Vol. 103, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 16, 1981 Pages 90-96

INDUCTION OF 2,3-BISPHOSPHOGLYCERATE SYNTHASE IN FRIEND LEUKEMIA CELLS

Hiroshi Narita, Shin-ichi Yanagawa, Ryuzo Sasaki, and Hideo Chiba

Department of Food Science and Technology, Faculty of Agriculture,

Kyoto University, Kyoto, 606 Japan

Received September 24, 1981

SUMMARY: Friend leukemia cells (clone 745A) induced to differentiate with dimethylsulfoxide showed at least a 10-fold increase of 2,3-bisphosphoglycerate synthase and concomitant accumulation of 2,3-bisphosphoglycerate. These changes paralelled that of the number of hemoglobin-positive cells. Accumulation of 2,3-bisphosphoglycerate was also induced by dimethylsulfoxide in the other clone C-10-6, but not in C-9-6 which is resistant to differentiation with dimethylsulfoxide. Induced activity of 2,3-bisphosphoglycerate synthase in clone 745A was neutralized by antiserum prepared from a rabbit which was immunized with human erythrocyte 2,3-bisphosphoglycerate synthase. By using this antiserum, biosynthesis of 2,3-bisphosphoglycerate synthase was detected in Friend cells only after induction by dimethylsulfoxide.

INTRODUCTION: Glycerate-2,3-P_2 is present in very high concentrations (4-11 mM) in the erythrocytes of most mammals, collaborating with hemoglobin in supplying oxygen to tissues (1,2). Several other roles for this compound in erythrocytes and reticulocytes have been found (3-5). In continuing studies on the metabolism of this compound, we have shown that induction of glycerate-2,3-P_2 synthase and concomitant accumulation of glycerate-2,3-P_2 are critical events in differentiation of rabbit erythroid cells. Glycerate-2,3-P_2 accumulates during erythroid differentiation in a linear relationship to the increase of hemoglobin (6) and this increase is primarily attributable to an increase in the *de novo* synthesis of glycerate-2,3-P_2 synthase protein (7).

Friend leukemia cells have been established that show a low level of spontaneous differentiation; when cultured with Me_2SO or certain other reagents, they are induced to erythroid differentiation at a much greater

Abbreviations: glycerate-2,3-P_2, 2,3-bisphosphoglycerate; Me_2SO, dimethylsulfoxide.

0006-291X/81/210090-07$01.00/0
Copyright © 1981 by Academic Press, Inc.
All rights of reproduction in any form reserved.

level (8). This differentiation involves accumulation of globin mRNA, heme, α-, β-globin, and enzymic, membrane, and morphological changes typical of normal erythroid differentiation *in vivo* (reviewed in Ref. 9).

To disclose the mechanism by which globin and glycerate-2,3-P_2 synthase are induced synchronously in differentiating erythroid cells, we have decided to manipulate Friend leukemia cells, because it appears difficult to synchronize normal erythroid precursors with respect to critical events in differentiation. In this paper, we show that glycerate-2,3-P_2 accumulation and *de novo* synthesis of glycerate-2,3-P_2 synthase in Friend cells are induced by Me_2SO.

EXPERIMENTAL PROCEDURES: Friend leukemia cells were obtained through the courtesy of Dr. Y. Ikawa (Cancer Institute, Tokyo). Clone 745A is an original cell line established from DBA/2 mouse (10). C-10-6 and C-9-6 are subclones of T-3-Cl-2 derived from DDD mouse (11). C-10-6 is susceptible to the induction by Me_2SO and C-9-6 is resistant. Cells were maintained in Ham's F-12 medium (MBA) supplemented with 10% heat-inactivated calf serum (Research Institute for Microbial Disease, Osaka University, Osaka), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The cultures were incubated at 37°C with 5% CO_2 flow in a humidified incubator. For induction, Me_2SO was added at day-0 to a final concentration of 1.8%. Every day thereafter the cultures were returned to the day-0 density ($5x10^5$ cells/ml) by adding fresh medium containing Me_2SO. A control culture was handled in the same way but without Me_2SO. Benzidine staining of hemoglobin-positive cells was examined by the method of Ikawa (11).

Cells were pelleted by centrifugation and then washed twice with 0.9% NaCl. For determination of glycerate-2,3-P_2, washed cells were sonicated in 5N $HClO_4$, centrifuged at 16,000*g* for 10 min and the supernatant was neutralized with 5N KOH. After removing the precipitated potassium perchlorate by centrifugation, glycerate-2,3-P_2 was determined as described previously (12). For determination of glycerate-2,3-P_2 synthase activity, washed cells were sonicated in 10 mM Tris-HCl buffer pH 7.5 containing 0.1 mM EDTA and 0.1 mM dithiothreitol. After centrifugation at 27,000*g* for 90 min, the supernatant was dialysed against the same buffer and the activity was determined by the method reported previously (13). Erythrocytes of DBA/2 mouse were treated in the same way.

Antiserum against human erythrocyte glycerate-2,3-P_2 synthase and control serum were made as in the previous paper (14). Neutralization and immunoprecipitation of glycerate-2,3-P_2 synthase were performed as described previously (7).

Noninduced and induced Friend cells (clone 745A) were labelled with ^3H-leucine (NEN). Induced cells ($7x10^8$ cells) grown in 1.8% Me_2SO for 7 days were washed once with Eagle's minimum essential medium minus leucine (Nissui) and then incubated in 70 ml of the same medium supplemented with 5 μCi/ml of ^3H-leucine and 10% heat-inactivated calf-serum, which was predialysed against phosphate-buffered saline. Noninduced cells were labelled in the same manner as above at the same population density as the induced cells. The labelled cells were washed twice with phosphate-buffered saline and lysed in ice-cold water by sonication. After centrifugation at 27,000*g* for 90 min, the resulting supernatant was subjected to immunoprecipitation with anti-glycerate-2,3-P_2 synthase antiserum or control serum. Protein A-bearing *Staphylococcus aureus* was used as an immunoadsorbent (15). About 10^7 cpm were incorporated into trichloroacetic acid-precipitable proteins in a lysate of 10^8 cells of both noninduced and induced cells and $4.5x10^6$ cpm each were subjected to immunoprecipitation. Electrophoretic analysis was performed on sodium dodecyl sulfate-polyacryl-

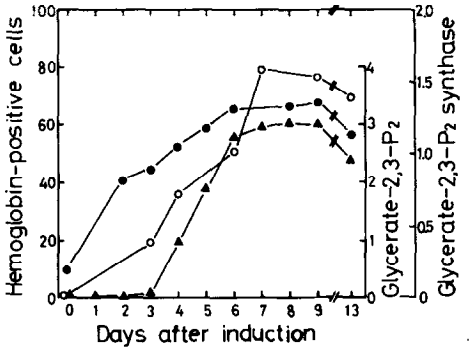

Figure 1. Kinetics of glycerate-2,3-P_2 and glycerate-2,3-P_2 synthase activity in Friend cells during exposure to 1.8% Me_2SO. (●) Glycerate-2,3-P_2 concentration (nmol/10^8 cells); (o) glycerate-2,3-P_2 synthase activity (unit/10^8 cells); (▲) hemoglobin-positive cells (%). Culture conditions and assay methods were as described in "EXPERIMENTAL PROCEDURES". For each assay, 10^8 745A cells at various days were used.

amide gels according to Laemmli (16). Gels were stained, sliced, and the radioactivity in the sliced gels was counted as in the previous paper (7).

RESULTS AND DISCUSSION: Fig.1 shows the time-course of Me_2SO-induced differentiation of clone 745A Friend cells and changes in glycerate-2,3-P_2 concentration and glycerate-2,3-P_2 synthase activity in these cells. Induced hemoglobin-positive cells increased after a 2-3 day lag and reached maximum at days 7 to 9, after which they decreased gradually. The decrease may be due to the lysis of fully induced cells in this system. Glycerate-2,3-P_2 is present at very low but certainly detectable level (0.5 nmol/10^8 cells) in noninduced cells. The level of this compound was increased by 7-fold at day-9 after incubation with Me_2SO. Glycerate-2,3-P_2 synthase activity was almost undetectable in noninduced cells but at least a 10-fold increase occurred during 6-9 days culture in the presence of Me_2SO. Levels of glycerate-2,3-P_2 and its synthase activity decreased after prolonged culture. When the cells were cultured in the absence of Me_2SO, no changes were observed (figure not shown). The time-course of induction of glycerate-2,3-P_2 synthase activity resembles that of hemoglobin, suggesting a coordinated regulatory mechanism in the synthesis of the two proteins.

Table I shows that the glycerate-2,3-P_2 level in clone C-10-6, which is a Me_2SO-inducible cell line, increases after induction by Me_2SO, although

Table 1. Glycerate-2,3-P_2 levels in Friend cell clones before and after induction by Me_2SO.
Erythrocytes used were prepared from DBA/2 mouse. Friend cells were cultured in the presence of Me_2SO for the indicated number of days.

Cells	Days	Glycerate-2,3-P_2 (nmol/10^8cells)	Glycerate-2,3-P_2 synthase activity (unit/10^8 cells)	Hemoglobin-positive cells (%)
Erythrocyte	—	32	1.5×10^{-2}	100
745A	0	0.5	nd	0.02
	9	3.4	1.5×10^{-4}	61
C-10-6	0	2.3	—	0.05
	3	3.6	—	0.5
	6	3.9	—	35
	9	3.6	—	48
C-9-6	0	1.7	—	0.02
	3	1.4	—	0.05
	6	1.3	—	0.06
	9	1.3	—	0.26

nd: not detectable

the increase is much less than that found for clone 745A. There is no increase in C-9-6, which is resistant to Me_2SO-induced differentiation. A result similar to that of clone C-10-6 with respect to glycerate-2,3-P_2 has been reported by Yoeh with clone 707T (17).

In order to clarify how glycerate-2,3-P_2 synthase activity increases in Friend cells after induction, we need a tool to identify this enzyme. We found that anti-human erythrocyte glycerate-2,3-P_2 synthase antiserum neutralized glycerate-2,3-P_2 synthase activity in 745A Friend cells after induction by Me_2SO (figure not shown). The synthase activity in the hemolysate prepared from DBA/2 mouse erythrocytes was neutralized as well.

It is noted that glycerate-2,3-P_2 phosphatase activity in Me_2SO-induced Friend cells and erythrocytes of DBA/2 mouse was neutralized with this antiserum just as the synthase activity was (figure not shown). Thus in these cells the two enzyme activities related directly to glycerate-2,3-P_2 metabolism are manifested by a single enzyme, glycerate-2,3-P_2 synthase-phosphatase, which has been shown to occur in erythrocytes of human (13,14) and others (18-21).

After hemolysate of erythrocytes from DBA/2 mouse was treated with anti-human erythrocyte glycerate-2,3-P_2 synthase antiserum, the resulting immuno-

precipitates were analysed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig.2A). Comparing this electrophoretic pattern with that obtained for the antiserum alone (figure not shown), a protein band with molecular weight of 27,000 was found to be attributable to glycerate-2,3-P_2 synthase in mouse erythrocytes. So far all glycerate-2,3-P_2 synthases from animal sources have been reported to be composed of two subunits with identical molecular weight of 27,000-30,000 (4). Most of other bands in Fig.2A are from immunoglobulins.

By using anti-human erythrocyte glycerate-2,3-P_2 synthase antiserum and glycerate-2,3-P_2 synthase immunoprecipitated from mouse erythrocytes, biosynthesis of glycerate-2,3-P_2 synthase in Friend cells was explored before and after induction by Me_2SO. The immunoprecipitates prepared from lysates of Friend cells which were labelled with ^3H-leucine before and after induction were analysed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig.2B and C). Before induction, no distinct radioactive peak was detectable by immunoprecipitation with either the specific antiserum or the control serum (Fig.2B). On the other hand, two peaks were detected when the lysate of Me_2SO-induced day 7 Friend cells was treated with anti-glycerate-2,3-P_2 synthase antiserum (Fig.2C). One peak with slower mobility seems to be a nonspecific precipitate, since this peak was detected when the lysate was treated with the control serum. The other peak has the same mobility as an authentic glycerate-2,3-P_2 synthase from mouse erythrocytes. Thus it is clear that the increase of glycerate-2,3-P_2 synthase activity seen in Fig.1 is due to *de novo* synthesis of the enzyme induced by Me_2SO. It is deduced from these results with Friend cells that the primary cause of high levels of glycerate-2,3-P_2 in the erythrocytes is the biginning or enhancing of synthesis of glycerate-2,3-P_2 synthase in erythroid precursor cells by the action of inducer. The most likely inducer *in vivo* is erythropoietin.

Friend 745A clone appears to be useful in exploring the mechanism by which the synchronous induction of glycerate-2,3-P_2 synthase and hemoglobin is

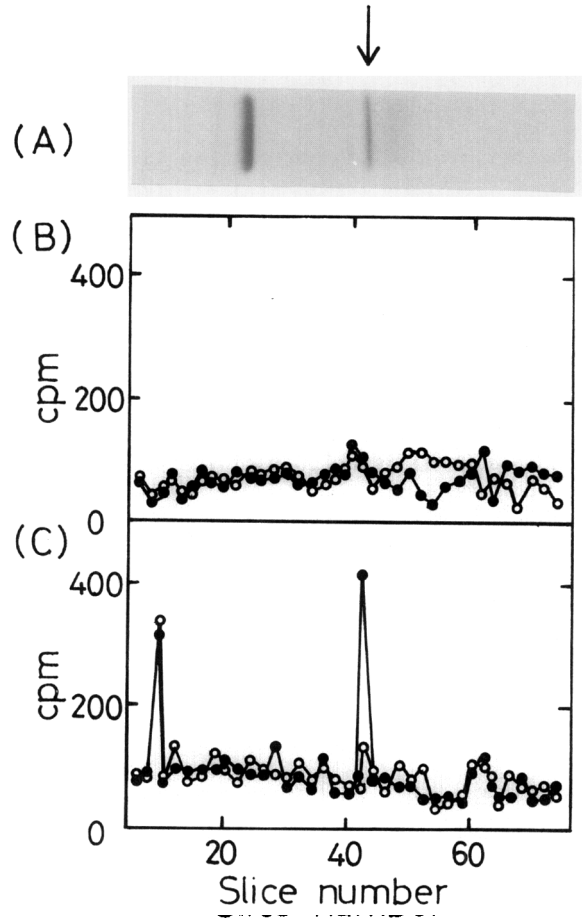

Figure 2. Biosynthesis of glycerate-2,3-P_2 synthase in Friend cells. Lysates prepared from 1.5×10^8 erythrocytes of DBA/2 mouse (A) and from 4.5×10^7 ^3H-labelled 745A cells before (B) and after (C) induction by Me_2SO were treated with anti-glycerate-2,3-P_2 synthase antiserum (●) or control serum (o). The resulting immunoprecipitates were analysed by electrophoresis on a 13.5% gel. Migration was from left to right. Proteins in (A) were stained with Coomassie blue. The patterns in (B) and (C) were depicted by measuring radioactivity of the sliced gels. The arrow indicates a protein of molecular weight of 27,000, the mouse erythrocyte glycerate-2,3-P_2 synthase subunit.

regulated at the transcriptional level. In this context, isolation of mRNA of this enzyme is worthwhile.

ACKNOWLEDGEMENTS: We wish to express our gratitude to Dr. Y. Ikawa (Cancer Institute, Tokyo) for his donation of Friend cells and to Dr. A. Ishimoto (Institute for Virus Research, Kyoto University, Kyoto) for his kind teaching of cell culture technique and stimulating discussions.

REFERENCES

1. Benesch,R.,and Benesch,R.E.(1967) Biochem.Biophys.Res.Commun. 26,162-167.
2. Chanutin,A.,and Churnish,R.R.(1967) Arch.Biochem.Biophys.121,96-102
3. Sasaki,R.,Ikura,K.,Katsura,S.,and Chiba,H.(1976) Agric.Biol.Chem. 40, 1797-1803.
4. Chiba,H.,and Sasaki,R.(1978) in Current Topics in Cellular Regulation (Horecker, B.L.,and Stadtman,E.R.eds) Vol.14,pp.75-116,Academic Press, New York.
5. Narita,H.,Ikura,K.,Sasaki,R.,and Chiba,H.(1979) Biochem.Biophys.Res.Commun. 86,755-761.
6. Narita,H.,Ikura,K.,Yanagawa,S.,Sasaki,R.,Chiba,H.,Saimyoji,H.,and Kumagai, N.(1980) J.Biol.Chem. 255,5230-5235.
7. Narita,H.,Yanagawa,S.,Sasaki,R.,and Chiba,H.(1981) J.Biol.Chem. 256, 7059-7063.
8. Friend,C.,Scher,W.,Holland,J.G.,and Sato,T. (1971) Proc.Natl.Acad.Sci. U.S.A. 68,378-382.
9. Marks,P.A.,and Rifkind,R.A.(1978) in Ann.Rev.Biochem. (Snell,E.E.,Boyer, P.D.,Meister,A.,and Richardson,C.C.eds) Vol.47,pp.419-448,Annual Reviews Inc.Palo Alto, Calfornia.
10. Scher,W.,Holland,J.G.,and Friend,C.(1971) Blood,37,428-437.
11. Ikawa,Y.,Aida,M.,and Inoue,Y. (1976) Gann, 67,767-770.
12. Sasaki,R.,Ikura,K.,Sugimoto,E.,and Chiba,H.(1974) Anal.Biochem. 61,43-47.
13. Sasaki,R.,Ikura,K.,Sugimoto,E.,and Chiba,H.(1975) Eur.J.Biochem. 50, 581-593.
14. Ikura,K.,Narita,H.,Sasaki,R.,and Chiba,H.(1978) Eur.J.Biochem. 89,23-31.
15. Kessler,S.W.(1975) J.Immunol. 115,1617-1624.
16. Laemmli,U.K.(1970) Nature, 227,680-685.
17. Yeoh,G.C.T.(1980) Nature, 285,108-109
18. Sasaki,R.,Ikura,K.,Narita,H.,and Chiba,H.(1976) Agric.Biol.Chem. 40, 2213-2221.
19. Rose,Z.B.,and Dube,S.(1976) Arch.Biochem.Biophys. 117,284-292.
20. Harkness,D.R.,Issacks,R.E.,and Roth,S.E.(1977) Eur.J.Biochem. 78,343-351.
21. Narita,H.,Utsumi,S.,Ikura,K.,Sasaki,R.,and Chiba,H.(1979) Int.J.Biochem. 10,25-38.