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RESPONSE OF A TRUE SLIME MOLD, *Physarum polycephalum*, TO SALT STRESS

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Summary

Under high salt conditions,haploid myxoamoebae of a true slime mold,*Physarum polycephalum* retract their pseudopodia and change their cell shape into disk-like form,after which they construct the cell wall to form their dormant type designated microcysts.We observed the change of the intracellular distribution of actin filaments associated with the cellular morphological changes.Staining with phalloidin showed that the actin filaments were almost uniformly distributed throughout the myxoamoebae cytoplasm.When these cells were exposed to salt stress,the actin structures changed into short rods or dots,and they localized along the periphery of the cell.An incubation of the myxoamoebae in high salt medium,caused the synthesis of several species proteins,among which a 66-kD protein (p66) was most prominently induced.We found that p66 was co-precipitated with polymerized actin and bound to ATP-agarose. A double staining of the disk-shaped cells with anti-p66 antibody and phalloidin revealed superimposable localization of p66 and actin filaments in the short rods or dots.And it was revealed that p66 was immunologically unrelated to the common heat shock protein,HSP70 and HSP90,those are highly conserved during evolution.From these results,p66 is supposed to be a novel stress protein and interact with actin to perticipate in the morphological changes of the cell induced by salt stress.

Introduction

Although human being can survive depending on plants and their products,we are destroying the forests,which are the producer in the ecosystem.This is the gravest problem that increases desert in our circumstances and it is feared that one third of the earth has already become the desolate and barren region.On the other hand,agricultural irrigation has been performed to provide the remarkably increasing population.The destruction of forests enhances the evaporation of water from the surface of soil,and leads an accumulation of salty components on soil surface by capillary action.Consequently the barren land is moreover increasing.Therefore,salt tolerant plants attract a considerable attention to gain productivity under such conditions.It is reported that variety of physiological changes,such as intracellular accumulation of saccarides,amino acids and glycine betaine,are found in salt tolerant plants.Though these changes are assumed to allow them to live under high salt circumstances,the mechanism that induces these physiological changes in plants is not yet clarified.Thus,it is important to know how salt tolerant plants acquired salt tolerance and how they adapted to salt stress.In order to elucidate the mechanism of salt tolerance and to establish a simplified system for the investigation on this problem,we employed haploid myxoamoebae of a true slime mold *Physarum polycephalum* and studied their defense action against salt stress.And the molecular mechanism of salt tolerance in this organism has been discussed.

Materials and Methods

Organisms. Myxoamoebae of *Physarum polycephalum*,strain J,were grown on a lawn of bacteria, *Aerobacter aerogenes*,on a nutrient agar medium.Ordinarily they were grown in the dark at 24℃ (1). Harvested myxoamoebae cells were applied to each experiments after washing with PBS.

Cyst formation study. To observe the morphological changes from myxoamoebae to microcysts by salt or heat stress,myxoamoebae were incubated in the buffers containing various concentration of salt for various times with shaking, or incubated in 0.025M potassium phosphate buffer at 24℃ or 40℃ with shaking. The disk-shaped cells and microcysts were counted under a phase contrast microscope. Microcysts were distinguished from other cells by staining with 1% Congo Red.And microcysts were also observed under a conventional electron microscope as described(2).

Labeling of newly synthesized proteins. Myxoamoebae were incubated in the buffer containing [^{35}S]methionine under various stress conditions. After appropriate times,cells were washed with a buffer containing non labeled methionine,then lysed in SDS sample buffer(3) to apply for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Gel electrophoresis and fluorography. One dimensional SDS-PAGE was performed according to the method of Laemmli(3) using 10% polyacrylamide gels.To visualize protein bands,gels were stained with Coomassie Brilliant Blue(CBB).The fluorogram was performed with Kodak X-Omat film(2).Two dimensional polyacrylamide gel electrophoresis was accomplished according to the method of O'Farrell(4).

Preparation of the anti-p66 antibody. The homogenate of stress treated myxoamoebae was centrifuged at 45,000×g for 30 min and the supernatant was applied to a CM-Sephadex column chromatography followed by an ATP-agarose column chromatography. The fraction containing p66 was electrophoresed in SDS-PAGE.The p66 band was recoverd from the gels,and rabbits were immunized with which to obtain the anti-p66 antibody(2).

Immunoblotting. Immunoblotting was carried out by the method of Towbin *et al.*(5) with anti-p66 antibody,anti-HSP70 antibody or anti-HSP90 antibody.

Immunofluorescence staining. Myxoamoebae were placed onto coverslips coated with poly-L-lysine and exposed to salt stress.After cells were permeabilized by 0.5% Triton-X100,they were treated with rhodamine-phalloidin and then with affiinity-purified anti-p66 antibody followed by the visualizing with FITC-conjugated anti-rabbit IgG antibody to detect actin filaments and p66, respectively, under a fluorescence microscope(2).

Co-precipitation with actin. The homogenate of myxoamoebae was centrifuged at 100,000×g and its supernatant was used for experiment of co-precipitation with actin. The resultant supernatants and precipitates were analyzed by SDS-PAGE(2).

Results and Discussion

Induction of morphological change from myxoamoebae into microcysts by salt stress. Fig.1 shows a life cycle of a true slime mold *Physarum polycephalum*. Haploid myxoamoebae ordinarily move around with their pseudopodia.When they are exposed to stresses such as salt stress,heat shock or starvation they retract their pseudopodia and construct the cell wall to become dormant cells designated microcysts. The microcysts are resistant to stress condition and survive for a long time until their environmental conditions improve. The rate of formation of microcysts was as follows: heat shock > salt stress > starvation = drying. The time course of cyst formation induced by salt stress is shown in Fig.2 and the electron microscopic image of a microcyst is shown in Fig.3.When the myxoamoebae exposed to 0.25M NaCl, approximately 80% of them changed into microcysts after 24 hours. The degree of morphological change depended on the concentration of NaCl

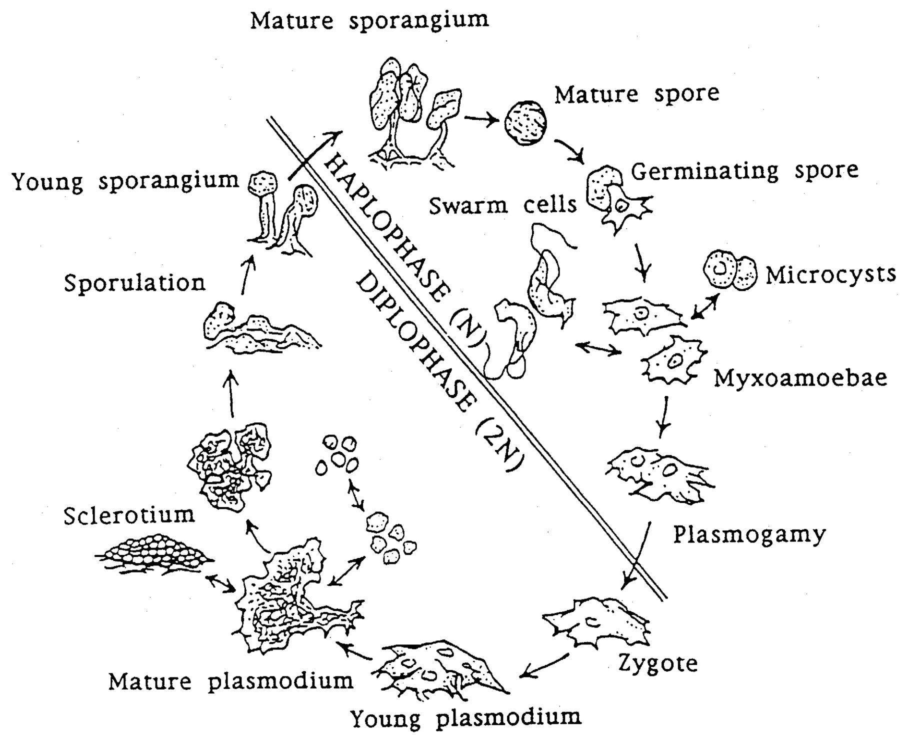

Fig.1 Life Cycle of *Physarum polycephalum*.

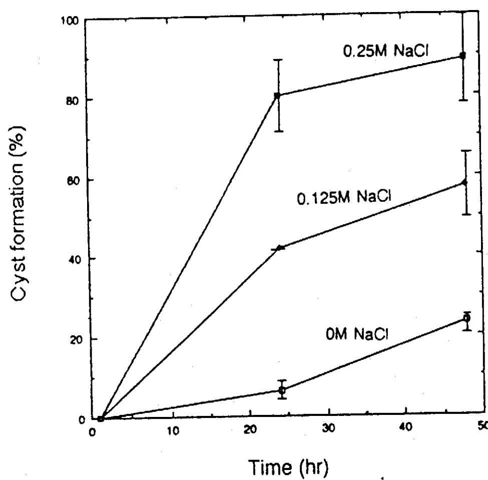

Fig.2 Time Course of Mycrocyst Formation Induced by Salt Stress.

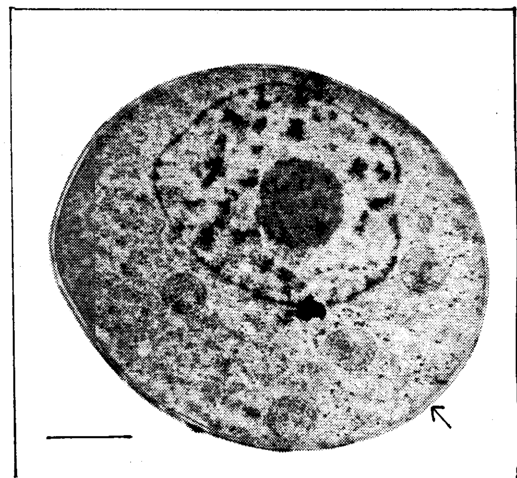

Fig.3 Electron Microscopic Observation of Mycrocyst. Arrow,cell wall;bar,1μm.

(Fig.2),and the microcysts formed the cell wall(Fig.3,arrow) around them to protect themselves from disadvantageous environmental conditions.

Changes of intracellular actin filament associated with microcyst formation. Mycrocyst formation was shown to be induced by various stresses.And it was revealed that myxoamoebae changed into microcyst through the process of disk-shaped cell (Fig.4).This morphological change suggests that the change of cytoskeleton was caused correspondently to the stress. The change of actin filaments was observed by rhodamin-phalloidin staining (Fig.4,lower).The actin filaments were almost uniformly distributed throughout the cytoplasm of the myxoamoebae particularly condensed in the microspike of pseudopodia.However, in the disk-shaped cells and microcysts, the actin filaments localized periphery of the cell in a thick and short fiber structure.It was supposed that the reconstitution of

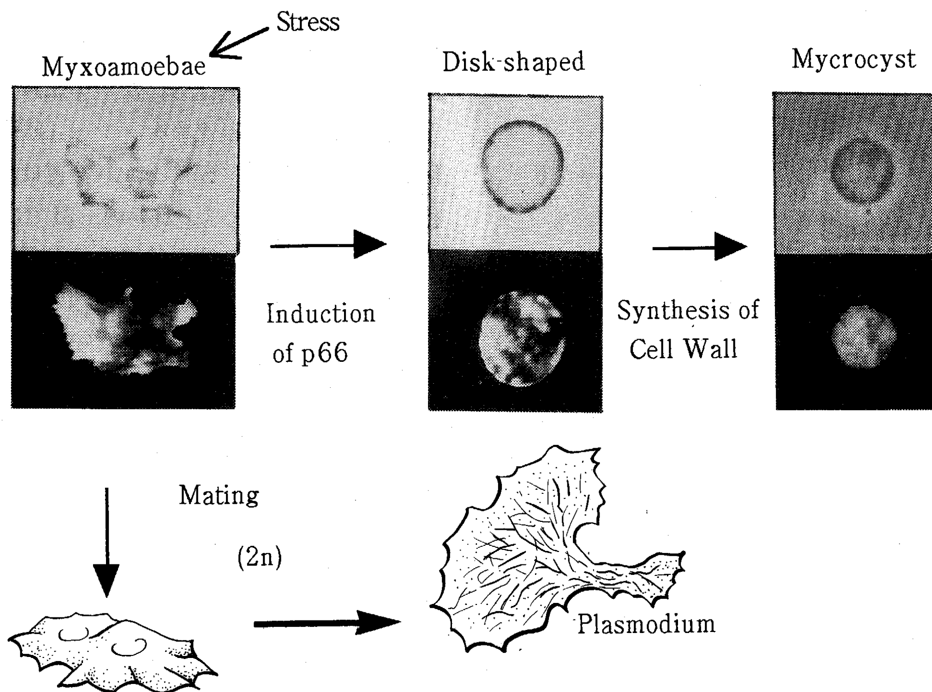

Fig.4 Morphological Change of *Physarum* Myxoamoebae under Some Stresses.
The upper row,phase contrast;the lower row,fluorescence,bar,5μm.

the actin filaments were closely related to the morphological change of the myxoamoebae associated with stresses, and the important role(s) of cytoskeleton in the cellular stress response was shown.

The induction of stress proteins. The induction of synthesis of several species proteins was observed in parallel with morphological change to microcyst. Five species of proteins were clearly induced by salt or heat stress (Table 1).After an hour of exposure to stresses, the synthesis of protein increased , and particularly remarkable increase was observed in 66-kD and 70-kD protein synthesis.Associated with the appearance of disk-shaped cells,66-kD protein (p66) was temporarily synthesized.The different mating types of myxoamoebae fuse together to differentiate into diploid plasmodia(Fig.1,4).The induction of the p66 was not shown when the plasmodia were exposed to stresses.This result suggests that the expression of p66 is regulated in each stage of cell differentiation.i.e.,it is assumed that p66 expresses in the specific phase of the process from myxoamoebae to microcysts.Thus,the p66 is considered to be a new type of stress protein that regulates cell differentiation.Furthermore, p66 is proved to be a single protein from the results of two dimensional polyacrylamide gel electrophoresis and immunoblotting (data not shown).

Co-precipitation with actin. When actin filaments and the p66 in the three types of the cells (myxoamoebae, disk-shaped cells,microcysts)were double stained by rhodamin phalloidin and anti-p66 antibody, respectively,they were deduced to localize superimposably in short rods and dots in disk-shaped cells(Fig.5),which suggested that p66 bound to actin filaments in these cells.To assess the proteins which might be involved in the change of distribution of actin during cyst formation,proteins were co-precipitated with actin filaments.As a result,p66 and polymerized actin and its associated protein were pelleted by centrifugation(Fig.6).By taking into consideration these results,the p66 is presumed to be one of the actin regulating protein,moreover,the interaction between actin and the p66 directly affects the morphological change of these cells corresponding to stresses.

A novel actin regulating protein,p42. The 42-kDa protein (p42) behaved together with p66 during the purification of p66,and it was revealed that this protein might interact with p66 *in vivo*. Then

Table 1. Induction of Stress Proteins by Salt Stress and Heat Shock.

98 k

[35 S]Met incorporation(dpm/cell)

	1 hr	6 hrs	24 hrs
control	0.027 (1)	0.125 (1)	0.060 (1)
heat shock	0.098 (3.62)	0.190 (1.52)	0.096 (1.6)
salt stress	0.111 (4.11)		0.054 (0.90)

66 k

[35 S]Met incorporation(dpm/cell)

	1 hr	6 hrs	24 hrs
control	0.030 (1)	0.194 (1)	0.067 (1)
heat shock	0.175 (5.83)	0.479 (2.46)	0.131 (1.95)
salt stress	0.132 (4.4)		0.061 (0.91)

76 k

[35 S]Met incorporation(dpm/cell)

	1hr	6hrs	24hrs
control	0.020 (1)	0.077 (1)	0.029 (1)
heat shock	0.073 (3.65)	0.137 (1.77)	0.070 (2.41)
salt stress	0.073 (3.65)		0.048 (1.65)

35 k

[35 S]Met incorporation (dpm/cell)

	1 hr	6 hrs	24 hrs
control	0.015 (1)	0.136 (1)	0.027 (1)
heat shock	0.054 (3.61)	0.191 (1.41)	0.065 (2.40)
salt stress	0.043 (2.84)		0.034 (1.25)

70 k

[35 S]Met incorporation(dpm/cell)

	1 hr	6 hrs	24 hrs
control	0.009 (1)	0.056 (1)	0.015 (1)
heat shock	0.037 (4.11)	0.095 (1.69)	0.031 (2.06)
salt stress	0.062 (6.88)		0.018 (1.2)

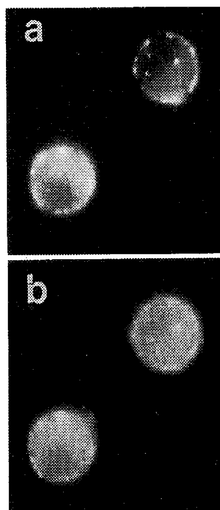

Fig.5 Co-loclization of Actin and p66 revealed by Double-labeled Immunofluorescence.The disk-shaped cells were visualized with rhodamin-phalloidin(a) and the anti-p66 antibody(b).

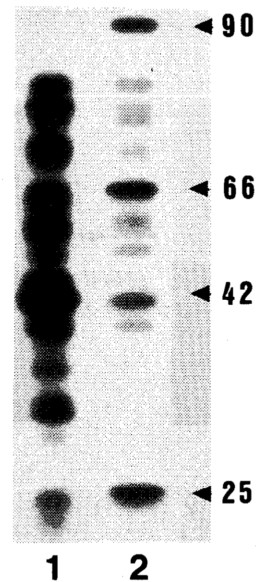

Fig.6 Co-precipitation Study of Cell Extract Polymerized Actin.The [^{35}S]mehionine-labeled cell extract was incubated with actin and centrifuged(2). The supernatant (lane1) and the pellet (lane2) were analyzed by SDS-PAGE,and fluorogram was made.

we carried out the immunoprecipitation experiment of the cell extract using anti-p66 antibody.Resulting precipitate contained both p66 and p42,therefore it is possible to consider that they also interact *in vivo*.Co-precipitation experiment of this mixture and actin of *Physarum* resulted in the precipitating both proteins with actin. And it is considered that the p42 mediates the binding of p66 and actin.Detailed investigation on these relationship is now under way.

In this study,we have clarified that the drastic change of structure of actin was closely involved in morphological change of myxoamoebae of *Physarum polycephalum* which subsequently protect themselves from salt stress, and on this occasion a novel protein designated p66 was induced in a stage-specific way,and regulated the structural change of actin filaments.

From now on,we will further investigate the structure of p66 and p42 genes and its expression systems,and elucidate the detailed regulation mechanism of actin in the cell differentiation,which give us the significant clues to clarify the relationship between the stress response and the induction of cell differentiation.

Acknowledgement

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