Specific Binding of 12-*O*-Tetradecanoylphorbol-13-acetate to Histone H1

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12-*O*-Tetradecanoylphorbol-13-acetate (TPA) binds specifically to histone H1 prepared from HeLa cells and to calf histone H1 with dissociation constants of 4.3×10^{-7} and 9.3×10^{-7} M, respectively. The TPA-binding to histone H1 was confirmed immunologically using a monoclonal antibody directed against an evolutionally conserved epitope of histone H1. This binding would influence DNA structure and the basal level of transcription, and may, in turn, account for the cell growth-regulatory activity of TPA.

Keywords 12-*O*-tetradecanoylphorbol-13-acetate; histone H1; tumor promoter

Phorbol esters are classical tumor promoters which potentiate the tumorigenic effect of a subcarcinogenic dose of an initiating carcinogen.^{1–3)} The most extensively studied phorbol ester is 12-*O*-tetradecanoylphorbol-13-acetate (TPA). In addition to its potent skin tumor-promoting activity, TPA elicits a wide variety of biological effects including irritation and regulation of cell growth and differentiation.³⁾ While the action of TPA as a tumor promoter and a cell differentiation inducer is very specific to cell types or species, its action as a growth modulator is applicable to a wide variety of cells. Therefore, the biological actions of TPA should be mediated by several different pathways.

In view of its variety of biological actions and modes of action, multiple initial target molecules of TPA are expected to exist. One of the most extensively studied target molecules is protein kinase C (PKC), which plays a role in mediating signal transductions.^{4,5)} We have also proposed a nuclear receptor-like molecule, cytosolic-nuclear tumor promoter-specific binding protein (CN-TPBP), which is initially present in cytosol but translocates into nuclei after binding to TPA.^{6,7)} Recently, we have identified at least four cellular TPA-specific binding proteins (TBP-1, -3, -4, and -5; TBP-2 is identical with CN-TPBP) in HeLa cells.⁷⁾ The apparent molecular weights and the dissociation constants with TPA estimated for binding proteins in HeLa cells are: PKC (85 kDa, $K_d = 0.68$ nM), CN-TPBP (66 kDa, $K_d = 0.07$ nM), TBP-4 (68 kDa, $K_d = 4.2$ nM), and TBP-5 (70 kDa, $K_d = 0.51$ nM).

Though the TPA-binding proteins so far known all initially exist in cytosol, we found that TPA also binds nuclear fractions with rather low affinity. Nuclear binding of TPA was suggested by Perrella *et al.*,⁸⁾ and induction of structural change of chromosomes by TPA was also demonstrated by Emerit and Cerutti,⁹⁾ though they did not characterize the binding molecule(s). During studies on the purification of TBPs, we have isolated other/additional TPA-binding proteins and identified them as histones H1a and H1d. Histone H1 is known to be related to chromosome structure, the basal level of transcription, DNA replication, and the cell cycle.^{10–13)} In this paper, we report the specific binding characteristics of TPA to

histone H1 prepared from HeLa cells and to calf histone H1.

MATERIALS AND METHODS

Peptide Sequencing Suspension-cultured HeLa cells provided by Ajinomoto Company were maintained in RPMI1640 medium supplemented with fetal calf serum (5% v/v) under 5% carbon dioxide at 37 °C. HeLa whole cell extract was prepared and separated as described previously.⁶⁾ The fractions containing TPA-specific binding protein(s) other than TBPs were further separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli.¹⁴⁾ After electrophoresis, Coomassie brilliant blue-stained protein bands were excised with a laser, and digested with 1 µg of lysyl-endopeptidase (Wako Pure Chemicals) in 500 µl of a solution containing 100 mM Tris–HCl (pH 9.0), 4 M urea at 37 °C for 6 h. The peptides eluted from the gels were separated on a C18 reverse-phase column (250 × 2.1 mm i.d., Vydac) with a linear gradient of CH₃CN–H₂O containing 0.1% CF₃COOH at a flow rate of 0.3 ml/min (Fig. 1). The collected peptides were sequenced on a model PPSQ-10 gas-phase protein sequencer (Shimadzu Co.).

Histone H1 Histone H1 was purified from the HeLa whole cell extract by a slight modification of the method described by Croston *et al.*¹⁵⁾ The extract was precipitated with 1.7 M ammonium sulfate, and the supernatant was subjected to Phenyl-Sepharose Fast Flow column chromatography (Pharmacia) eluted with a linear gradient of 1.7–0.1 M ammonium sulfate in 50 mM sodium phosphate buffer (pH 7.0). The histone H1-containing fractions were collected, and further separated by Mono Q HPLC (Pharmacia) eluted with a linear gradient of 0–1.0 M NaCl in 20 mM Tris–HCl (pH 8.0) and a HiTrap Heparin column (Pharmacia) eluted with a linear gradient of 0–2.0 M NaCl in 50 mM sodium phosphate (pH 7.0). At each step, fractions were checked by SDS–PAGE (5–20% gradient gel purchased from Atto Company) and TPA-binding activity was measured (*vide infra*). The obtained electrophoretically pure histone H1 fractions were confirmed to contain histone H1a and H1d by peptide se-

quencing and Western analysis (*vide infra*) and were used as histone H1. Calf histone H1 was purchased from Boehringer Mannheim.

Monoclonal Antibody against Histone H1 The monoclonal antibody of clone 5F3 belongs to a series of such antibodies raised against the histone H1 proteins of the dipteran insect, *Chironomus thumi*.¹⁶ It crossreacts with histone H1 from other organisms¹⁷ and is directed against the sequence SGSFK (A. Steuernagel, unpublished results), which is evolutionarily conserved in histone H1 of plants and animals; it corresponds to amino acid residues 104–108 in human histone H1a and 101–105 in human histone H1d, respectively.

SDS-PAGE and Western Blott Analysis Samples for analysis were prepared according to the protocol described by Wrange *et al.*¹⁸ SDS-PAGE, using 5–20% gradient gel (Atto Company) was performed by the method recommended by the gel supplier. The separating gel was soaked in transfer buffer (100 mM Tris, 192 mM glycine, 5% methanol, 0.1% SDS) for 20 min, and then placed together with an Immobilon P membrane (Millipore) in a sandwich. Proteins were transferred for 90 min at 300 mA. Immunodetection was performed using 5F3 anti-histone H1 antibody and a blotting detection kit for mouse antibodies (Amersham: second antibody, biotinylated goat anti-mouse IgG; colour detection, streptavidin-alkaline phosphatase conjugate/5-bromo-4-chloro-2-indolyl phosphate system) according to the protocol recommended by the kit supplier.

TPA-Binding Assay and Immunoprecipitation Assay TPA-binding activity was assayed by the dextran-coated charcoal method as described previously.⁶ Briefly, samples were mixed with 0.1 volume of dimethyl sulfoxide (DMSO) and incubated with 4 nM [³H]12-*O*-tetradecanoylphorbol-13-acetate ([³H]TPA, 469.9 GBq/mmol, NEN) in the presence or absence of a 400-fold excess amount of TPA for 16 h at 4°C. For the Scatchard analysis, various concentrations of [³H]TPA were incubated with histone H1 samples. In the case of immunoprecipitation assay,¹⁶ samples were incubated with 5F3 anti-histone H1 antibody (16 h, 4°C), and then protein A Sepharose (Pharmacia, 15 mg/ml) was added and the mixture was shaken for 8 h at 4°C. The suspension was centrifuged and the supernatant was used for the assay. Samples incubated with [³H]TPA were mixed with 3/10 volume of 1% charcoal–0.1% dextran in 0.6 M NaCl–20 mM Tris (pH 8.0)–10% DMSO, and stood on ice for exactly 15 min. Then, the suspension was centrifuged and the radioactivity of the supernatant in Atomlight (DuPont) was measured with a liquid scintillation counter.

RESULTS AND DISCUSSION

HeLa cells have been found to possess at least six TPA-specific binding proteins: PKC and TBP-1–5.^{6,7} During experiments on the separation/purification of TBPs, we have found a TPA-binding protein(s) other than PKC or TBPs.⁷ This protein(s) was partly co-eluted with TBP-5 on Mono Q HPLC, though the protein's affinity for TPA was much lower than that of TBP-5.⁷ The protein was further separated by repeated Mono Q HPLC. SDS-

PAGE analysis of the fractions containing this TPA-binding protein showed two major bands around 30 kDa (higher molecular size band A and lower molecular size band B, data not shown). These two bands were sequenced as described in Materials and Methods. Because both components were blocked at the N-terminal, the internal peptide sequences were analyzed after digestion with lysyl endopeptidase. The digestion mixture of each band was separated by reversed-phase HPLC (Fig. 1), and the appropriate peak(s) was analyzed by a gas-phase peptide sequencer. The higher molecular size band A (Fig. 1a) was confirmed to contain partial internal sequences of GTLVQTK (a common sequence for histone H1a–H1d subtypes) and ALAAGGYDVEK (a specific sequence for histone H1a).¹⁹ The lower molecular size band B (Fig. 1b) was confirmed to contain an internal sequence of KPAAATVTK (a specific sequence for histone H1d).¹⁹ The newly found TPA-binding proteins were thus suggested to be histones H1a and H1d, and we next investigated the interaction of TPA with histone H1s.

Human histone H1 was prepared from suspension-cultured HeLa cells by a modification of the method described by Croston *et al.*,¹⁵ i.e., by four successive steps of ammonium sulfate precipitation, hydrophobic interaction chromatography on Phenyl Sepharose Fast Flow, ion exchange chromatography on Mono Q, and affinity chromatography on Hitrap Heparin. The SDS-PAGE analysis of histone H1 fraction thus prepared showed two major bands around 30 kDa (Fig. 2a, bands A and B) whose electrophoretic mobilities corresponded to histones H1a and H1d (Fig. 1), respectively. Both the bands were

Fig. 1. Reversed-Phase HPLC of TPA-Binding Protein Digested with Lysyl-Endopeptidase

TPA-binding proteins were prepared and analyzed as described in Materials and Methods. Digests of the higher molecular size band A (a) and the lower molecular size band B (b) are shown, as are peptide sequences of analyzed peaks. The mark (▽) indicates a peak containing the N-terminal blocked fragment.

Fig. 2. SDS-PAGE and Western Analysis of Histone H1 Fraction Prepared from HeLa Cells

a: Two histone H1 fractions was electrophoresed on SDS-PAGE with molecular size markers and stained with Coomassie brilliant blue. b: Result of Western blotting analysis (see Materials and Methods). Positions of molecular size markers are shown.

recognized by anti-histone H1 monoclonal antibody in the Western blot (Fig. 2b), indicating that the histone H1 fraction thus prepared is a mixture of histone H1a and H1d. Purchased calf histone H1 showed basically the same electrophoretic pattern on SDS-PAGE as that presented in Fig. 2 (data not shown).

Histone H1 samples were assayed for TPA-specific binding activity by the dextran-coated charcoal method.⁶⁾ As shown in Fig. 3a, histone H1 prepared from HeLa cells bound specifically to TPA. The specific binding was more than 75% of the total binding under the experimental conditions used. This TPA-specific binding was greatly decreased by successive treatment of the sample with anti-histone H1 monoclonal antibody and protein A Sepharose (Fig. 3c). The results indicate that the observed TPA-specific binding is due to histone H1, not contaminating factor(s), if any, even though the histone H1 subtypes (H1a and H1d) could not be discriminated by this experiment. Calf histone H1 also showed TPA-specific binding activity (Fig. 3b). The TPA-binding to histone H1 was efficiently competed with by 3,5-dihydroxycholestan-6-one (yakkasterone, a specific ligand for CN-TPBP which does not bind PKC),^{7,20)} but not by diacylglycerol (a specific ligand for PKC which does not bind CN-TPBP).

The TPA-binding affinity of human and calf histone

Fig. 3. TPA-Binding Activity of Histone H1

The assay procedure is described in Materials and Methods. Also see the text. a: Total and non-specific binding of [³H]TPA to histone H1 prepared from HeLa cells. □, [³H]TPA 4 nM; ■, + TPA 400 fold. b: TPA-specific binding to HeLa histone H1 in the presence or absence of anti-histone H1, mouse IgG and/or protein A Sepharose. c: Total and non-specific binding of [³H]TPA to calf histone H1. □, [³H]TPA 4 nM; ■, + TPA 100 fold. d and e: Scatchard analysis of TPA-binding to HeLa and calf histone H1, respectively. (d) $K_d = 4.3 \times 10^{-7}$ M. $r = 0.982$. (e) $K_d = 9.3 \times 10^{-7}$ M. $r = 0.906$.

H1's was measured by Scatchard analysis (Fig. 3d and e). In both cases, good linearity was obtained by the least-squares methods ($r=0.982$ for human histone H1 and 0.906 for calf histone H1). The dissociation constants of human and calf histone H1 to TPA were calculated to be 4.3×10^{-7} and 9.3×10^{-7} M, respectively, under the experimental conditions used. The values suggest that the affinity of histone H1 for TPA is weaker than that of PKC by three orders of magnitude. However, cells generally contain approximately 1000-fold higher amounts of histone H1 molecules compared to PKC. Therefore, binding of TPA to histone H1 cannot be ignored, especially when cells in various fundamental biological assay systems are treated with TPA at several hundred nanomolar concentration levels.

In conclusion, we found that TPA binds histone H1, implying that histone H1 might be one of the target molecules of TPA mediating some of the general/non-specific biological activities of TPA. Since histone H1 plays a role in the packaging of DNA into chromatin and in DNA-replication,^{10,11)} its interaction with TPA might influence the structure of chromatin. In addition, histone H1 is known to be a general repressor of transcription.^{12,21,22)} On the other hand, certain H1 subtypes might not interfere with this process, as histone H1 has been shown to be present in highly active genes.²³⁾ Moreover, TPA was reported to induced phosphorylation of histones which leads to enhancement of template activity of DNA.²⁴⁾ This phosphorylation might be induced by binding of TPA to histone H1. Therefore, TPA-binding to histone H1 might change the basal level of transcription.

Numerous organisms and cell types have been shown to possess several different subtypes of histone H1,¹⁹⁾ and indirect evidence suggests a functional role of this diversity.²⁵⁾ Intranuclear differences in the distribution of functionally divergent H1 subtypes have recently been shown to exist^{16,23)} and appear to be correlated to differences in chromatin architecture.²⁶⁾ If TPA binds only one (or a few) histone H1 subtype(s), it might modify the gene expression of some specific gene group(s).

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