Induction of a Novel Nuclear Protein (p54) by Phorbol Esters in Mouse Erythroleukemia (Friend) Cells

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ABSTRACT

Tumor-promoting phorbol esters including 12-O-tetradecanoylphorbol-13-acetate (TPA), specific inhibitors for erythroid differentiation in mouse Friend cells, induce a newly identified nuclear protein with a molecular weight of 54,000 (p54) in Friend cells. Phorbol, an analogue of TPA with neither tumor-promoting nor differentiation-inhibitory activity, did not induce p54. In a variant cell line of Friend cells which exhibits resistance to TPA in erythroid differentiation, p54 was not induced by TPA. The induction of p54 by TPA was counteracted by erythroid-inducing agents including dimethyl sulfoxide, hexamethylenesiamide, and actinomycin D. Throughout these experiments, we have observed an inverse relationship between p54 induction and cellular potential for erythroid differentiation.

INTRODUCTION

Friend cells (mouse erythroleukemia cells) (1) have been studied extensively as a model for cellular differentiation. They exhibit terminal differentiation into erythroid cells in vitro when exposed to differentiation-inducing agents such as DMSO (2), butyric acid (3, 4), HMBA (5), or actinomycin D (6). Within several days after treatment with these agents, the cells begin to express various cellular functions characteristic of normal erythropoesis. These include hemoglobin and heme synthesis (2, 7–9), appearance of erythrocyte membrane antigens (10), and cessation of cell division (2). The in vitro differentiation in Friend cells is inhibited by phorbol esters with tumor-promoting activity such as TPA (11, 12). Numerous experiments with this in vitro differentiation system have provided a great deal of insight into our understanding of the mechanism of cellular differentiation, especially that of the terminal differentiation of hematopoietic cells.

Recently, we analyzed the erythroid induction processes in Friend cells by cell fusion experiments (13). These experiments have revealed that commitment to erythroid differentiation is a result of 2 fundamentally different cellular reactions, one originating from inhibition or disturbance of DNA replication and the other involving a transmembrane reaction triggered by most of the inducing agents such as DMSO or HMBA. In subsequent experiments, we characterized the latter membrane-mediated reaction in more detail. It has become quite evident that at a very early stage of the membrane-mediated reaction there is a site or reaction at which biologically active phorbol esters competitively with DMSO or HMBA to affect induction of erythroid differentiation (14).

Based upon these and other results, several models for the mechanism of erythroid differentiation have been proposed (13, 15–17). However, the mechanism at a molecular level by which erythroid-inducing agents affect Friend cells and eventually lead to the induction of erythroid differentiation still remains to be elucidated. Probably one of the most attractive working hypotheses for the mechanism of erythroid differentiation at the molecular level would be to postulate a regulatory protein which itself or in concert with others controls erythroid differentiation. Inducing agents and inhibitors would affect synthesis of the protein or activity associated with it. In this respect, Keppel et al. (18) reported that a histone protein H1° (IP25) appears at the early stage of the erythroid differentiation. This suggests that the induction of IP25 is closely associated with the differentiation, although subsequent experiments showed that the appearance of IP25 is still observed under a condition in which the erythroid differentiation is blocked (15).

We report here that a new nuclear protein (p54) is induced following treatment of Friend cells with biologically active phorbol esters. In a variant cell line with an altered sensitivity (less sensitivity) to phorbol esters in erythroid differentiation, p54 was not induced by TPA. Furthermore, the induced synthesis of p54 is inhibited by erythroid-inducing agents. Throughout these experiments, an inverse relationship between erythroid differentiation and synthesis of p54 was observed.

MATERIALS AND METHODS

Materials. TPA was obtained from Dr. P. Borchert (Eden Prairie, MN). Phorbol was a gift from Dr. M. Terada and Dr. T. Sugimura. PDD, 2-acetamido-5-fluorouracil, benzene (pyrene), and mitomycin C were purchased from Sigma Chemical Co. (St. Louis, MO). HMBA was a generous gift from Dr. T. Yamane. Actinomycin D was obtained from Merck & Co. Eagle's MEM (in powder) was purchased from Nissui Seiyaku (Tokyo, Japan). FCS was obtained from Flow Laboratories, and [3H]methionine was purchased from CEA (France). Antibodies against p53 and actin were kindly provided by Dr. N. Yamaguchi and Dr. I. Yahara, respectively. All the reagents used were reagent grade.

Cells. A Friend (murine erythroleukemia) cell line (DS19) was obtained from Dr. M. Terada. The cell line was derived from cell line 745 (2). A TPA-resistant variant (IAM102) was selected among IAM102 which was kindly provided by Dr. R. A. Rifkind.

Labeling of Proteins. Friend cells were cultured in MEM (supplemented with heat-inactivated 12% FCS) at 37°C in a humidified atmosphere containing 5% CO2 in air. The cells at late exponential phase (1 to 1.5 × 10⁶ cells/mL) were diluted into a fresh medium to a cell density of 5 × 10⁵ cells/mL. After mitomycin C treatment (0.2 mg/mL), the culture medium was replaced with fresh FCS-free medium containing 1 μg/mL PDD and 200 μg/mL phorbol 12,13-diacetate. After 24 h, the cells were collected by centrifugation (450 x g, 5 min), washed twice with (2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4): 140 mM NaCl; 5 mM KCl; 2 mM MgCl2; 2 mM CaCl2;...
buffer, and resuspended in methionine-free MEM (12% FCS) at a cell density of 1 x 10^6 cells/ml. To this, [35S]methionine (100 to 200 μCi/ml; specific activity, >600 Ci/mmol) was then added and incubated for 4 h unless otherwise stated. After the labeling, the cells were washed twice with phosphate-buffered saline (137 mM NaCl:4.2 mM KCl:8.6 mM Na2HPO4:1.1 mM KH2PO4). There were no appreciable differences in the total radioactivities incorporated into control and TPA-treated cells.

Isolation of Nuclei, Membranes, and Cytoplasm Fraction. For isolation of nuclei, the labeled cells were resuspended in approximately 5-fold volumes of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4):1 mM MgCl2:1 mM CaCl2:10 mM NaCl buffer and, after 10 min, homogenized by a Dounce homogenizer (20 strokes). The cell homogenate was then centrifuged (800 x g, 5 min). Nuclei were obtained from the sediment by 2 more successive washings (with the above buffer) and centrifugation (800 x g, 5 min). Membrane and cytoplasm fractions were prepared from the supernatant fraction. After addition of 1 M sucrose (final concentration, 0.25 M), the supernatant fraction was centrifuged (100,000 x g, 120 min). The resultant supernatant fraction and precipitates were used as cytoplasm and membrane fractions, respectively. All manipulations were carried out at 0-4°C unless otherwise specified.

Immunoprecipitation. Nuclei isolated from 2 x 10^6 cells, which had been pulse-labeled (4 h) with [35S]methionine after 24 h incubation with TPA (100 ng/ml), were mixed with a buffer (0.3 ml) containing 50 mM Tris-HCl (pH 8.0), 0.5% NP40, 120 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride. After standing for 5 min at 0°C, the lysate was centrifuged (11,000 x g, 5 min). To the supernatant, antisera against actin (5 μl) and p53 (5 μl) (19) were added, and the sample was left overnight. At 30 min after addition of 40 μl of purified Pansorbin (10% Staphylococcus aureus cells), the precipitate was centrifuged (5000 x g, 5 min) and washed twice with Buffer A [50 mM Tris-HCl (pH 7.5):150 mM NaCl:1% NP40] and once with Buffer B [50 mM Tris-HCl (pH 7.5):150 mM NaCl]. The precipitate was then subjected to 2-dimensional gel electrophoresis. All the manipulations were carried out at 0-4°C.

Two-Dimensional Gel Electrophoresis and Radioautography. Two-dimensional gel electrophoresis was carried out according to the method of O'Farrell et al. (20). Before electrophoresis, proteins in each sample (labeled nuclei, membrane, cytoplasm) were precipitated by TCA (10%) and washed once with TCA (5%) and twice with acetone. The precipitates were then dissolved in sample lysis buffer [2% Pharmalyte (pH 3 to 10):5% 2-mercaptoethanol:9.2 M urea:4% NP40] and subjected to electrophoresis (20). After electrophoresis, the gels were fixed with 12% TCA, treated with Enhance (NEN), dried, and exposed to Kodak XRP-1 films generally for 5 days at -80°C.

Assay of Erythroid Differentiation. Hemoglobin synthesis was assayed according to the method of Orkin et al. (7) by benzidine staining. In short, a cell suspension (200 μl) was mixed with 20 μl of freshly prepared benzidine solution (10:1 mixture of 0.2% 3,3′-dimethoxybenzidine in 0.50 M acetic acid and 30% hydrogen peroxide), and stained cells were scored under a microscope.

RESULTS

Induction of a Nuclear Protein (p54) by TPA. When Friend cells (DS19) were pulse-labeled with [35S]methionine (4 h) after incubation with a phorbol ester (TPA) for 24 h and patterns of nuclear proteins synthesized during the pulse were examined by 2-dimensional electrophoresis, we detected a spot which was not present or which was present at a much lower level in extracts from TPA-treated cells and those from control (without TPA treatment) (A). TPA-treated cells (B), membrane (m) proteins from TPA-treated cells (C) and cytoplasmic (c) proteins from TPA-treated cells (D). Arrowhead, TPA-induced protein (p54). Molecular weight markers (Amersham) were used as the reference to determine the molecular size of p54. For details, see “Materials and Methods.” MW, molecular weight; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NEPHGE, nonequilibrated pH gradient gel electrophoresis.

Fig. 1. Two-dimensional gel electrophoresis of labeled protein in subcellular fractions following TPA treatment. Mouse Friend cells (DS19) were incubated in the presence of TPA (100 ng/ml) for 24 h and pulse-labeled with [35S]methionine (100 μCi/ml) for 4 h. The cells (total, 1.0 x 10^7 cpm) were fractionated into subcellular fractions, and total proteins in each subcellular fraction were subjected to 2-dimensional gel electrophoresis (20). Nuclear (n) proteins were from control cells (without TPA treatment) (A), TPA-treated cells (B), membrane (m) proteins from TPA-treated cells (C) and cytoplasmic (c) proteins from TPA-treated cells (D). Arrowhead, TPA-induced protein (p54). Molecular weight markers (Amersham) were used as the reference to determine the molecular size of p54. For details, see “Materials and Methods.” MW, molecular weight; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NEPHGE, nonequilibrated pH gradient gel electrophoresis.
cells. The molecular weight of the protein was tentatively determined to be 54,000 based on its mobility in sodium dodecyl sulfate:gel electrophoresis, and its estimated $p_I$ was between 7.8 and 8.0. The protein (p54) was exclusively present in the nuclei, since we could not detect the p54 spot in membrane or cytoplasmic fractions from TPA-treated cells (Fig. 1, C and D). On the other hand, we detected p54 in unfractionated whole-cell extracts from TPA-treated Friend cells but not in the extracts from control cells (Fig. 2), indicating that the presence of the labeled p54 in the nuclei is not a result of translocation of the protein to the nuclei from the other fractions by TPA. The incorporation of $[35S]$methionine into p54 stimulated by TPA apparently accompanies a net increase in the protein, for an increase in the protein concentration at the p54 spot by TPA was also detected by the silver staining of the samples instead of fluorography (data not shown).

In Fig. 3, we show the induction of p54 as a function of TPA concentration in the medium. As seen from the figure, p54 was induced at a concentration of TPA as low as 10 ng/ml, and the induction seemed to reach a plateau at 20 to 50 ng/ml. These concentrations (10 to 50 ng/ml) are equivalent to the concentrations which exhibit substantial (more than 50%) inhibition for erythroid differentiation in Friend cells.

Although there are several reports that biologically active phorbol esters induce specific proteins (21–26), this is the first case demonstrating that a phorbol ester induces a specific nuclear protein. It should be noted, however, that the apparent induction of p54 could be a result of the modification of other $[35S]$methionine-labeled proteins like the other TPA-inducible proteins reported previously. This point will be discussed later.

The induction of p54 was also observed following treatment with PDD, another phorbol ester with a differentiation-inhibitory activity and tumor-promoting activity (data not shown). On the other hand, phorbol, which has a molecular structure similar to that of TPA but neither tumor-promoting nor differentiation-inhibitory activity, did not show any inducing activity of p54 at a concentration of as high as 500 ng/ml (data not shown). Other agents including tumor initiators [2-acetamidofluorene (5 µg/ml) and benzo(a)pyrene (5 µg/ml)] and a DNA-damaging agent [mitomycin C (0.1 µg/ml)] were tested, but they did not induce p54 (data not shown).

The induction of p54 by TPA as a function of time of incubation was studied by pulsing (4 h) the cells with $[35S]$methionine at a

![Fig. 2. Two-dimensional gel electrophoresis of total labeled proteins. Mouse Friend cells (DS19) were incubated in the presence of TPA (100 ng/ml) for 24 h and pulse-labeled with $[35S]$methionine (100 µCi/ml, 4 h). The cells were lysed, and total proteins (approximately $1 \times 10^6$ cpm) were subjected to 2-dimensional gel electrophoresis (20). Total proteins from control cells (without TPA) (A) and TPA-treated cells (B). Arrowhead, TPA-induced protein (p54). For details, see "Materials and Methods."](image-url)
PHORBOL ESTER-INDUCIBLE PROTEIN

different time after the addition of TPA (100 ng/ml). As seen in Fig. 4, an appreciable amount of incorporation of [35S]methionine into p54 was first detected at 8 h, reaching a maximum at 24 h after addition of TPA. The rate of incorporation then decreased, and only a trace of incorporation was detected after 48 h of incubation with TPA.

The half-life of TPA-induced protein (p54) was estimated from pulse and chase experiments. From the rate of the decrease of radioactivities in the p54 spot measured at different chasing periods with cold methionine, we estimated the half-life of p54 to be roughly 8 to 10 h. Typical patterns of labeled proteins in control (0-h chase) and chased (8-h chase) samples are shown in Fig. 5.

p54 induction in a TPA-resistant Variant Friend Cell Line.

We further examined p54 induction by TPA in a variant cell line of Friend cells with an altered sensitivity to TPA. For this purpose, we isolated Friend cells (IAM102) which expressed substantial resistance to TPA in its induction of erythroid differentiation by DMSO as shown in Chart 1. In contrast to the parental strain, p54 was not induced by TPA in IAM102 (Fig. 6), confirming that the induction of p54 is a result of a specific cellular response to biologically active phorbol esters.

Effect of Erythroid-inducing Agents on p54 Induction by TPA.

The possible role of p54 in erythroid differentiation was further investigated by examining whether the induction of p54 by TPA was counteracted by typical erythroid-inducing agents such as DMSO, HMBA, or actinomycin D. For this, the cells were incubated with TPA (100 ng/ml) together with either DMSO (1.8%, v/v), HMBA (4 mw), or actinomycin D (10 ng/ml), and after 24 h they were pulse-labeled with [35S]methionine (for 4 h).

Under these conditions, erythroid differentiation, which is normally observed at least among 80% of the cells when treated by the inducing agent alone, was observed among only less than 10% of the treated cells because of the presence of TPA. When nuclear proteins were analyzed by electrophoresis, it was found that all the inducing agents tested almost completely blocked the induction of p54 by TPA (Fig. 7). A similar inhibitory effect of DMSO on p54 synthesis was observed when total cellular proteins (rather than nuclear proteins) were examined (data not shown). The inhibitory effect of inducing agents on p54 induction was not a result of direct inhibitory effect of these agents on p54 synthesis. We have found that the presence of DMSO only during the pulsing period (4 h) with [35S]methionine did not exhibit any effect and that DMSO must be present from the beginning of the incubation with TPA to express its effect (data not shown).

These results indicate that there is an inverse relationship between the actions of the inducing agents and the inhibitors in inducing p54 as observed in their effects on erythroid differentiation. On the other hand, it is quite clear that the induction of p54 is not necessary for TPA to block induced erythroid differentiation.

CANCER RESEARCH VOL. 45 AUGUST 1985

3839
PHORBOL ESTER-INDUCIBLE PROTEIN

DISCUSSION

At present, we do not know by what molecular mechanism biologically active phorbol esters induce p54 and how the erythroid-inducing agents counteract the induction. It was proposed that pleiotropic effects of phorbol esters on cultured mammalian cells are mediated by cascades of reactions which originate from a single reaction, activation of protein kinase C by phorbol ester (27). Therefore, it is possible that the apparent induction of p54 involves modification (e.g., phosphorylation) of a protein by a protein modification enzyme(s). Although the absence of major protein spots with higher pI than that of p54 (pI 7.8 to 8.0) with control samples in the 2-dimensional gel seems to exclude the possibility of at least protein phosphorylation as a cause of the p54 induction, from our experimental results alone one cannot determine by what mechanism p54 is apparently induced in response to biologically active phorbol esters. The use of antibodies against p54 will give a definite answer to this question.

There are several reports in which phorbol esters induce specific proteins in cultured mammalian cells (21-26). However, it seems quite unlikely that p54 is identical with any of the phorbol ester-induced proteins reported previously. In addition to the unique intracellular location (nuclei), p54 did not correspond to any of the other TPA-inducible proteins with respect to molecular weight.Recently, a considerable interest has been focused on p53 (28, 29). Although p53 is not reported as a TPA-inducible protein, the proximity of its molecular weight to p54 and a possible role of p53 in erythroid differentiation suggested by Shen et al. (30) prompted us to examine the structural similarity between p53 and p54. The extracts labeled with [35S]methionine from TPA-treated cells were immunoprecipitated with antibodies against p53 (19) and actin (as a reference), and the precipitates were then analyzed by gel electrophoresis. We have found that p53 migrated at a different position from where p54 was supposed to migrate (data not shown), thus indicating that p54 is structurally different from p53.

At the present moment, the biological significance of p54 induction by TPA and its inhibition by erythroid-inducing agents is not clear. However, the converse relationship between erythroid differentiation and p54 synthesis, especially the inhibition of p54 induction by erythroid-inducing agents, may suggest the possibility that p54 is directly or indirectly involved in the commitment of erythroid differentiation. For example, one could speculate that intracellular p54 concentration is maintained by constitutive synthesis of p54, sufficient to keep the cells from committing to erythroid differentiation. The reduction of the intracellular concentration of p54 below a threshold level by erythroid-inducing agents would then derepress expression of genes which are necessary, although possibly not sufficient, for erythroid differentiation. Biologically active phorbol esters counteract the lowering of p54 concentration by their inducing activity of p54. Such a mechanism is compatible with the stochastic model proposed by Gusella et al. (18) for erythroid differentiation. In this respect, it will be interesting to examine the effect of erythroid-inducing agents on the constitutive level of p54 in the cells by using more sensitive and quantitative assay procedure.

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CANCER RESEARCH VOL. 45 AUGUST 1985
3840
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**Fig. 4.** Induction of p54 as a function of time of incubation with TPA. Mouse Friend (DS19) cells were incubated for different time periods with TPA (100 ng/ml) and pulse-labeled with [35S]methionine (100 μCi/ml) for 4 h. Nuclei were isolated, and nuclear proteins (approximately 1 x 10^8 cpm) were subjected to 2-dimensional gel electrophoresis (20). Nuclear proteins from 0 h (A), 8 h (B), 16 h (C), 24 h (D), 32 h (E), 48 h (F), and 72 h (G) incubation with TPA. Arrowhead, p54. For details, see "Materials and Methods."

**Fig. 5.** Turnover of pulse-labeled p54. Mouse Friend cells (DS19) were incubated in the presence of TPA (100 ng/ml) for 24 h, and the cells were pulse-labeled with [35S]methionine (100 μCi/ml) for 4 h. The cells were washed twice with phosphate-buffered saline by centrifugation (450 x g, 4 min) and incubated in MEM (1 x 10^6 cells/ml) supplemented with 12% FCS and 1 mm methionine for an additional 8 h. Total nuclear proteins (approximately 1 x 10^6 cpm) were then subjected to 2-dimensional gel electrophoresis (20). Nuclear proteins were from control cells (0 h chase) (A) and chased cells (8 h) (B). Arrowhead, p54. For details, see "Materials and Methods."
Fig. 7. Effect of erythroid-inducing agents on p54 induction by TPA. Mouse Friend cells (DS19) were incubated with TPA (100 ng/ml) and an erythroid-inducing agent which included DMSO (1.8%, v/v), HMBA (4 mu), and actinomycin D (ActD) (10 ng/ml). After 24 h, the cells were labeled with [35S]methionine (100 μCi/ml) for 4 h. Nuclei were isolated, and total nuclear proteins (approximately 1 × 10⁶ cpm) were subjected to 2-dimensional gel electrophoresis (20). Nuclear proteins were from cells without treatment (A), treated with TPA (B), treated with TPA and DMSO (C), treated with TPA and HMBA (D), and treated with TPA and actinomycin D (E). Arrowhead, p54. For details, see "Materials and Methods."
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