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EFFECT OF TEMPERATURE ON ppp(A2'p)$_n$A BINDING PROTEIN ACTIVITIES IN RABBIT RETICULOCYTE LYSATES AND OTHER MAMMALIAN EXTRACTS

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SUMMARY: Lysates of rabbit reticulocytes and other mammalian cells are known to contain an activity which binds with high specificity ppp(A2'p)$_3$A,3'-[^{32}P]pCp. The binding activity shows a marked dependence on preincubation of lysates at different termperatures (4°C - 45°C). For example, binding was increased 50% by preincubation of rabbit reticulocyte lysates at 37°C for 60 minutes. An identical preincubation of mouse brain extracts results in a greater than 90% loss of binding activity. Fractionation of rabbit reticulocyte lysates into the postribosomal supernatant (PRS) and the ribosomal salt wash (RSW), followed by heparin-agarose column chromatoraphy, showed that with the PRS fraction, most of the binding activity is eluted with 600 mM KCl. With the RSW fraction, more than 50% of the binding activity is eluted with 250 mM KCl. These data suggest that multiple ppp(A2'p)$_n$A binding protein activities exist in mammalian cells.

Double-stranded RNA (dsRNA), one of the most potent inhibitors of protein synthesis in mammalian cell extracts, appears to act through at least three enzymatic activities: a dsRNA-activitated protein kinase which inhibits initiation factor eIF-2; a dsRNA-activated (2'-5')A$_n$ synthetase which synthesizes a series of 2',5'-linked oligoadenylates (2'-5')A$_n$; and a (2'-5')A$_n$-dependent endoribonuclease which cleaves rRNA and mRNA on the 3'-end of UpN sequences to yield UpN terminated products (1-5).

Much information has been obtained on the (2'-5')A$_n$ synthetase. The enzyme has been partially purified from unfractionated rabbit reticulocytes (6); purified to apparent homogenity in interferon-treated Ehrlich ascites tumor cells (7) and HeLa cells (8). In addition to converting ATP into (2'-5')A$_n$ by a nonprocessive mechanism (9), the synthetase also catalyzes the nucleotidyl transfer from different nucleoside 5'-triphosphates into the 2'-OH of appropriate receptors such as the 5'-adenylic acid on the 3'-

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terminus of tRNA, NAD^+, ADP-ribose and Ap_4A (20,10,11). The cellular level of $(2'-5')A_n$ synthetase has been shown to be markedly affected by interferon (12), glucocorticoids (13), sodium n-butyrate, dimethyl sulfoxide (14), and by processes of cellular differentiation (15,16).

The properties of the $(2'-5')A_n$-dependent endoribonuclease are less well understood. Work on this very important aspect of $(2'-5')A_n$ system is greatly facilitated by the development of a radiobinding assay which is based on the high specificity and affinity of $ppp(A2'p)_3A[^{32}P]pCp$ for a $ppp(A2'p)_nA$ binding protein. This binding protein is thought to be the $(2'-5')A_n$-dependent endoribonuclease (17-19). Using this assay, we have investigated the effects of preincubation of mammalian extracts at different temperatures (4°C -45°C) on the subsequent interaction of $ppp(A2'p)_nA$ binding protein with $ppp(A2'p)_3A[^{32}P]pCp$ at 4°C. Our results show that mammalian extracts exhibit markedly different thermal stability profiles.

MATERIALS AND METHODS

Rabbit reticulocyte lysates were purchased from Clinical Convenience Products (Madision, Wisconsin). $ppp(A2'p)_3A,3'-[^{32}P]pCp$ (3000 Ci/mmol) was from Amerisham Corporation. NP-40 was from Calbiochem-Behring Corporation. Nitrocellulose filters (2.5 cm, Type HA, 0.45μM pore size) were purchased from Millipore corporation. Heparin-agarose was obtained from Sigma. $ppp(3'dA)_4$ was obtained from Dr. R. Suhadolnik, Temple University and was a gift from Dr. P. Torrence, NIH.

Preparation of Cell-free Extracts. Mouse or rabbit tissues were homogenized (5 ml lysis buffer (10 mM Hepes, 90 mM KCl, 1.5 mM $Mg(OAc)_2$, 1 mM dithionthreitol, 0.5% (v/v) NP-40, 10% glycerol, pH 7.5) per gram wet tissue) in a 40 ml glass homogenizer fitted with a teflon pestle with 4-5 strokes at a setting of 2000 rev/min. The homogenate was centrifuged in an Eppendorf microcentrifuge for 2 min. The supernatant was pipetted off and the pellet discarded. For cell extracts from cultured cells, 1×10^7 cells were lysed by freezing and thawing three times in liquid N_2.

Assay of $ppp(A2'p)_3A,3'-[^{32}P]pCp$ Binding Proteins. The reaction mixture (50 μl) contained 17,000-60,000 cpm of $ppp(A2'p)_3A,3-[^{32}P]pCp$ (6.6 fmol in 10 μl), 10 μl of buffer (20 mM Tris-HCl, pH 7.5, 85 mM KCl, 5 mM Mg $(OAc)_2$, 1 mM ATP and 5% glycerol), and varying amounts of proteins (see each experiment). Incubations were on ice for 60 min. The reaction was terminated with 1 ml of ice cold buffer (20 mM Tris-HCl, pH 7.5, 80 mM KCl, 5 mM $Mg(OAc)_2$, 5% (v/v) glycerol), filtered through a nitrocellulose membrane and washed 3 times with 1 ml aliquots of buffer. The filters were dried under a heat lamp, placed in glass scintillation vials with 4.5 ml scintillant (OCS, Amersham) and counted in a LS-7000 Beckman scintillation counter.

Preparation of Postribosomal Supernatant (PRS) and Ribosomal Salt Wash (RSW) from Rabbit Reticulocyte Lysates. Rabbit reticulocyte lysates were centrifuged at 135,000 x g for 4 h. The PRS was removed and diluted with

an equal volume of Buffer A (20 mM Tris, pH 7.6, 80 mM KCl, 4 mM $Mg(OAc)_2$ and 1 mM ATP). The diluted PRS was made 50% saturated with ammonium sulfate by the addition of an equal volume of a saturated solution of $(NH4)_2$ SO4 (prepared in 20 mM Tris HCl, pH 7.6, 4 mM $(MgOAc)_2$ and 1 mM ATP). After stirring for 30 min in the cold, the suspension was centrifuged (10,000 x g, 20 min), the pellet was resuspended in Buffer A with a tenth of the volume of the initial diluted PRS. The dissolved protein was dialyzed overnight against Buffer A supplemented with 5% glycerol. After dialysis, the protein solution was centrifuged (10,000 x g, 5 min) to remove aggregated proteins. The protein solution was stored in 2-ml portions at -85°C. To obtain the ribosomal salt wash, ribosomes were resuspended in 0.25 M sucrose, 1 mM dithiothreitol, pH 7.0 to give 300 OD_{260}/ml. 3 M KCl was added to a final concentration of 0.5 M KCl. After stirring in the cold for 60 min, the ribosomes were centrifuged at 135,000 x g for 4 h. The ribosomal salt wash was made 50% saturated with ammonium sulfate as described above.

Chromatography on Heparin-Agarose. Heparin agarose (2.5 x 15 cm) was equilibrated with Buffer A supplemented with 5% glycerol. The 50% ammonium sulfate precipitated PRS (18 ml) was passed through the column and eight fractions (12.5 ml) were collected. The column was eluted with Buffer B supplemented with 600 ml KCl. Aliquots (10-20 µl) of each fraction were assayed for binding activity. An identical procedure was used in the chromatography of ammonium sulfate precipitated RSW (20 ml from 450 ml of rabbit reticulocyte lysates).

RESULTS AND DISCUSSION

Preincubation at Different Temperatures and ppp(A2'p) A_n Binding Activities in Mammalian Cell Extracts. Table 1 shows the $ppp(A2'p)_nA$ binding activities of a number of mammalian cell extracts. Of all the tissues examined, interferon-treated guinea pig macrophages or mouse brain at day 17-18 of gestation shows the highest specific activity. When the cell extracts are preincubated for 30 min at temperatures ranging from 4°C to 45°C, a marked tissue-specific, temperature-dependence in binding at 4°C is observed. For example, while the binding activity of rabbit lung or kidney is not affected by preincubation at any temperature, the rabbit liver and heart activities decrease by 24% and 58%, respectively, upon preincubation at 30°C. In contrast, when mouse tissue extracts are preincubated at 30°C for 30 min, the binding activities of liver, lung and kidney show a 50% decrease while virtually no decrease was detected with the heart. At 37°C, a significant decrease in binding activities was observed with all four tissues. In the mouse brain, a 90% loss of activity is observed by preincubation at 37°C. A number of explanations may be considered for the different thermal stability of $ppp(A2'p)_nA$ binding activities in mammalian extracts. One possibility is that during the

TABLE 1
Effects of preincubation at different temperatures on binding activities in mammalian extracts

Tissue[a]	Preincubation Temperature (°C)				
	4	23	30	37	45
	ppp(A2'p)$_3$A3-[^{32}P]pCp Bound (fmol)				
Rabbit					
Liver	2.36	2.67	2.48	1.80	0
Lung	0.84	1.00	1.00	0.71	0
Heart	0.26	0.26	0.26	0.11	0
Kidney	2.60	2.87	2.92	2.53	0
Mouse					
Liver	0.53	0.46	0.27	0.18	0
Lung	1.49	1.42	0.79	0.50	0
Heart	0.19	0.22	0.17	0.11	0
Kidney	0.57	0.50	0.30	0.20	0
Mouse Brain					
17-18 d gestation	2.95	2.52	2.12	0.34	0
2-3 month	0.21	0.13	0.08	0.06	0
3T3 cells - IF	1.85	1.72	1.61	1.18	0
+100μ/ml IF	3.32	3.52	3.13	2.81	0
L cell - IF	1.41	1.32	0.97	0.40	
+100μ/ml IF	1.55	1.84	1.45	1.11	
Guinea Pig - IF macrophage	1.65	1/74	1.47	0.68	
+100μ/ml IF	3.19	3.31	3.22	1.91	
Human fibroblast	0.64	0.57	0.37	0.07	
Placenta	1.93	1.72	1.49	0.17	

[a]proteins used for each 50 μl assay were as follows:
Mouse extracts : 7-10 μg
Rabbit extracts : 40-50 μg
Tissue cultures
3T3, L, human fibroblasts : 20-30 μg
Guinea pig macrophages : 2-3 μg
Human placenta : 0.3 - 0.4 mg

[a]Tissue extracts were incubated for 30 min. at the indicated temperatures.

preincubation step, a significant amount of (2'-5')A_n may be synthesized from ATP by the endogenous (2'-5')A_n synthetase known to be present in mammalian extracts (1). This does not appear to be the case since the synthetase is known to have an almost absolute requirement for double-stranded RNA for activity (1). Also, chromatography of heat-denatured extracts after preincubation on DEAE-cellulose does not show a detectable amount of (2'-5')A_n in all extracts examined (data not shown). A second

possibility is that there may be differential activation of a 2'-phosphodiesterase activity during the preincubation. Such a possibility was ruled out by incubation of radioactive $ppp(A2'p)_3$ A with extracts (rabbit reticulocyte, brain or macrophage extracts) preincubated at different temperatures and showing that there was no difference in the rate of degradation of $ppp(A2'p)_3A$ during the subsequent incubation on ice. The most likely explanation is that there may be a change in the conformation of the binding proteins or a selective loss of other factors which interact with the binding proteins. Similarly, cultured cell extracts also show a significant loss of binding activity by a 30 min incubation of lysates at 30°C or 37°C. In general, interferon-treated cell lysate binding activities are more resistant to thermal inactivation. For example, in L cell extracts, a 30 min preincubation at 30°C results in a 31% and 7% loss of binding activities in control or interferon-treated cells. Whether this is related to the establishment of the antiviral state in interferon-treated mammalian cells remains to be be determined.

Thermal Stability of $ppp(A2'p)_nA$ Binding Protein in Rabbit Reticulocyte Lysates. Figure 1 shows the effects of preincubating rabbit reticulocyte lysates at different temperatures. At 30°C or 37°C, a 50% increase (20%-120% depending on lysates) in binding is observed. Maximal activation is usually obtained after 30-60 min heat treatment of the lysates. At 45°C, the binding activity is rapidly inactivated with a $t_{1/2}$ of 20-30 min.

Multiple $ppp(A2'p)A_n$ Binding Protein Activities in the Postribosomal Supernatant (PRS) or Ribosomal Salt Wash (RSW) of Rabbit Reticulocyte Lysates. Rabbit reticulocyte lysates are separated into PRS and RSW by ultracentrifugation. When each fraction is passed through a heparin-agarose column, results in Figure 2 are obtained. In the PRS, all of the binding activity is retained on the column and is eluted with 600 mM KCl (PRS_{600}). In contrast, chromatography of RSW on heparin-agarose results in the resolution of three peaks of binding activities, eluting with 80 mM, 250 mM and 600 mM KCl, respectively. The most active fractions are eluted

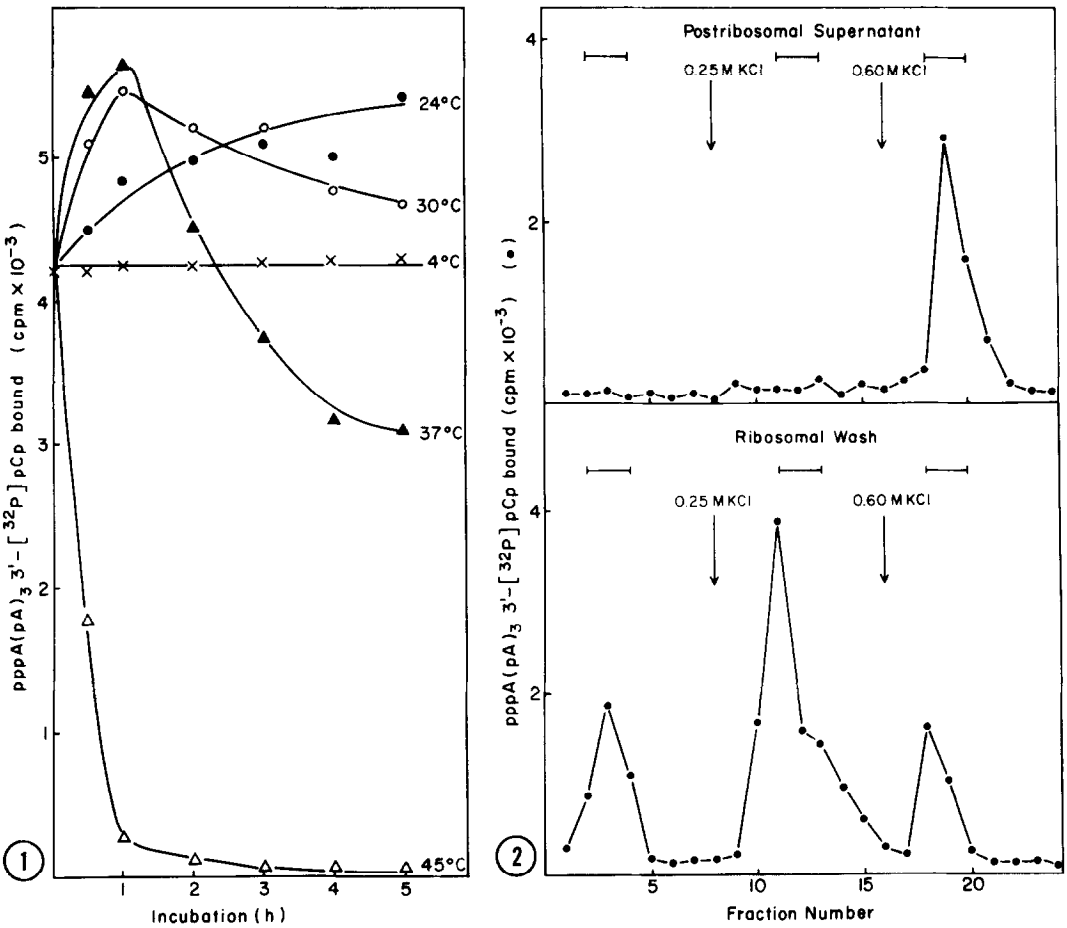

Figure 1. Effects of preincubating rabbit reticulocyte lysates at different temperatures on subsequent binding activity at 4°C. Lysates were incubated, aliquots were removed at specific times and binding determined as described in Materials and Methods. x-x, preincubated at 4°C; ●-●, preincubated at 24°C; o-o, preincubated at 30°C; ▲-▲, preincubated at 37°C; Δ-Δ, preincubated at 45°C.

Figure 2. Chromatography of PRS or RSW on heparin-agarose columns. For experimental details, see Materials and Methods.

with 250 mM KCl (RSW_{250}). PRS_{600} or RSW_{250} are pooled separately, concentrated with ammonium sulfate, and studied with respect to (1) heat inactivation at 42°C, (2) inhibition by aurintricarboxylic acid (ATA), (3) inhibition by cibacron blue (CB), (4) inhibition by heparin, and (5) displacement by $pppA(pA)_3$ or $ppp3'dA(p3'dA)_3$. Aurintricarboxylic Acid has been shown to prevent the binding of mRNA to the 40S subunit (21-23). Since the $(2'-5')A_n$-dependent endonuclease is expected to interact with

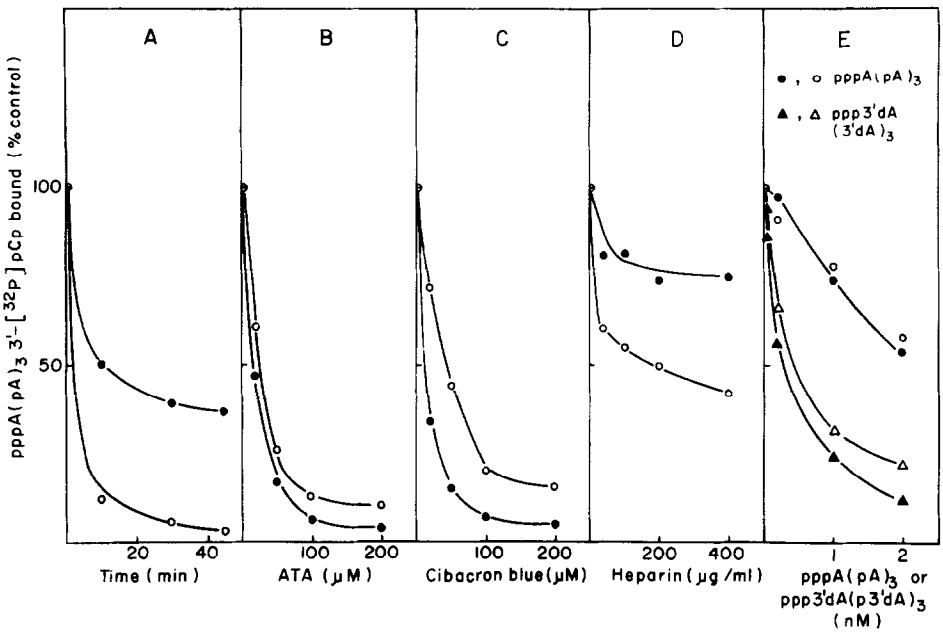

Figure 3. Some properties of PRS_{600} or RSW_{250}. For each 50 μl assay, 3.5 μg of protein was used. The open symbols represent RSW_{250}. The closed symbols represent PRS_{600}. A. Effects of preincubation at 42°C; B. Effects of aurintricarboxylic acid; C. Effects of Cibacron blue; D. Effects of heparin; E. Effects of $pppA(pA)_3$ or $ppp3'dA(p3'dA)_3$.

mRNA, it is of interest to examine the effects of ATA on $ppp(A2'p)_nA$ binding activity. Similarly, cibacron blue or heparin has been shown to affect the activities of a number of proteins which interact with nucleotides (24-28). Results of these studies are shown in Figure 3. PRS_{600} is more resistant to thermal inactivation (Fig. 3A) and is more sensitive to inhibition by ATA or CB (Figs. 3B,C). It is inhibited to a smaller extent by heparin (Fig. 3D) and is displaced to a similar extent as RSW_{250} by $pppA(dA)_3$ or $ppp3'dA(3'dA)_3$.

In conclusion, the thermal stability profiles, the elution pattern on heparin-agarose columns, and the differential inhibition by compounds such as ATA, CB, or heparin, all are consistent with the possibility that multiple $ppp(A2'p)_nA$ binding protein activities exist in mammalian cells.

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