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RESPONSE OF A TRUE SLIME MOLD, *Physarum polycephalum*, TO SALT STRESS II
The Effects of The Inhibitors for RNA and Protein Syntheses on Stress-induced Microcyst Formation

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Summary

As reported before, 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, microcysts. We found the change of the intracellular distribution of actin filaments associated with the cellular morphological changes from myxoamoebae to microcysts, and the induction of syntheses of some species of stress proteins, among which 66kD protein, p66 was most prominently induced (1,2). But we did not know whether the induction of these proteins are completely necessary for the process of cyst formation. In this report, we examined the effects of the inhibition of RNA and protein syntheses using some inhibitors for their syntheses on stress-induced microcyst formation to know whether the expression of new RNA and proteins are necessary for the induction of morphological changes. And we showed that newly synthesized RNA and protein(s) were necessary for the stress-induced microcyst formation.

Introduction

Recently, salt tolerant plants attract a considerable attention to gain productivity under desert 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 a simple, single-cell organism, myxoamoebae of a true slime mold *Physarum polycephalum* ,and studied their defense action against salt stress.

Before, we found that the myxoamoebae changed their shape into microcysts under high salt conditions, and the induction of a novel stress protein, p66, was associated with this change (12). The same morphological change occurred under some other stresses, *e.g.*heat shock, starvation, high osmotic pressure (2). And when the cells were exposed to heat shock, very rapid changes in membrane lipids (3) and protein kinase activity (4) were observed. Under high salt conditions, the same changes in membrane lipids were also observed (5).

In this report, we examined the necessity of newly synthesized RNA and protein(s) for the process

of microcyst formation which is induced by salt stressusing some inhibitors for RNA and protein synthesis.

Materials and Methods

Organisms. Haploid myxoamoebae of *Physarum polycephalum* were grown on a lawn of bacteria, *Aerobacter aerogenes*, on a nutrient agar medium. Ordinarily, they were grown in the dark at 24°C (6). The cells were applied to each experiments after washing with phosphate buffered saline (PBS : 136mM NaCl,2.6mM KCl,2.7mM Na_2HPO_4,1.4mM KH_2PO_4).

Cyst formation study in the presence or absence of inhibitors. To observe the effects of of inhibitors on morphological changes from myxoamoebae to microcysts caused by salt, myxoamoebae were incubated in the buffers containing 0.25 M concentration of salt and various concentration of inhibitors at 24°C with shaking. Myxoamoebae and microcysts were counted under a phase contrast microscope. Microcysts were distinguished from other cells by staining with 1% Congo Red.

Labeling of newly synthesized proteins. Myxoamoebae were incubated in KPB containing [^{35}S]methionine under various stress conditions. After appropriate times,cells were washed with KPB containing non labeled methionine,then lysed in SDS sample buffer (7) 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 (7) 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 (8).

Results and Discussion

Effects of inhibitors of RNA syntesis and protein synthesis on the induction of morphological change from myxoamoebae into microcysts by salt stress. Haploid myxoamoebae of a true slime mold, *Physarum polycephalum*, ordinarily move around with their pseudopodia, and 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 conditions and survive for a long time until their environmental conditions improve. The course of cyst formation induced by salt stress was studied before (1), and when the myxoamoebae exposed to 0.25M NaCl, more than 90% of them changed into microcysts after 24-hour incubation. The degree of morphological change depended on the concentration of NaCl, and the microcysts formed the cell wall around them to protect themselves from disadvantageous environmental conditions. 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 stress,and particularly remarkable increase was observed in 66-kD and 70-kD protein synthesis (1,2). The induction of the p66 was not shown when the diploid plasmodia were exposed to stresses. This result suggests that the expression of p66 is regulated in each stage of cell differentiation, and 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). And former studies show that 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.

We here examined the effects of inhibition of RNA and protein syntheses on this process to know

the necessity of newly synthesized RNA and protein(s) for the salt-induced microcyst formation. To inhibit RNA synthesis we used some RNA synthesis inhiboitors. We examined actinomycin D, proflavin and 8-azaguanine from 1 x 10^{-6} M to 1 x 10^{-2} M. Only 8-azaguanine showed the blocking effect on the transformation from myxoamoebae to microcysts at 1 x 10^{-3}M, but other two inhibitors showed no inhibition even at high concentration as 1 x 10^{-2}M (Table I). It is considered that most of inhibitors can not penetrate into inside of the cell, but the synthesis of RNA might be necessary for the process of cyst formation as shown in the case of 8-azaguanine. In the presence of 1 x 10^{-3}M 8-azaguanine, apparent decrease of p66 synthesis was observed (data not shown).

As the protein synthesis inhibitors, we examined streptomycin, puromycin and cycloheximide from 1 x 10^{-7} to 1 x 10^{-2}M. Streptomycin inhibited 25% of cyst formation at the concentration of 10^{-4}M and 50% at 10^{-2}M, puromycin 33% at 10^{-3}M and 97% at 5 x 10^{-3}M. Cycloheximide showed very strong inhibitory effect on the transformatin to microcysts. At 10^{-6}M concentration, 23% of cyst formation was inhibited and at 10^{-4}M, nearly 100% (96%) inhibition was observed. From these results, newly synthesized protein(s) are considered to be necessary for the transformation from myxoamoebae to microcysts(Fig.1). In the presence of protein synthesis inhibitors, p66 synthesis was apparently decreased (data not shown).

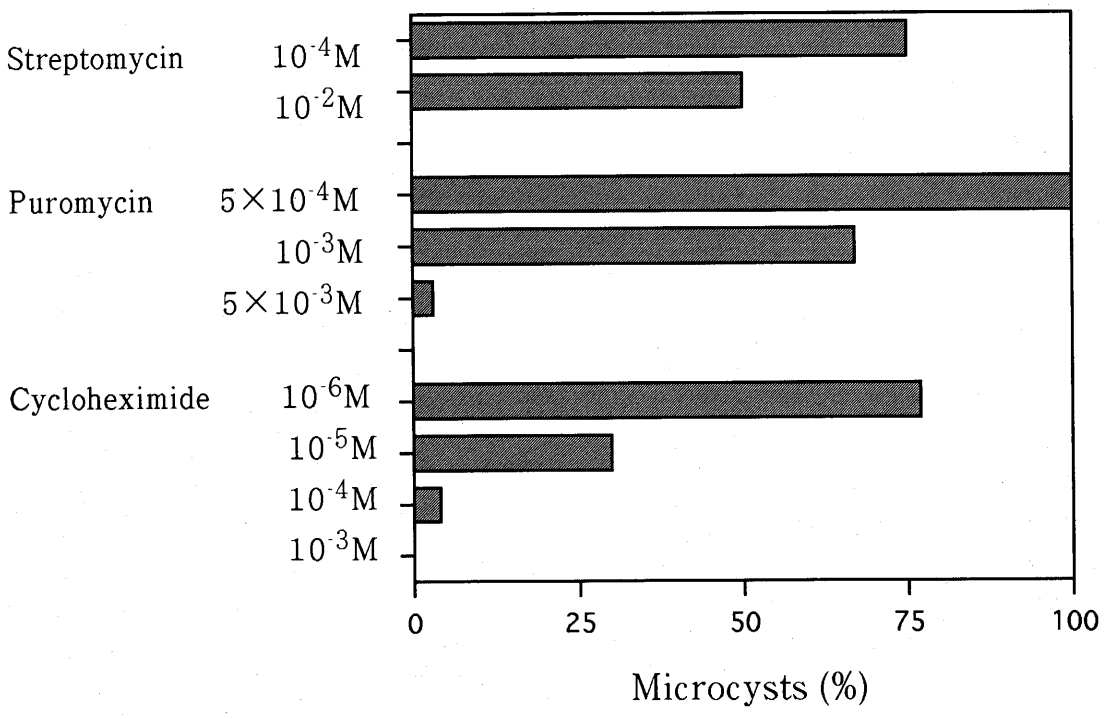

Fig.1. Effects of inhibitors for protein synthesis on microcyst formation.

We also examined the effects of some other substances known as SH-reagents or inhibitors for respiration. As shown in Table I, SH-reagents showeed the inhibition of microcyst formation, but the effects were not continue long (data not shown). Iodoacetic acid and sodium fluoride, known as inhibitors for glycolysis, showed almost no inhibition of microcyst formation, but inhibitors for oxidative phosphorylation, potassium cyanide, sodium azide and 2,4-dinitrophenol, showed apparent inhibition of stress-induced transformation of myxoamoebae to microcysts.These results show that *Physarum* cells use ATP produced in the oxidative phosphorylation as the energy source for this process.

Class of inhibitors	Compound	Effect	Lowest conc. blocking transformation	Highest conc. tested
RNA synthesis	8-Azaguanine	+	1×10^{-3}M	
	Actinomycin D	±		1×10^{-2}M
	Proflavin	±		1×10^{-2}M
Protein synthesis	Cycroheximide	+	1×10^{-6}M	
	Streptomycin	+	1×10^{-4}M	
	Puromycin	+	1×10^{-3}M	
Respiration	Sodium Azide	+	5×10^{-4}M	
	2,4-Dinitrophenol	+	5×10^{-4}M	
	Potassium Cyanide	+	1×10^{-3}M	
	Sodium Fluoride	+		1×10^{-2}M
	Iodoacetic acid	+		1×10^{-2}M
SH-reagent (SH groups)	2-Mercaptoethanol	+	1×10^{-3}M	
	PCMB	+	1×10^{-4}M	

Table 1. Effect of various inhibitors on microcyst formation

In this study, we have clarified that the newly synthesized RNA and proteins are necessary for the process of cyst formation which is caused by salt stress. An expression of a novel protein designated p66, which is induced in a stage-specific way and regulated the structural change of actin filaments, might be the one of the targets of these inhibitors.

We will further investigate the structure of p66 gene and regulation mechanism of its expression, 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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