ABSTRACT

The synthesis of a unique protein with a molecular weight of 32,000 (p32) in BALB/c 3T3 cells has been shown previously to increase after treatment with potent tumor-promoting phorbol esters (Hiwasa et al., Proc. Natl. Acad. Sci. U. S. A., 79: 1800, 1982). In the present study, two new classes of tumor promoters which are structurally different from phorbol esters were investigated for their potencies to enhance p32 synthesis. Teleocidin, dihydroteleocidin B, and lyngbyatoxin A, which are indole alkaloid tumor promoters, enhanced p32 synthesis to the same extent that 12-O-tetradecanoylphorbol-13-acetate did. However, no increase was observed by treatment with the biologically inactive hydrolysate of teleocidin. Polycate tumor promoters such as aplysiatoxin and debromoaplysiatoxin also stimulated p32 synthesis, but their effective concentrations were higher than those of 12-O-tetradecanoylphorbol-13-acetate.

When 3T3 cells were treated with a combination of two of the three tumor promoters, TPA, teleocidin, and aplysiatoxin, no synergistic effect of p32 synthesis was observed. This implies that these tumor promoters enhance the synthesis of p32 through the same mechanism.

INTRODUCTION

Recently, teleocidin, an indole alkaloid, was found to be a potent tumor promoter in mouse skin carcinogenesis (8, 9, 26, 28). Based on a 2-stage carcinogenesis concept (1), TPA has been widely used as a model compound of potent tumor promoters and has been reported to cause pleiotropic effects in the promotion stage (see Ref. 4 for review). Teleocidin, which is structurally unrelated to TPA, caused effects similar to those of TPA, such as induction of ODC activity (7, 8), induction of human lymphoblastoid cell (NL-3) aggregation (14), inhibition of terminal differentiation of Friend erythroleukemia cells by DMSO (6), activation of Epstein-Barr virus expression in Epstein-Barr virus genome-carrying human lymphoblastoid cells (5, 16), specific binding on membrane receptors (24, 30), and enhancement of viral transformation and anchorage-independent growth (6, 15). Lyngbyatoxin A is also an indole alkaloid which induced biological activities similar to those of TPA and teleocidin (5, 8, 21, 26).

Recent studies indicated that aplysiatoxin was as strong as TPA in promotion of mouse skin tumors but that debromoaplysiatoxin was a weaker promoter than aplysiatoxin (10, 26).

We have recently shown that the synthesis of one polypeptide (p32) increased after the addition of tumor-promoting phorbol esters to BALB/c 3T3 mouse fibroblasts and that the increase was the earliest change of gene expression caused by tumor promoters (13). In this report, we have studied the effects of indole alkaloid and polycate tumor promoters on the synthesis of p32 in order to discern whether or not the enhancement of p32 synthesis is a general phenomenon caused by potent tumor promoters. It was shown that these tumor promoters also enhanced p32 synthesis and that the ability of each compound to enhance p32 synthesis reflected its tumor-promoting activity.

MATERIALS AND METHODS

Cells. BALB/c 3T3 A31 mouse fibroblasts were cultured in Eagle's MEM supplemented with 10% newborn calf serum on plastic tissue culture dishes.

Chemicals. TPA was purchased from Consolidated Midlands Co. (New York, N. Y.). Teleocidin was isolated from Streptomyces medici-dicus (29). In our laboratory, Drs. H. Fujiki and T. Sugimura found recently that teleocidin is a mixture of teleocidin A and teleocidin B (9). We call this preparation "teleocidin." Dihydroteleocidin B was obtained by catalytic reduction of teleocidin B. Lyngbyatoxin A, aplysiatoxin, and debromoaplysiatoxin were isolated from Lyngbya majuscula (3, 19, 20). We are indebted to Dr. R. E. Moore of the University of Hawaii, Honolulu, Hawaii, for his generous gift of lyngbyatoxin A, aplysiatoxin, and debromoaplysiatoxin. All of these test compounds were dissolved in DMSO and stored at -20°C.

Preparation of Cell Extract and Gel Electrophoresis. Preparation of cell extract was performed as described previously (13). Various test compounds dissolved in DMSO were added to the medium, and the final concentration of DMSO was adjusted to 0.1%. Cells were incubated at 37°C for 2 hr in the presence or absence of test compounds. The medium was then replaced by methionine-free medium containing 1% newborn calf serum, [35S]methionine (20 µCi/ml; 1000 to 1200 Ci/mmol; New England Nuclear, Boston, Mass.), and test compounds or a solvent. The cells were incubated for 30 min, washed twice with phosphate-buffered saline (0.137 M NaCl, 2.7 mm KCl, 8.3 mm Na2HPO4, 1.47 mm KH2PO4, 0.9 mm CaCl2, and 0.5 mm MgCl2), scraped with a rubber policeman, and washed with phosphate-buffered saline once more. The pelleted cells were suspended in a solution containing 0.5% Nonidet P-40, 10 mm NaCl, 10 mm Tris-HCl (pH 7.4), 3 mm MgCl2, 1 mm phenylmethylsulfonyl fluoride, and 1 mm dithiothreitol and incubated for 3 min at 0°C. The cell suspension was centrifuged at 700 x g for 3 min. An appropriate amount of the supernatant was lyophilized for gel electrophoresis. One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 2-dimensional gel electrophoresis were carried out by the methods of Laemmli (17) and O'Farrell et al. (22, 23), respectively (detailed in a previous paper (13)). Autoradiography was performed on Fuji RX medical film.

Measurement of Radioactivity of p32. After the autoradiography, the gel portion corresponding to p32 was cut out and solubilized by
incubation with 1 ml of Protosol (New England Nuclear) at 55° for 3 hr. The radioactivity released was then measured with a toluene-based scintillator.

**Determination of Degradation Rate of p32.** A31 cells were prelabeled with [35S]methionine for 1 hr, washed with MEM, and then treated with TPA or DMSO for 2 hr in the presence of cycloheximide (5 µg/ml). Cell extract was prepared, and the radioactivity of p32 was measured as described above.

**Determination of Methionine Pool Size.** The intracellular pool size of methionine was determined according to the methods of Schultz et al. (25) and West and Holtzer (31). A31 cells grown in 33-mm dishes were pretreated with TPA or DMSO for 2 hr followed by incubation with [35S]methionine in MEM, the methionine concentration of which was adjusted to 10, 1, or 0.1 µM. After 1 hr, cells were harvested and acid-soluble and acid-insoluble 35S radioactivities were measured. The intracellular free methionine pool was calculated by solving the following pair of equations for \( P \) (25).

\[
dl_1/\text{dt} = R \times L_1/(P + G_1)
\]

\[
dl_2/\text{dt} = R \times L_2/(P + G_2)
\]

where \( dl_1/\text{dt} \) and \( dl_2/\text{dt} \) are dpm of [35S]methionine incorporated into acid-insoluble material at 2 different methionine concentrations, \( L_1 \) and \( L_2 \) are dpm of [35S]methionine in acid-soluble material, \( G_1 \) and \( G_2 \) are pmol of methionine taken up by the cells, \( P \) is the size of the active endogenous free methionine pool, and \( R \) is a conversion constant.

**RESULTS**

Among various changes of gene expression caused by tumor promoters reported thus far (2, 12, 18), the enhancement of the synthesis of one polypeptide (p32) in BALB/c 3T3 cells is remarkable because this enhancement takes place as early as 2 hr after the addition of TPA (13). The radioactivity of [35S]methionine incorporated into p32 in 3T3 cells treated with TPA for 2 hr was approximately twice as high as that in control cells (13). In order to comprehend the stimulation of p32 synthesis, the degradation rate of p32 and intracellular methionine pool size were determined. After 2 hr treatment with TPA, the degradation rate of p32 increased by approximately 20%. Intracellular methionine pool sizes of control cells, as shown in Table 1. Almost the same degree of enhancement of p32 synthesis was observed by treatment with telolecidin at concentrations higher than 20 ng/ml (data not shown).

Aplysiatoxin and debromoaplysia toxin also induce ODC activity, and the tumor-promoting activity of aplysia toxin is stronger than that of debromoaplysia toxin (8, 10, 11). The effect of these 2 polycatexes on p32 synthesis was also examined, and the results showed that aplysia toxin and debromoaplysia toxin caused the same degree of enhancement of p32 synthesis. The minimal concentrations of aplysia toxin and debromoaplysia toxin to cause maximal effect were 100 ng/ml and 1 µg/ml, respectively (Fig. 3; Table 1).

In order to analyze the foregoing results more quantitatively, the radioactivities of p32 synthesized after the treatment with tumor promoters were measured. Because p32 turns over more rapidly than do the other proteins (13) and also because the synthesis of p32 increased spontaneously in untreated 3T3 cells cultured for more than 4 weeks (data not shown), the radioactivity of p32 of treated cells was expressed as percentage of that of control cells, as shown in Table 1. Almost the same degree of enhancement of p32 synthesis was obtained by treatment with TPA, telolecidin, dihydroteleocidin B or lynybytxa toxin A at 20 ng/ml; with aplysia toxin at 100 ng/ml; or with debromoaplysia toxin at 1 µg/ml. No significant enhancement was observed after hydrolyzed teleocidin treatment. Under the same conditions, these compounds caused no meaningful effects on the synthesis of polypeptides except p32.

When cells were incubated with TPA and teleocidin simultaneously, no additional enhancement of p32 synthesis was obtained compared with that observed in cells treated with TPA or teleocidin alone (Table 1). Likewise, no synergistic effect was seen with the combination treatments of TPA and aplysia toxin or teleocidin and aplysia toxin (Table 1). These results suggest that 3 classes of tumor promoters, TPA, teleocidin, and aplysia toxin, enhance the synthesis of p32 through a common pathway.

**DISCUSSION**

Although a number of studies concerning the effects of tumor promoters have accumulated recently, little is known about the action mechanism of tumor promoters at the molecular level. We have previously shown that the synthesis of p32 in BALB/c 3T3 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as shown in Fig. 1, teleocidin, dihydroteleocidin B, and lynybytxa A enhanced the synthesis of a M, 32,000 protein almost as much as did TPA. No enhancement was observed when cells were treated with hydrolyzed teleocidin which was produced by cleavage of an amide bond in teleocidin molecule. This hydrolyzed substance has no effect on induction of ODC activity (8) or on induction of aggregation of NL-3 cells (14).

To analyze proteins synthesized after the treatment with various tumor promoters in more detail, 2-dimensional gel electrophoresis was carried out according to the method of O'Farrell (22). As shown in Fig. 2, the synthesis of only one polypeptide (p32), indicated by arrows, increased reproducibly when cells were treated with TPA or teleocidin. The pattern of the 2-dimensional gel electrophoretogram of the cell extract treated with dihydroteleocidin B or lynybytxa A was the same as that of the cell extract treated with teleocidin; also, hydrolyzed teleocidin did not cause any stimulation of the synthesis of p32 (data not shown). No further enhancement of p32 synthesis was observed by treatment with teleocidin at concentrations higher than 20 mg/ml (data not shown).

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Enhancement of p32 Synthesis by Tumor Promoters in 3T3 Cells

cells is specifically stimulated by tumor-promoting phorbol esters such as TPA and that the stimulation is regulated at the transcriptional level (13). There are some other proteins the synthesis of which decreased slightly after TPA treatment (Fig. 2, Lanes a and b). However, only the synthesis of p32 increased after the treatment with tumor promoter, and this change was the most prominent, reproducible and statistically significant. No further differences were found even when more basic proteins were analyzed by nonequilibrium isoelectric focusing gel electrophoresis (23) (data not shown).

In the present study, stimulation of p32 synthesis has been proven not to be restricted to phorbol esters but to be a general phenomenon also caused by tumor-promoting indole alkaloids and polyacetates. The effective concentration of indole alkaloids to induce maximal stimulation of p32 synthesis was very similar to that of TPA, i.e., 20 ng/ml. Potent tumor-promoting indole alkaloids were reported not only to promote skin tumors in 7,12-dimethylbenz(a)anthracene-treated mouse skin but also to possess biological potencies similar to those of TPA in a number of assay systems (6–9, 14–16, 28, 32). These 2 different classes of tumor promoters, phorbol ester and indole alkaloids, are considered to induce their effects via a similar receptor system (24, 30). The present observation that TPA and teleocidin did not enhance the synthesis of p32 synergistically is consistent with previous reports (24, 30). After utilizing the same receptor system, these 2 classes of tumor promoters are thought to exert their effects on the transcriptional system for p32 synthesis since the enhancement by either group of compounds did not take place in the presence of actinomycin D (Ref. 13; data not shown).

Aplysiatoxin and debronymplaxiosaioxin, which is a debrinated form of alysiatoxin, had similar activities in skin irritation and the induction of ODC activity, while the tumor-promoting activity of debronymplaxiosaioxin was weaker than that of alysiatoxin (8, 10, 26). The percentages of mice which bore tumors promoted by TPA, alysiatoxin, and debronymplaxiosaioxin for 30 weeks were 100, 93, and 53, respectively (11). The effective dose of debronymplaxiosaioxin needed to cause adhesion of HL-60 cells or aggregation of NL-3 cells was much higher than that of alysiatoxin (10). The same tendency was observed with respect to the effective concentrations of alysiatoxin and debronymplaxiosaioxin in the activity of enhancing p32 synthesis.

The relationship between the synthesis of p32 and the state of cell growth was also examined. Cells at an exponentially growing phase synthesized slightly more p32 than those at a stationary phase (data not shown). The synthesis of p32, however, did not change during cell cycle, and it was stimulated at an early time after release from treatment with Colcemid, excess thymidine, or hydroxyurea (data not shown). Some growth factors such as epidermal growth factor, fibroblast growth factor, and insulin did not stimulate p32 synthesis. These results suggest that p32 plays some role in the growth of cells independent of cell cycle.

Although the function of p32 remains to be determined, a good correlation between the enhancement of p32 synthesis and tumor-promoting activities of varying tumor promoters suggests that p32 plays an important role in the stage of tumor promotion. Our unpublished result revealed that N-methyl-N'1-nitro-N-nitrosoguanidine, which is a complete carcinogen (27) and therefore is supposed to have both initiating and promoting activities, also specifically enhances the p32 synthesis in BALB/c 3T3 cells (4). Presently, we are employed not only in elucidating the action mechanism of tumor promoters at the molecular level but also in the screening unidentified promoters in the environment using the system described herein.

4 T. Hiwasa and S. Sakiyama, manuscript in preparation.

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative % of radioactivity of p32</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO (0.1%)</td>
<td>100 ± 6.6</td>
</tr>
<tr>
<td>TPA (20 ng/ml)</td>
<td>200 ± 2.3</td>
</tr>
<tr>
<td>Teleocidin (20 ng/ml)</td>
<td>215 ± 17.2</td>
</tr>
<tr>
<td>Dihydroleocidin (B 20 ng/ml)</td>
<td>198 ± 2.5</td>
</tr>
<tr>
<td>Lyngbyatoxin A (20 ng/ml)</td>
<td>202 ± 12.2</td>
</tr>
<tr>
<td>Aplysiatoxin (20 ng/ml)</td>
<td>200 ± 12.2</td>
</tr>
<tr>
<td>Aplysiatoxin (100 ng/ml)</td>
<td>194 ± 4.2</td>
</tr>
<tr>
<td>Debronymplaxiosaioxin (1 μg/ml)</td>
<td>195 ± 9.5</td>
</tr>
<tr>
<td>Hydrolyzed teleocidin (1 μg/ml)</td>
<td>114 ± 8.6</td>
</tr>
<tr>
<td>TPA (20 ng/ml) + teleocidin (20 ng/ml)</td>
<td>208 ± 7.6</td>
</tr>
<tr>
<td>TPA (20 ng/ml) + alysiatoxin (100 ng/ml)</td>
<td>199 ± 4.2</td>
</tr>
<tr>
<td>Teleocidin (20 ng/ml) + alysiatoxin (100 ng/ml)</td>
<td>198 ± 11.2</td>
</tr>
</tbody>
</table>

* Normalized to 100.
* Mean ± S.E. (n ≥ 3).
* p < 0.001 versus DMSO.
* p < 0.01 versus DMSO.
* p > 0.1 versus DMSO, not significant.
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REFERENCES


Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cell extracts treated with various tumor promoters. BALB/c 3T3 cells were treated for 2 hr with DMSO, 0.1% (a); teleocidin, 5 ng/ml (b); teleocidin, 20 ng/ml (c); dihydroteleocidin B, 5 ng/ml (d); dihydroteleocidin B, 20 ng/ml (e); lyngbyatoxin A, 5 ng/ml (f); lyngbyatoxin A, 20 ng/ml (g); TPA, 5 ng/ml (h); TPA, 20 ng/ml (i); and hydrogenated teleocidin, 20 ng/ml (j) and then labeled with [35S]methionine for 30 min. Cell extracts were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis. Right ordinate, molecular weight. Arrow, position of p32. Right ordinate, molecular weight. SDS, sodium dodecyl sulfate.
Enhancement of p32 Synthesis by Tumor Promoters in 3T3 Cells

Fig. 1

Fig. 2a

Fig. 2b

Fig. 2c
Increase in the Synthesis of a \( M_r 32,000 \) Protein in BALB/c 3T3 Cells Treated with Tumor-promoting Indole Alkaloids or Polyacetates

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