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Stimulation of Mammalian and Yeast Phosphoprotein Phosphatases by Megamodulins from Various Sources

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SUMMARY: Phosphoprotein phosphatases (PPase) were isolated from either the rabbit cerebral cortex or Baker's yeast by excluding endogenous megamodulin with histone, and then by desalting cations with Bio-Gel P-6DG filtration. The stimulation of PPase in the presence of Mn^{2+} was greatly enhanced by megamodulins prepared from various sources including rabbit brain, Baker's yeast, wheat germ, and Escherichia coli (E. coli). Moreover, the augmented activity of PPase was also demonstrated in the presence of [megamodulin-Mn^{2+}] complex.

INTRODUCTION: Stimulatory protein kinase modulators (1 - 3), a group of protein factors, have recently been designated as 'megamodulins' (4 - 6) because of their similarities in large molecular weight in aggregated 'holo' form (1 - 7), ubiquitous nature (1 - 6), and possible roles in regulating a broad spectrum of cellular activities. However, their regulatory functions, being demonstrated so far in vitro, only include the following: stimulation of mammalian and arthropodous guanosine 3':5'-monophospate-dependent protein kinases (1 - 3, 8, 9), mammalian megamodulin-dependent protein kinases (4 - 6, 10) and E. coli RNA polymerase (11); binding with a variety of cations (4 - 6, 12, 13); and interaction with various basic proteins (4 - 6, 14). Therefore, further exploration on other megamodulin-dependent cellular activities is very desireable. In the present study, we report on the effect of megamodulin on another class of enzyme, PPase.

MATERIALS AND METHODS: Baker's yeast (Saccaromyces cerevisiae) was obtained from Nabisco Brands, Inc., Bio-Gel P-6DG was from Bio-Rad. Arginine-rich histone (HA) was purchased from Worthington. Other histones and wheat germs were from Sigma, and [γ-^{32}P] ATP was from New England Nuclear.

With slight modification, the preparation of phosphoprotein was basically the same as that described by others (15). Briefly, in a final volume of 9 ml,

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the incubation mixture contained the following: 0.5 mg of muscle adenosine 3':5'-monophosphate-dependent protein kinases and megamodulin-dependent protein kinases from the step of DEAE-cellulose (5, 16); 30 mM Tris-HCl, pH 7.4; 3 mM theophylline; 5 mM $MgCl_2$; 6 mg of various histones; 5 mM adenosine 3':5'-monophosphate; 10 nmoles of [γ-^{32}P] ATP containing approximately 1.1 x 10^7 cpm. The incubation was performed at 30°C for 50 minutes, and phosphorylation was terminated by the addition of 2.5 ml of 100% trichloroacetic acid. The resultant precipitate was centrifuged, washed twice by dissolving in water and reprecipitated with trichloroacetic acid, and then redissolved in 2 ml of water. Finally, it was dialyzed against deionized water for 24 hours with 3 changes of water.

Phosphoprotein phosphatase (PPase) was prepared either from the rabbit cerebral cortex or Baker's yeast. Approximately 1 g of rabbit cerebral cortex was homogenized in 3 volumes of deionized water with a glass-Teflon homogenizer. The homogenate was centrifuged at 47,000 rpm, and at 4°C for 20 minutes, using a Beckman SW-50.1 rotor. Then, the resultant supernatant was mixed with 100 µg of arginine-rich histone, and the mixture was centrifuged again for 60 minutes. The final supernatant was collected and applied to a Bio-Gel P-6DG column (1.5 x 38 cm) which was eluted with deionized water. The volume sized for each fraction was 2.5 ml. Fractions 15 and 16 were pooled and used as PPase source. Likewise, Baker's yeast was homogenated with a bead-beater, followed by histone-treatment. Finally, fraction 13 from Bio-Gel P-6DG filtration was collected and used as yeast enzyme source.

The preparation of crude megamodulin by boiling and tricholoroacetic acid-precipitation of tissue or cell homogenate was essentially the same as that described by Appleman et al. (17) and Walsh et al. (18) for rabbit skeletal muscle and by Donnelly et al. (19) for lobster tail muscle. The subsequent purification of crude megamodulin was modified over past studies (1 - 3) by employing Bio-Rad P-6DG desalting gel filtration followed by either Sephadex G-100 or G-150 gel filtration (4 - 6).

The preparation of [megamodulin - Mn^{2+}] complex was essentially the same as that for Mg^{2+} (or other cations) complex (4 - 6, 12). Briefly, the pre-incubated

mixture of purified megamodulin and $MnCl_2$ was applied to a Bio-Gel P-6DG column which was eluted with deionized water. Subsequently, the protein peak fraction was collected as the source of this complex.

For modified standard PPase assay, the reaction mixture was in a final volume of 0.32 ml consisting of the following: 30 mM Tris-HCl buffer, pH 7.4; 1 mM dithiothreitol; 60 µg of ^{32}P-labeled histones containing approximately 1.5×10^4 cpm; 11 or 15 µg of enzyme protein; with or without appropriate amount of megamodulin or [megamodulin - Mn^{2+}] complex; with or without varied amount of $MnCl_2$. The incubation was carried out at 30°C for 10 minutes and terminated by the addition of trichloroacetic acid. Finally, PPase activity was determined by measuring the radioactive orthophosphate released from the ^{32}P-labeled phosphohistone (15).

RESULTS AND DISCUSSION: The non-kingdom (phylum, genus, or species) specific stimulation of PPase was demonstrated in the presence of various megamodulins prepared from either *E. coli*, wheat germ, Baker's yeast or rabbit brains (Tables 1 and 2). However, there were differences in the potency of activation. The less potency in the presence of yeast megamodulin (peaks 2 and 3) or wheat germ megamodulin may be due to either the contaminant inhibitory factor(s) in megamodu-

TABLE 1. Stimulatory effect of various megamodulins on phosphoprotein phosphatase (PPase) from rabbit cerebral cortex.

Preparation of PPase (50 µg)	Megamodulin (50 µg)	Relative Activity of PPase (%): $-Mn^{2+}$	$+Mn^{2+}$ (3mM)
Crude cytosol	None	100	118 ± 4
	Baker's yeast:		
	Peak 1	174 ± 9	219 ± 12
	Peak 2	113 ± 3	140 ± 6
	Peak 3	100 ± 2	123 ± 2
	Rabbit brain	243 ± 17	275 ± 15
Bio-Gel P-6DG eluate	None	100	103 ± 4
	E. coli	293 ± 23	317 ± 19
	Wheat germ	150 ± 8	159 ± 6
	Baker's yeast (peak 1)	196 ± 7	331 ± 15
	Rabbit brain	249 ± 10	338 ± 11

Each value shown represents the mean ± (standard error) of three to five samples. Controls, expressed by 100 in relative activity, for crude cytosal and Bio-Gel P-6DG eluate were 612 and 983 cpm respectively.

TABLE 2. Stimulatory effect of various megamodulins on PPase from Baker's yeast

Megamodulin (50 μg)	Relative activity of PPase: $-Mn^{2+}$	$+Mn^{2+}$ (0.3 mM)
None	100	478 ± 19
E. coli	171 ± 8	1980 ± 142
Wheat germ	158 ± 7	1982 ± 135
Baker's yeast (peak 1)	213 ± 13	1880 ± 103
Rabbit brain	107 ± 2	2129 ± 111

Each value shown represents the mean ± (standard error) of three to five samples. PPase (11 μg) was purified up to the step of Bio-Gel P-6DG filtration. Control, expressed by 100, was 646 cpm.

lin preparation or excessively pre-existing Mn^{2+} in crude cytosol preparation (Table 1). After PPase was further purified by both histone-treatment to remove large portion of endogenous megamodulins and Bio-Gel P-6DG filtration to exclude excessive cations, greatly enhanced enzyme activity was observed (Tables 1 and 2). Moreover, yeast enzyme, prepared from these steps, was exceptionally sensitive to megamodulins at very low concentrations of Mn^{2+} (Table 2 and Figure 1). In the presence of yeast megamodulin, the apparent Km of PPase for Mn^{2+} was decreased from 0.45 mM to approximately 0.075 mM (Figure 1).

The augumented activity of yeast PPase in the presence of various [megamodulin - Mn^{2+}] complexes freshly prepared was shown in Table 3. However, it is unclear whether the binding of Mn^{2+} with megamodulins (to form complexes) can induce the conformational changes of megamodulins to become active forms so that complexes can interact with phosphoproteins and PPase.

The linear time course of PPase activity, either in the presence or absence of megamodulin (or Mn^{2+}), up to 10 minutes of incubation time was illustrated in Figure 2. Nevertheless, at very high concentrations of substrate, the stimulatory effect of megamodulin was no longer detectable (data not shown). In addition, when megamodulin was replaced by another (other) protein(s), such as pyruvate

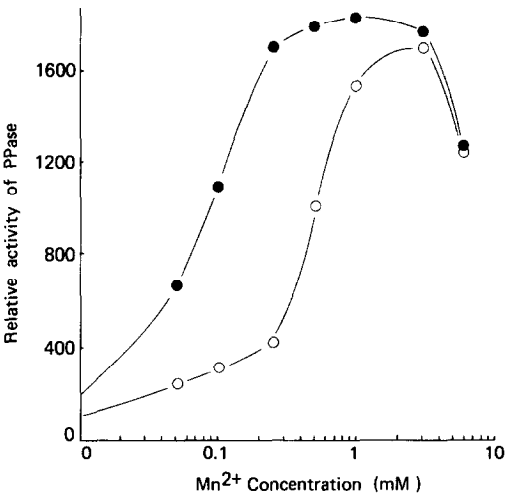

Figure 1. Effect of MN^{2+} concentration on yeast PPase activity. Each value shown represents the mean of triplicate samples in the presence (●) or absence (o) of yeast megamodulin (peak 1, 50 μg). PPase (11 μg) was purified up to the step of Bio-Gel P-6DG filtration. Control, expressed by 100 in relative activity of PPase, was 671 cpm.

kinase or bovine serum albumin, no activation of PPase was found (data not shown), indicating the unique regulatory role of megamodulins.

Some of the differences between megamodulin and calmodulin have been preliminarily compared (13). Calmodulin, which functions mainly as a Ca^{2+}-mediator in eukaryotes (20, 21), may not exist in prokaryotes because of ambiguous findings (20 - 22). Moreover, calmodulin, which is much smaller in molecular

TABLE 3. Activation of PPase from Baker's yeast by [megamodulin - Mn^{2+}] complexes

[Megamodulin - Mn^{2+}] complex	Relative activity of PPase
None	100
Baker's yease (peak 1)	738 ± 47
E. coli	795 ± 26
Rabbit brain	863 ± 34

Each value shown represents the mean ± (standard error) of triplicate samples in the presence or absence of [megamodulin - Mn^{2+}] complex (35 μg). PPase (11 μg) was purified up to the step of Bio-Gel P-6DG filtration.

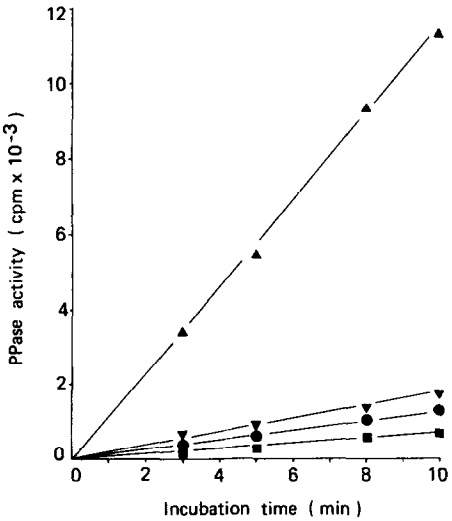

Figure 2. Phosphoprotein phosphatase (PPase) activity as a function of incubation time. Eleven µg of yeast PPase from Bio-Gel P-6DG was used. PPase activity: basal, ■; megamodulin (45 µg), ●; Mn^{2+} (0.3 mM), ▼; megamodulin $+Mn^{2+}$, ▲.

weight (13), was well separated from megamodulin during gel filtration (23) employed in this study. Therefore, it is very unlikely that the observed stimulation of PPase at low concentrations of Mn^{2+} was enhanced by contaminant calmodulin.

Cytosol phosphoprotein phospatases prepared from the step of Bio-Gel P-6DG can be further resolved into three active fractions by DEAE-cellulose chromatography (15), and all three phosphatases were affected by megamodulins (Kuo, unpublished data). Therefore, results in this study indicated the combined stimulatory effect of megamodulin on these three phospatases. It is of interest that megamodulins activate both megamodulin-dependent protein kinases (4 - 6, 10) and PPase. Despite totally opposite functions carried out by both enzymes, the time sequence for the actions of megamodulin-dependent protein kinases and PPase may be different. Therefore, there may be no contradictory antagonism *in vivo* between these two classes of enzymes. Nevertheless, the real mechanism to avoid such concomitant actions of these enzymes remains unclear. In our latest experiments, we also have successfully isolated and purified yeast megamodulin-dependent protein kinases which utilize yeast endogenous protein(s) as substrate(s) (Kuo, unpublished data). Further studies on the role of mega-

modulin on regulating PPase in yeast, by using yeast endogenous phosphorylated protein(s) to replace mammalian histones (in this study), are being carried out in our laboratory.

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REFERENCES

1. Kuo, W. N. and Kuo, J. F. (1976) J. Biol. Chem. 251, 4283-4286
2. Kuo, W. N., Shoji, M. and Kuo, J. F. (1976) Biochem. Biophys. Acta. 437, 142-149.
3. Kuo, W. N., Shoji, M. and Kuo, J. F. (1976) Biochem. Biophys. Res. Commun. 70, 280-286.
4. Kuo, W. N. (1983) Cytobios 36, 175-186.
5. Kuo, W. N. (1983) Microbios (in press).
6. Kuo, W. N., Liu, L. P., Brown, K. F. and Hardy, J. L. (1983) Microbios (in press).
7. Shoji, M., Brackett, N. L., Tse, J., Shapira, R. and Kuo, J. F. (1978) J. Biol. Chem. 253, 3427-3434.
8. Yamamotot, M., Takai, Y., Hashimoto, E., and Nishizuka, Y. (1977) J. Biochem. (Tokyo) 81, 1857-1862.
9. Chihara-Nakashima, M., Hashimoto, E., and Nishizuka, Y. (1977) J. Biochem. (Tokyo) 81, 1863-1867.
10. Kuo, W. N. (1981) Experientia 37, 235-237.
11. Sen, S., Rahmani, M. A., Kuo, W. N. and Aikens, I. Microbios (in press)
12. Kuo, W. N. (1982) Microbios Letters 20, 141-146.
13. Kuo, W. N. (1982) Cytobios 33, 183-196.
14. Kuo, W. N. (1983) Experientia 39, 60.
15. Maeno, H. and Greengard, P. (1972) J. Biol. Chem. 247, 3269-3277.
16. Beavo, J. A., Bechtel, P. J. and Kreb, E. G. (1974) Method in Enzymology 38, 299-308.
17. Appleman, M. M., Birnbaumer, L. and Torres, H. N. (1966) Arch. Biochem. Biophys. 116, 39-43.
18. Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fischer, E. H. and Krebs, E. G. (1971) J. Biol. Chem. 246, 1977-1985.
19. Donnelly, T. E., Jr., Kuo, J. F., Reyes, P. L., Liu, Y. P. and Greengard, P. (1973) J. Biol. Chem. 248, 190-198.
20. Cheung, W. Y. (1982) Scientific American 246, 62-70.
21. Lin, Y. M. (1982) Molecular and Cellular Biochemistry 45, 101-112.
22. Iwasa, Y., Yonemitsu, K., Matsui, K., Fukunaga, K. and Miyamoto, E. (1981) Biochem. Biophys. Res. Commun. 98, 656-660.
23. Kuo, W. N. (1983) Cytobios 36, 31-38.