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# IDENTIFICATION OF PROTEIN PHOSPHATASES DEPHOSPHORYLATING mRNP PROTEINS FROM CRYPTOBIOTIC GASTRULAE OF THE BRINE SHRIMP A. SALINA

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In the cytosol of A. salina cryptobiotic gastrulae at least five protein phosphatases active on phosphorylase a have been detected by ion exchange chromatography on DEAE-cellulose. Only two of these enzymes (PP-X and PP-Y) are active in mRNP dephosphorylation. Both enzymes are insensitive to inhibitor-1 and -2 and stimulation of enzymatic activity (2.5-fold with PP-X and 6.5-fold with PP-Y) can be accomplished by ethanol treatment of the native enzymes, or freeze-thawing in the presence of 1.7% (v/v) 2-mercaptoethanol. These properties allow PP-X and PP-Y to be classified as type-2A enzymes according to the nomenclature of Cohen.
This paper is the first report of protein phosphatases capable of dephosphorylating mRNP proteins. 

It is generally accepted that messenger RNA is associated with specific proteins to form messenger ribonucleoproteins (mRNP) (1). It is often proposed that these proteins have a function in translational control (2). Posttranslational modification of mRNP associated proteins by phosphorylation has been observed frequently, and is proposed to play a role in the regulation of mRNA metabolism (3-6). Dephosphorylation of mRNP proteins has also been suggested to have a function in translation (4,7) although no experimental results have been published so far.

Artemia salina cryptobiotic gastrulae are characterized by the presence of stored non-polysomal mRNP (8). A casein kinase II activity which phosphorylates several mRNP proteins, is associated with the

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latter mRNP (5). "In vitro", the poly(A) binding proteins P38 and P23.5 are the best phosphate acceptors (9).

In this communication we report the presence and identification of 2 phosphoprotein phosphatases able to dephosphorylate mRNP proteins.

## MATERIALS AND METHODS

### 1. Preparation of mRNP proteins and associated casein kinase II.

Postmitochondrial supernatant was prepared from cryptobiotic gastrulae of A. salina (Macau, Brazil) in 10 mM Hepes pH 7.2, 250 mM KCl, 0.1 mM DTT, 0.1 mM PMSF. Poly(A)-containing non-polysomal mRNP was obtained from the postmitochondrial supernatant (8). mRNP proteins were prepared by a 1.5 M KCl salt wash of oligo(dT)-cellulose bound mRNP, dialyzed against 10 mM Hepes pH 7.2, 100 mM KCl, 5 mM 2-mercaptoethanol, and concentrated by ultrafiltration on a PM 10 membrane (Amicon, Oosterhout, The Netherlands). The isolation of mRNP associated casein kinase II was as described previously (5).

### 2. Preparation of protein phosphatases.

Postribosomal supernatant was prepared from the postmitochondrial supernatant by differential centrifugation in a Beckman R60 Ti rotor for 1 h at 250,000 xg. The supernatant was brought to 30% saturation in $(NH_4)_2SO_4$. The precipitate was collected by centrifugation and discarded. The remaining supernatant was brought to 60% (w/v) $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, dissolved in 20 mM Tris pH 7.2, 0.1% (v/v) 2-mercaptoethanol (buffer A) and dialyzed against buffer A. The dialysate was chromatographed on DEAE-cellulose as described in the legend to fig. 1.

### 3. Phosphoprotein phosphatase assay.

Phosphorylase b, inhibitor-1 and inhibitor-2 were purified to homogeneity from rabbit skeletal muscle as in (19). [$^{32}$P]-labelled phosphorylase a was prepared from phosphorylase b using phosphorylase kinase (10). Purified mRNP proteins were labelled with 6 µM [$\gamma$-$^{32}$P] ATP (specific activity 110 Ci/mmol, Amersham International, Buckinghamshire, England) and 8 units of mRNP associated casein kinase II (5). Reactions were stopped by gelfiltration on Sephadex G50 in buffer A containing 100 mM NaCl to remove ATP. Protein phosphatases were assayed by measuring the release of [$^{32}$P]-phosphate from [$^{32}$P]-labelled phosphorylase a (3900 cpm/µg) or purified mRNP proteins (2400 cpm/µg). The assay (30 µl) contained 17.5 mM Tris-HCl pH 7.0, 0.03 mM EGTA, 0.03% (v/v) 2-mercaptoethanol, 0.35 mg bovine serum albumin/ml, 10 µg [$^{32}$P]-labelled subtrate, appropriate amounts of divalent ions, as described in the legend of fig. 1 and protein phosphatase. 5 mM caffeine was included when phosphorylase a was used as a substrate. Reactions were initiated with [$^{32}$P]-substrate, terminated and analyzed as in (11-12). In experiments where inhibitor-1 and inhibitor-2 were present, protein phosphatase and inhibitor protein were preincubated for 10 min and the reactions initiated with substrate.

## RESULTS AND DISCUSSION

Recent studies in different mammalian tissues have demonstrated that cytosolic protein phosphatases that are active against phosphoseryl

and phosphothreonyl-protein substrates may be classified into two groups, designated type 1 and 2 (16-17). The latter can be separated by chromatography on DEAE-cellulose into different subclasses such as $2A_0$, $2A_1$ and $2A_2$ (18-19, 25).

As shown in fig. 1, at least five protein phosphatases present in the postribosomal supernatant of A. salina cryptobiotic gastrulae were resolved by DEAE-cellulose chromatography. Protein phosphatase activity was determined under three assay conditions i.e. in the absence of divalent ions and in the presence of 5 mM $MgCl_2$ or 1 mM $MnCl_2$. The rationale behind these experiments was that several protein phosphatases show a different metal ion dependency (13, 16, 20-21). With phosphorylase a as a substrate (fig. 1B), five different phosphatase activities, numbered 1 to 5 in order of their elution from the column, could be detected, consistent with the results obtained using chicken brain extracts (25) or rat liver cytosol (18). Since we were interested in mRNP specific protein phosphatases, no attempts were made to further characterize these five phosphorylase phosphatase activities.

Only two major peaks of mRNP protein phosphatase activity were detected after chromatography on DEAE-cellulose by assays carried out with [$^{32}$P]-labelled mRNP proteins (fig. 1C). In order to obtain a protein phosphatase assay that was linear with respect to time and specific activity, DEAE-cellulose fractions had to be diluted 65 times when [$^{32}$P]-mRNP proteins were used as a substrate. The major peak of activity eluted at 330 mM NaCl (designated PP-X) and dephosphorylated mRNP proteins 56 times more efficiently than phosphorylase a, based on the % [$^{32}$P] released by the same amount of enzyme. A second mRNP protein phosphatase eluted at 230 mM NaCl (designated PP-Y) and dephosphorylated mRNP proteins 198 times more efficiently than phosphorylase a.

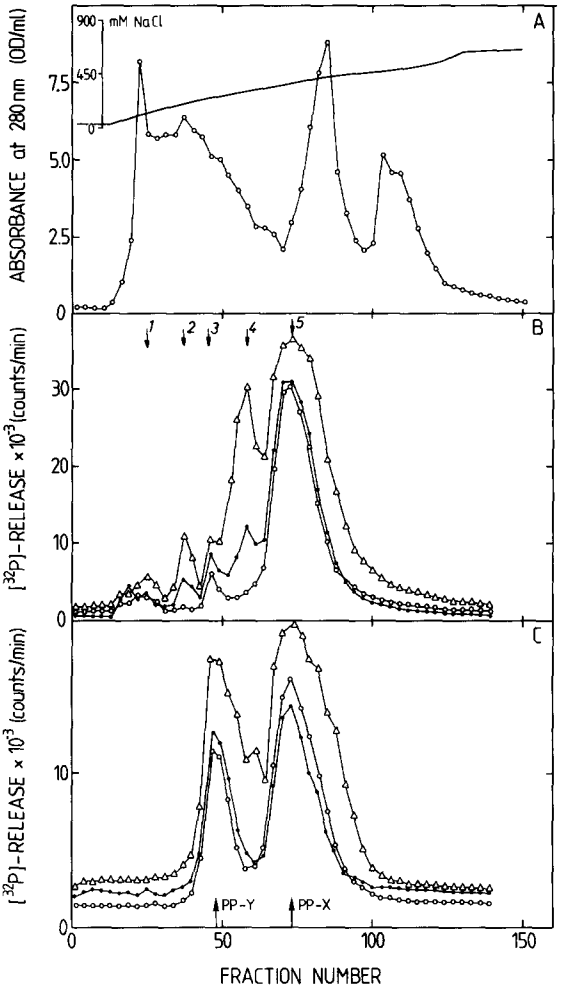


Fig. 1 : Resolution of A. salina cytosolic phosphatases by chromatography on DEAE-cellulose.
The column (60 x 3 cm) was equilibrated with buffer A. After application of the dialyzed 60% $(NH_4)_2SO_4$ precipitate (300 ml), the column was washed with buffer A (1000 ml). Bound proteins were eluted with a 2000 ml linear gradient of 0.05-0.9 M NaCl in buffer A. The flow rate was 140 ml/h and fractions of 10 ml were collected. Phosphatase assays were performed as described in Materials and Methods. (A) (———) NaCl salt concentration; (0——0) absorbance at 280 nm. (B) Aliquots of the indicated fractions were assayed for phosphorylase phosphatase activity in the presence of (●——●) 5 mM $MgCl_2$; (△——△) 1 mM $MnCl_2$ or the absence of divalent ions (0——0). The position of the protein phosphatases, numbered 1 to 5, are as indicated. (C) Aliquots of the indicated fractions were diluted 65 times and assayed for mRNP protein phosphatase activity in the presence of (●——●) 5 mM $MgCl_2$; (△——△) 1 mM $MnCl_2$ or the absence of divalent ions (0——0). The position of PP-X and PP-Y is as indicated.

In the protein phosphatase assay used, activity was assessed by measuring the appearance of trichloroacetic acid-soluble radioactivity. A potential artefact might be that low molecular weight peptides,

Table I : Demonstration that protein phosphatases-X and -Y (PP-X, PP-Y) are not proteinases

| Phosphatase fraction | [$^{32}$P]-radioactivity released in standard assay (cpm) | Phosphate released in the presence of NaF | | [$^{32}$P]-radioactivity extracted into acidic molybdate (cpm) |
|---|---|---|---|---|
| | | mM | cpm | |
| PP-X | 14461 | 5 | 3445 | 14480 |
| | | 10 | 1910 | |
| | | 50 | 1627 | |
| PP-Y | 9685 | 5 | 4526 | 8659 |
| | | 10 | 2433 | |
| | | 50 | 1722 | |

Assays were performed as described in Materials and Methods using mRNP proteins as substrate. Incubations were at 30°C for 30 min. In the presence of NaF, fractions were preincubated at 4°C for 30 min. The incorporation of inorganic [$^{32}$P]-phosphate into acidic molybdate was performed, as described (13).

soluble in trichloroacetic acid, are generated by proteinase action. Control experiments were therefore performed to examine this possibility (Table I). Sodium fluoride is known to be a potent inhibitor of phosphatases without having a significant effect on proteinases. Both mRNP specific protein phosphatases are inhibited by NaF with an $I_{50}$ of 3 mM and 4 mM for PP-X and PP-Y respectively. That both mRNP specific phosphatases are not proteinases (or contaminated by proteinases), was established by the demonstration that the [$^{32}$P]-radioactivity liberated during the reaction could be removed quantitatively as a [$^{32}$P]-molybdate complex (Table I).

The mRNP specific protein phosphatases were classified as type 2 protein phosphatases by measurement of their sensitivity to inhibitors-1 and -2 (Table II). No inhibition of either PP-X and PP-Y - activity could be observed with both inhibitors excluding the possibility that the mRNP protein phosphatase activities are type 1 phosphatases. However, one should take into account that the lack of effect of inhibitors-1 and -2 might be due to the fact that the mammalian inhibitors may not affect the shrimp enzymes. When the PP-X- and PP-Y-fractions from DEAE-cellulose were treated with 80%

Table II : Classification of protein phosphatases -X and -Y as type -2A enzymes

| | PP-X | | PP-Y | |
|---|---|---|---|---|
| control assay | 8939 | (100) | 7510 | (100) |
| + 30 nM $I_1$ | 9296 | (104) | 7420 | (99) |
| + 30 nM $I_2$ | 8921 | (99.8) | 7247 | (96) |
| ethanol treatment | 27165 | (304) | 46742 | (622) |
| freeze-thawing | 21176 | (237) | 53088 | (707) |

Assays were performed as described in Materials and Methods using phosphorylase a as substrate. Incubations were at 30°C for 30 min. Treatment of the phosphatases with ethanol was as reported by Brandt et al. (14). The freeze-thawing procedure was similar to that of Kobayashi et al. (15).

aqueous ethanol at room temperature or were frozen and thawed in the presence of 1.7% (v/v) 2-mercaptoethanol, a 2.5- and 6.5-fold stimulation in mRNP protein phosphatase activity was measured for PP-X and PP-Y respectively (Table II). After ethanol treatment or freeze-thawing, the high molecular weight enzymes PP-X (Mr = 224,000) and PP-Y (Mr = 346,000) were dissociated into a low molecular weight species (Mr = 35,000) as demonstrated by HPLC gelfiltration (data not shown). Taking into account these characteristics both mRNP protein phosphatases can be classified as protein phosphatases 2A. The latter treatments produced a substantial increase in the phosphatase activity of PP-Y and a smaller increase in PP-X, suggesting that PP-X is protein phosphatase $2A_2$ and PP-Y is protein phosphatase $2A_1$ (22-24). This classification is in agreement with the elution behaviour of the corresponding mammalian enzymes on DEAE-cellulose (19, 25). Experiments are in progress to purify these mRNP specific protein phosphatases to near homogeneity in order to study the effect of mRNP dephosphorylation on the regulation of mRNP metabolism.

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