Vol. 97, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 28, 1980 Pages 480-486

STIMULATION OF POLYPEPTIDE SYNTHESIS BY SPERMIDINE AT THE LEVEL OF INITIATION IN RABBIT RETICULOCYTE AND WHEAT GERM CELL-FREE SYSTEMS

KAZUEI IGARASHI, MASAHARU KOJIMA, YASUHIRO WATANABE, KATSUMI MAEDA, and SEIYU HIROSE

Faculty of Pharmaceutical Sciences, Chiba University, Yayoi-cho, Chiba 260, Japan

Received October 8,1980

SUMMARY: It is shown that the stimulation of eukaryotic polypeptide synthesis by spermidine is due to the stimulation at the level of initiation by following reasons. The incorporation of formylmethionine into polypeptides was stimulated by spermidine at the same degree to the incorporation of leucine into polypeptides. Fluorography of the polypeptides formed showed that the number of chains of individual protein synthesized was larger when spermidine was added. The formation of the complex of Met-tRNA$_f$, globin mRNA and 40-S ribosomal subunits was stimulated by spermidine.

It is known that polyamines can enhance protein synthesis in prokaryotic and eukaryotic cell-free systems (1-6). In an Escherichia coli cell-free systems, we have reported that the stimulation of polypeptide synthesis is due mainly to a stimulation of the initiation complex formation of polypeptide synthesis (4,6,7). In the eukaryotic cell-free systems, however, the contradictory mechanisms on polyamine stimulation of polypeptide synthesis have been presented. Konicki et al. (2) have reported that polyamines stimulate polypeptide synthesis at initiation level in the rabbit reticulocyte cell-free system, but Hunter et al. (5) have reported that polyamines stimulate polypeptide synthesis at elongation level in the wheat germ cell-free system.

In this communication, we have studied the effect of spermidine on polypeptide synthesis in both rabbit reticulocyte and

0006-291X/80/220480-07$01.00/0

wheat germ cell-free systems in detail and have found that polyamines stimulate polypeptide synthesis at initiation level.

MATERIALS AND METHODS

Preparation of cell-free extracts - Reticulocyte lysate was prepared from the blood of rabbits which had been made anemic by acetylphenylhydrazine injection according to the method of Hunt and Jackson (8). Nuclease-treated lysate was prepared from the reticulocyte lysate by the method of Pelham and Jackson (9). The incubated 30,000 x g supernatant (IS-30) of wheat germ was prepared by the method of Roberts and Paterson (10).

Preparation of polysomes, ribosomes, mRNA, and aminoacyl-tRNAs - Reticulocyte polysomes were obtained by centrifugation of the reticulocyte lysate at 150,000 x g for 2 h. Yeast polysomes were prepared by the method of Gasior et al. (11). Reticulocyte ribosomes were obtained by centrifugation of the nuclease-treated lysate at 150,000 x g for 2.5 h. Globin mRNA and yeast mRNA were prepared from their respective polysomes according to the method of Krystosek et al. (12) using oligo(dT)-cellulose (Boehringer Mannheim GmbH). Transfer RNAs were prepared from Escherichia coli, rat liver and wheat germ 100,000 x g supernatants by the procedure of Zubay (13). Separation of E. coli $tRNA_f^{Met}$ from $tRNA_m^{Met}$ was performed by DEAE-Sephadex A-50 and subsequent BD-cellulose chromatography according to the method of Seno et al. (14). The preparation of E. coli f[^{3}H]Met-$tRNA_f$ was carried out by the method of Nakamoto and Kolakofsky (15) using 2 μM [^{3}H]methionine (specific activity, 3.3 Ci/mmol). [^{35}S]Met-$tRNA_f$ was prepared by charging deacylated rat liver tRNA with [^{35}S]methionine (specific activity, 100-200 Ci/mmol) using partially purified E. coli aminoacyl-tRNA synthetase as described by Smith and Henshaw (16). When rat liver f[^{35}S]Met $tRNA_f$ was prepared, 0.15 mM folinic acid was added to the reaction mixture.

Assay for polypeptide synthesis - The reaction mixture (0.05 ml), which contained 20 mM Hepes-KOH (pH 7.6), 15 μM hemin, 100 mM potassium acetate, 2 mM dithiothreitol, 1.5 mM ATP, 8 mM creatine phosphate, 7.5 μg of creatine kinase (Boehringer Mannheim GmbH), 20 μg of tRNA, 15 μl of reticulocyte nuclease-treated lysate, 0.05 μCi of [^{14}C]leucine (specific activity, 351 mCi/mmol, Radiochemical Centre Amersham), 30 μM each of 19 other amino acids, 0.5 μg of mRNA, and magnesium acetate and spermidine at the specified concentrations, was incubated at 30° C for 20 min. A 0.04 ml aliquot of each reaction mixture was placed on a paper disc (25 mm diameter) and the hot trichloroacetic acid insoluble radioactivity was assayed with a liquid scintillation spectrometer. When wheat germ IS-30 (1.8 A_{260} units) was used in place of reticulocyte nuclease-treated lysate, the reaction mixture contained 30 mM KCl and 60 mM potassium acetate instead of 15 μM hemin and 100 mM potassium acetate.

SDS-polyacrylamide gel electrophoresis - The reaction mixture (0.05 ml) for the determination of chain length by SDS-polyacrylamide gel electrophoresis was the same to that for the polypeptide synthesis except that 2 μCi of [^{35}S]-methionine (specific activity, 1150 Ci/mmol) was added instead of [^{14}C]leucine. Electrophoresis was carried out according to the method of Laemmli (17). Fluorography was performed by the method of Laskey et al. (18,19).

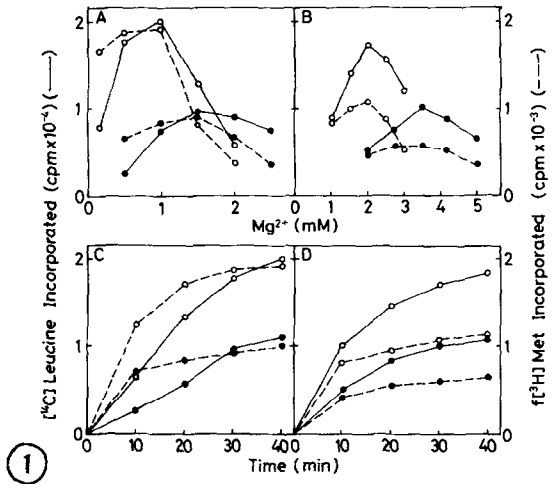
Assay of Met-tRNA$_f$ binding to rabbit reticulocyte ribosomes - The reaction mixture (0.05 ml), which contained 20 mM Hepes-KOH (pH 7.6), 100 mM KCl, 2 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 0.1 mM sparsomycin, rat liver [^{35}S]Met-tRNA$_f$ (25,000 - 30,000 cpm), 0.5 μg of globin mRNA, 1.5 A_{260} units ribosomes, and magnesium acetate and spermidine at the specified concentrations, was incubated at 30° C for 5 min. The amount of [^{35}S]Met-tRNA$_f$ bound to ribosomes was measured by the procedure of Nirenberg and Leder (20).

Sucrose gradient centrifugation analysis of the initiation complex of globin mRNA, [^{35}S]Met-tRNA and ribosomes - After incubation of 0.4 ml of the above mentioned reaction mixture at 30° C for 5 min, a 0.37 ml aliquot was taken and placed on the top of a 15 to 30% sucrose gradient (4.7 ml) in 50 mM Tris-HCl (pH 7.5), 100 mM KCl, and 5 mM magnesium acetate. The tube was centrifuged in a Hitachi RPS-50 rotor for 110 min at 48,000 rpm. After centrifugation, 8 drop fractions were collected from the bottom of the tube. Absorbance at 260 nm of each fraction was measured after a 10-fold dilution with water. The radioactivity was measured using a 0.3 ml aliquot of each fraction.

RESULTS AND DISCUSSION

Effect of spermidine on polypeptide synthesis - The stimulation degree by spermidine of formylmethionine incorporation and leucine incorporation into polypeptide was compared using globin mRNA in rabbit reticulocyte and wheat germ cell-free systems. As shown in Fig. 1, the stimulation degree of formylmethionine incorporation into polypeptide from *E. coli* fMet-tRNA$_f$ was almost equal to that of leucine incorporation, although optimal Mg^{2+} concentration for formylmethionine incorporation was slightly lower than that for leucine incorporation. Similar results were obtained with rat liver fMet-tRNA$_f$, although the incorporation of radioactivity into polypeptide was low.

Effect of antibiotics was examined using reticulocyte lysate (Fig. 2). The stimulation degree by spermidine of polypeptide synthesis decreased gradually as the concentration of aurintricarboxylic acid, an inhibitor of initiation, was increased, but the addition of cycloheximide, an inhibitor of elongation, did not influence significantly the stimulation degree by spermidine. Then, [^{35}S]methionine-labeled products of yeast mRNA directed



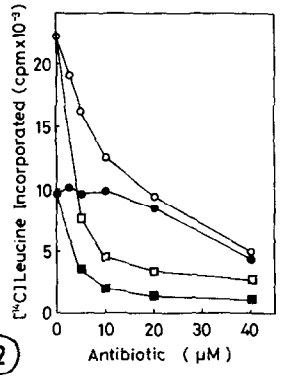
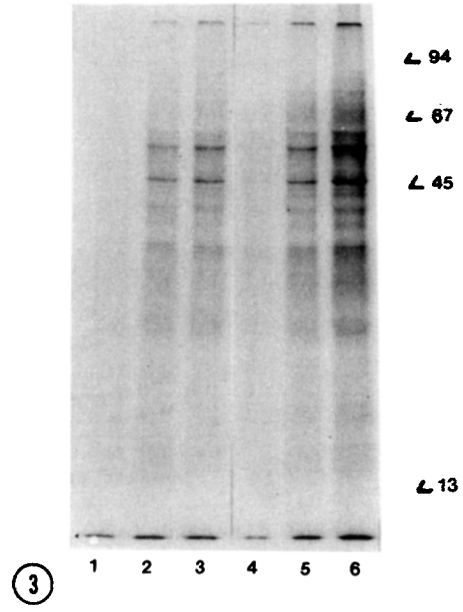


Fig. 1. Effect of spermidine on globin mRNA directed formylmethionine and leucine incorporations into polypeptides in rabbit reticulocyte (A,C) and wheat germ (B,D) cell-free systems. The assay was carried out under standard conditions unless stated below. (----) Radioactivity of f[^{3}H]Met-tRNA$_f$ incorporated (*E. coli* f[^{3}H]Met-tRNA$_f$ (7 μg, 20,000 cpm) and non-labeled leucine (30 μM) were added to the reaction mixture instead of [^{14}C]leucine); (——) radioactivity of [^{14}C]leucine incorporated (*E. coli* non-labeled fMet-tRNA$_f$ (7 μg) was added to the reaction mixture); (A and B, •) no spermidine; (A and B, o) 0.5 mM spermidine. (C, •) 1.5 mM Mg^{2+} and no spermidine; (C, o) 1 mM Mg^{2+} and 0.5 mM spermidine; (D, •) 3.75 mM Mg^{2+} and no spermidine; (D, o) 2 mM Mg^{2+} and 0.5 mM spermidine.

Fig. 2. Effect of aurintricarboxylic acid and cycloheximide on spermidine stimulation of polypeptide synthesis using rabbit reticulocyte lysate. The assay was carried out under standard conditions except that the reaction mixture contained antibiotics specified in the figure and reticulocyte lysate (15 μl) instead of nuclease-treated lysate and mRNA. (•) Aurintricarboxylic acid and 1.5 mM Mg^{2+}; (o) aurintricarboxylic acid, 1 mM Mg^{2+} and 0.5 mM spermidine; (■) cycloheximide and 1.5 mM Mg^{2+}; (□) cycloheximide, 1 mM Mg^{2+} and 0.5 mM spermidine.

polypeptide synthesis in a rabbit reticulocyte cell-free system were analyzed by fluorography. As shown in Fig. 3, the number of chains of individual protein synthesized was larger when spermidine was added, while the extension of the chain length was not significantly influenced by spermidine. Similar results were obtained with wheat germ IS-30. These results strongly suggest that polyamines stimulate polypeptide synthesis at the level of initiation.



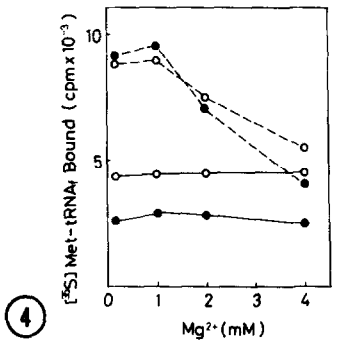


Fig. 3. Sodium dodecylsulfate polyacrylamide gel autoradiogram of [^{35}S]methionine-labeled products of yeast mRNA directed polypeptide synthesis in a rabbit reticulocyte cell-free system. SDS-polyacrylamide slab gel electrophoresis and fluorography were performed as described in Materials and Methods. (1) 1.5 mM Mg^{2+}, 10 min incubation; (2) 1.5 mM Mg^{2+}, 20 min incubation; (3) 1.5 mM Mg^{2+}, 30 min incubation; (4) 1 mM Mg^{2+} plus 0.5 mM spermidine, 10 min incubation; (5) 1 mM Mg^{2+} plus 0.5 mM spermidine, 20 min incubation; (6) 1 mM Mg^{2+} plus 0.5 mM spermidine, 30 min incubation.

Fig. 4. Effect of spermidine on rat liver Met-tRNA$_f$ binding to rabbit reticulocyte ribosomes. The assay was carried out under standard conditions except that the addition of mRNA was changed as specified below. (o——o) No mRNA and no spermidine; (o----o)no mRNA and 1 mM spermidine; (●——●)0.5 µg of globin mRNA and no spermidine; (●----●)0.5 µg of globin mRNA and 1 mM spermidine.

Effect of spermidine on the formation of initiation complex -

As shown in Fig. 4, Met-tRNA$_f$ binding to reticulocyte ribosomes without mRNA was stimulated by spermidine. Met-tRNA$_f$ binding in the absence of spermidine was inhibited by the addition of globin mRNA. The addition of spermidine stimulated greatly Met-tRNA$_f$ binding in the presence of globin mRNA. The similar results were obtained when GTP was replaced by GMP-PCP (data not shown), suggesting that the stimulation of the formation of initiation complex by spermidine may be at the level of the formation of

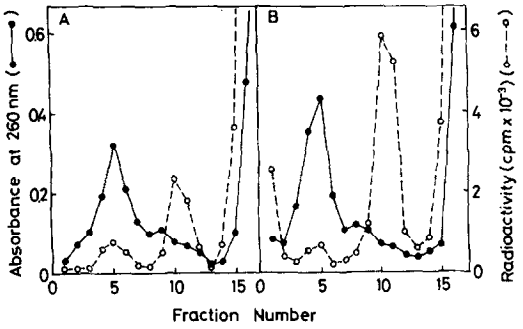


Fig. 5. Sedimentation behavior of the complex of globin mRNA, [^{35}S]Met-tRNAf, and reticulocyte 80S ribosomes. Sucrose gradient centrifugation and measurement of radioactivity were carried out as described in Materials and Methods. (A) 2 mM Mg^{2+}; (B) 1 mM Mg^{2+} and 0.5 mM spermidine.

the complex of globin mRNA, Met-tRNA$_f$ and 40S ribosomal subunits, because GTP hydrolysis occurs at the formation of the complex of globin mRNA, Met-tRNA$_f$ and 80S ribosomes (21). This was confirmed by sucrose gradient centrifugation analysis of the initiation complex. As shown in Fig. 5, stimulation of Met-tRNA$_f$ binding by spermidine occurred on the 40S ribosomal subunit region. These results confirm that polyamines stimulate polypeptide synthesis at the level of initiation.

Discrepancy between the results of Hunter et al.(5) and our results may be due to the difference of mRNA used, since TMV RNA is unieque among eukaryotic mRNAs.

ACKNOWLEDGEMENT

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. Igarashi, K., Sugawara, K., Izumi, I., Nagayama, C. and Hirose, S. (1974) Eur. J. Biochem. 48, 495-502.
2. Konicki, D., Kramer, G., Pinphanichakarn, P. and Hardesty, B. (1975) Arch. Biochem. Biophys. 169, 192-198.

3. Atkins, J. F., Lewis, J. B., Anderson, C. W. and Gesteland, R. F. (1975) J. Biol. Chem. 250, 5688-5695.
4. Igarashi, K., Yabuki, M., Yoshioka, Y., Eguchi, K. and Hirose, S. (1977) Biochem. Biophys. Res. Commun. 75, 163-171.
5. Hunter, A. R., Farrell, P. J., Jackson, R. J. and Hunt, T. (1977) Eur. J. Biochem. 75, 149-157.
6. Igarashi, K., Watanabe, Y., Nakamura, K., Kojima, M., Fujiki, Y. and Hirose, S. (1978) Biochem. Biophys. Res. Commun. 83, 806-813.
7. Igarashi, K., Matsuo, Y., Mitsui, K. and Hirose, S. (1980) Biochem. Biophys. Res. Commun. 93, 360-368.
8. Hunt, T. and Jackson, R. J. (1974) in Modern Trends in Human Leukemia (Neth, R., Gallo, R. C., Spiegelman, S. and Stohlman, F., eds.) pp. 300-307, J. F. Lehmanns Verlag, Munich.
9. Pelham, H. R. B. and Jackson, R. J. (1976) Eur. J. Biochem. 67, 247-256.
10. Roberts, B. E. and Paterson, B. M. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2330-2334.
11. Gasior, E., Herrera, F., Sadnik, I., McLaughlin, C. S. and Moldave, K. (1979) J. Biol. Chem. 254, 3965-3969.
12. Krystosek, A., Cawthon, M. L. and Kabat, D. (1975) J. Biol. Chem. 250, 6077-6084.
13. Zubay, G. (1962) J. Mol. Biol. 4, 347-356.
14. Seno, T., Kobayashi, M. and Nishimura, S. (1968) Biochim. Biophys. Acta, 169, 80-94.
15. Nakamoto, T. and Kolakofsky, D. (1966) Proc. Natl. Acad. Sci. U.S.A. 55, 606-613.
16. Smith, K. E. and Henshaw, E. C. (1975) Biochemistry, 14, 1060-1067.
17. Laemmli, U. K. (1970) Nature, 227, 680-685.
18. Bonner, W. M. and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83-88.
19. Laskey, R. A. and Mills, A. D. (1975) Eur. J. Biochem. 56, 335-341.
20. Nirenberg, M. and Leder, P. (1964) Science, 145, 1399-1407.
21. Benne, R. and Hershey, J. W. B. (1978) J. Biol. Chem. 253, 3078-3087.