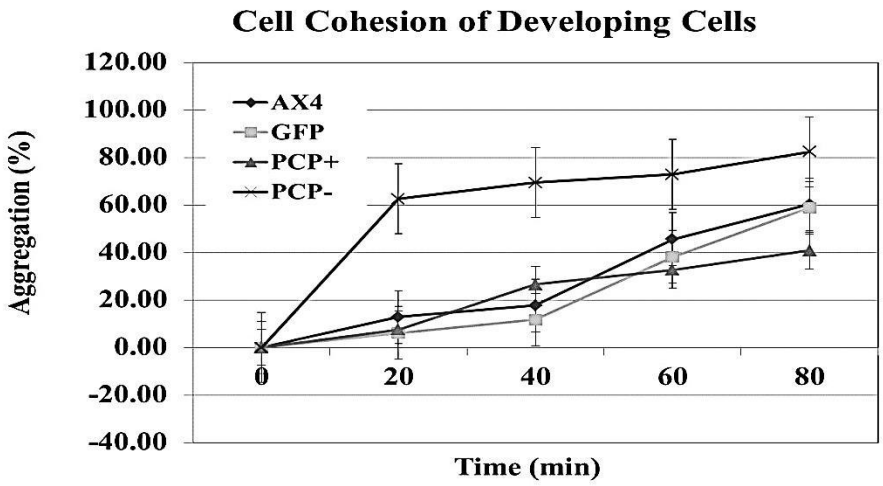

Figure 5- Cell–cell adhesion in the developmental cells. Mutant forms of Pcp show an initial increase in cell-cell adhesion. Total adhesion during development of control (WT-Ax4; GFP), Pcp over-expressing (▲) and *pcp*- (×) cell lines during the 1 h of development. Values presented are the mean (±SD) of three experiments. Adhesivity of the GFP-pcp and *pcp*- mutants was different from that of the control at 1 h.

The Ca^{2+} dependent gp24 is expressed in vegetative cells [12]. We also characterized adhesivity in the vegetative cells in the absence (figure - 6) and presence of EDTA (figure -7) to assess the contribution of such Ca^{2+}-dependent CAMs. There were no differences in the adhesiveness of parental AX4 and GFP-control cells. It was observed that during vegetative growth cells expressing GFP-pcp were less adhesive than GFP-control cells, whereas cells expressing *pcp*- were more adhesive than GFP-control cells (figure - 6). The differences, however, were not statistically significant.

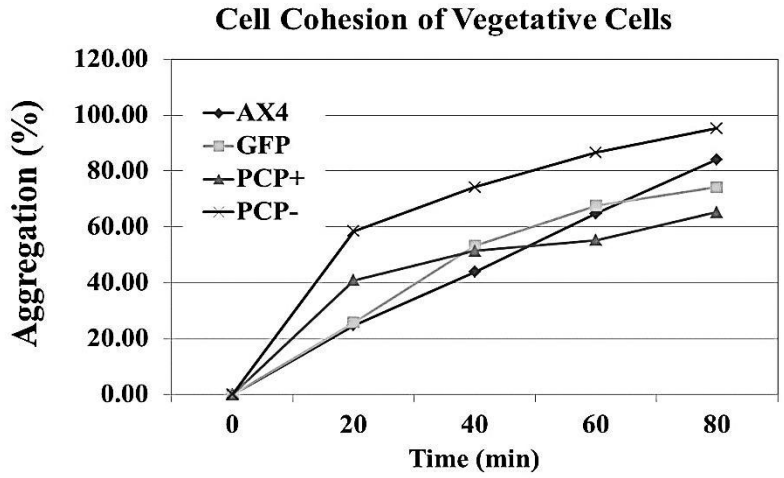

Figure 6- Cell–cell adhesion in vegetatative cells. Mutant forms of Pcp show an initial increase in cell-cell adhesion. Total adhesion during vegetation of control (WT-Ax4; GFP), Pcp over-expressing (▲) and *pcp*- (×) cell lines during the 1 h of development. Values presented are the mean (±SD) of three experiments. Adhesivity of the GFP-pcp and *pcp*- mutants was different from that of the control at 1 h.

Importantly, there were no detectable differences in adhesivity among the cell lines in the presence of EDTA (figure -7), suggesting that pcp may regulate cell–cell adhesions via a Ca2+-dependent CAM such as gp24. This would explain the difficulty of Pcp overexpressing cells have with the aggregation process.

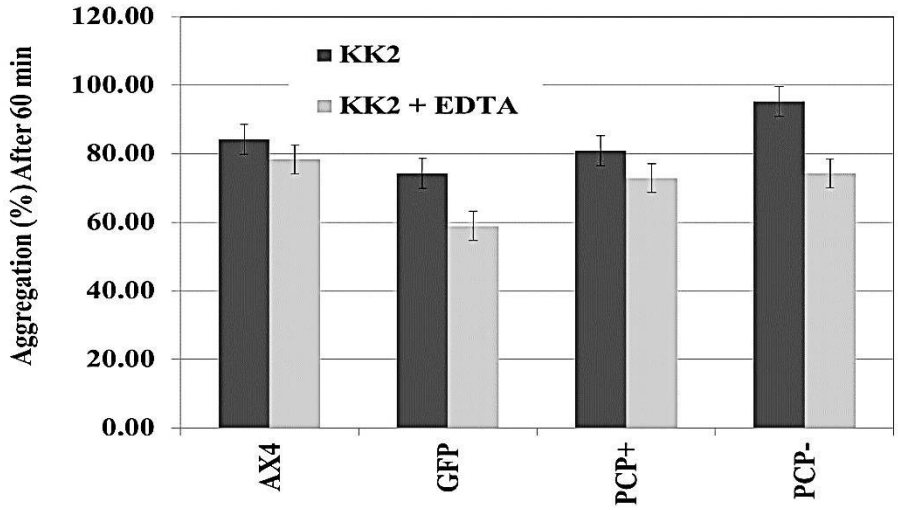

Figure 7- Cell–cell adhesion in vegetative cells in the presence of EDTA. Adhesion of control and mutant vegetative cells in the absence (black bars) or presence (gray bars) of EDTA. Values presented are the mean (±SD) of three experiments. Adhesivity of the GFP-pcp and *pcp*- mutants compared to the control was different. Cell lines show a slight decrease in cell-cell adhesion in the presence of EDTA.

Caspases are cysteine proteases, which are essential for apoptotic cell death regulation in animals [25]. These enzymes are activated in many processes other than apoptosis, including inflammation, cell proliferation, and cell differentiation [26]. In *Dictyostelium*, it has been reported that the paracaspase-null mutant (*pcp*-) cells do not alter the process of developmental stalk cell death and vacuolar autophagic cell death compared to wild type cells [4]. Recently, a study have been suggested that the eukaryotic caspase-like proteins, Pcp, in *Dictyostelium* have a role in the formation, regulation, and/or function of the contractile vacuolar system, an organelle critical for maintaining the normal morphology of the cell [5]. This led us to investigate the role of Pcp in cellular functions other than cellular death. When cells assayed for cell size differences, it was found that the mean size does change among the mutant cells and the control. The cells with *pcp*- mutant have shown a smaller size than the wild type. Analysis of the data suggests that the effects of the mutations on the CV system affect the overall cellular metabolism [5]. Certainly, it can involve internal osmotic changes.

D. discoideum is a unicellular organism that is frequently exposed to stressful conditions and therefore needs to develop a survival mechanism [27]. Interestingly, the mutant cells of Pcp in our study were unable to initiate early development of cells when we placed the cells under starvation conditions in a layer of developmental buffer. On the other hand, we saw an enhanced development phenomenon in *pcp*- mutant cells compared to the controls. This result supports a previous study which has demonstrated that developmental cell death can occur in the absence of *pcp* [4] however, they displayed an increase in the rate of development, forming fruiting bodies after only 16 hours. Pcp overexpressing and the *pcp*- expressing cells both displayed alterations in the developmental process and cell-cell adhesion. Upon starvation, *Dictyostleium* cells will signal each other by releasing cAMP and aggregate together via chemotaxis to form a mound. Some *Dictyostelium* mutants are unable to aggregate normally by chemotaxis and do so by random motion and adhesivity [28, 29, 30]. A possible explanation for the increased rate of aggregation observed in the *pcp*- cells was due to altered cell-cell adhesion. We observed an initial increase in cell-cell adhesion of almost 60% after 20 minutes in *pcp*- cell line whereas in AX4

cells, the cell-cell adhesion percentage was 12%. Interestingly, Pcp overexpressing and the *pcp*- expressing cells both did not show differences in EDTA-resistant cell-cell adhesion compare with the control cell lines. Failure to form EDTA-resistant adhesion could be due to impaired expression on the cell surface of the csA glycoprotein [31]. It has been shown by Bozzaro that a mutant defective in EDTA-stable adhesion, but displaying rather normal aggregation on agar, is, with very high probability, defective in functional csA. We believe that the defects in cell-cell adhesion seen in the Pcp overexpressing cell line is due to improper signaling of downstream effectors. It is indeed interesting that, in addition to their role in in the formation, regulation, and/or function of the contractile vacuolar system [5], Pcp protein regulate the filopodia formation. During *D. discoideum* development, membranes of streaming cells first make contact through filopodia that are rich in gp24 [32]. Our results have been shown that the membranes of streaming cells make contact through many filopodia structures. It is interesting that a developmentally regulated Pcp protein might regulate the formation of these filopodia. The severity of the aggregation defect observed the Pcp overexpressing cell line may be the result of combined defects in filopodia formation.

Conclusion

During development, actin cytoskeletal changes and cell–cell adhesion are important in controlling multicellular structure formation. We have shown that overexpression of Pcp resulted an inhibition of aggregation during starvation. These phenotypic changes were specific to Pcp overexpression, by comparing, cells with *pcp*- did not induce the same alterations. These studies identify for the first time a caspases-like protein may regulate several of these processes simultaneously. Taking into consideration the suggestion of this study that paracaspase protein is a possible target for treatment against parasitic amoeba infections since this protein contributes to the development signal pathway.

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