Cytosolic-nuclear Tumor Promoter-specific Binding Protein: Association with the 90 kDa Heat Shock Protein and Translocation into Nuclei by Treatment with 12-O-Tetradecanoylphorbol 13-Acetate

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Suspension-cultured HeLa cells possess a cytosolic-nuclear tumor promoter-specific binding protein (CN-TPBP) which lacks protein kinase C activity. This CN-TPBP existed in cytosol of HeLa cells, but translocated into nuclear fraction of the cells after treatment of the cells with 12-O-tetradecanoyl-phorbol 13-acetate (TPA). The translocation of CN-TPBP induced by TPA became apparent within 10 min after the treatment with TPA, and was completed within 3 h. CN-TPBP bound TPA with the association constant of 1.4×10^{10} M^{-1}, and also bound teleocidin B, debromoaplysiatoxin, and thapsigargin in a mutually competitive manner. The binding affinity order of synthetic analogs of teleocidin B correlated with the adhesion-inducing potency order of the compounds toward human leukemia cell line HL-60. The apparent molecular weight of CN-TPBP under non-denaturing conditions was estimated to be 66–68 kDa. CN-TPBP forms a complex with the 90 kDa heat shock protein, and the complex was stabilized by the presence of molybdate. These characteristics of CN-TPBP are similar to those of the nuclear receptors of glucocorticoid and dioxin. These findings suggested that CN-TPBP acts as a nuclear receptor for tumor promoters, and that tumor promoters may exert their biological effects by binding to CN-TPBP.

Key words: Tumor promoter receptor — 12-O-Tetradecanoylphorbol 13-acetate — 90 kDa Heat shock protein

Tumor promoters potentiate the tumorigenic effect of a subcarcinogenic dose of an initiating carcinogen,[1–3] and elicit biological effects such as irritation, induction of ornithine decarboxylase, and induction of terminal differentiation of some cultured cell lines.[3–5] One of the most extensively studied tumor promoters is 12-O-tetrade-canoylphorbol 13-acetate (TPA), which is considered to elicit its effects by inducing an altered program of gene expression. Among the genes whose transcription is induced by TPA are cellular oncogenes such as c-*fos*,[6, 7] c-*jun*,[8] c-*myc*[6, 9] and c-*sis*,[10] which could be responsible for the loss of growth control and the acquisition of cell immortality. Other known targets for TPA induction are the collagenase and stromelysin genes,[11, 12] which may have a role in tumor invasiveness, metastasis and angiogenesis.[13, 14] TPA also induces κ immunoglobulin enhancer-binding protein, NF-κB.[15] Some genes whose transcription is induced by TPA are known to possess a palindromic DNA element referred to as the TPA-response element (TRE).[16, 17] TRE is a binding site of the transcription factor AP1, the constituents of which include Fos and Jun proteins.[18, 19] The AP1 is also inducible by TPA.[6–8, 17] A response element which is commonly recognized by AP1 and receptors for vitamins A and D has recently been reported.[20]

The currently prevailing hypothesis concerning the molecular mechanism of tumor promotion is that tumor promoters elicit their biological effects by binding and activating protein kinase C (PKC).[21, 22] However, there exist potent tumor promoters such as thapsigargin which do not bind or activate PKC.[3, 23, 24] The pathway responsible for transducing the signal generated by the activation of PKC to the transcriptional machinery is not known, and no direct evidence for the participation of PKC in tumor promotion or the biological effects elicited by tumor promoters has been reported.

On the other hand, TPA acts synergistically with vitamin A or D, or steroids in some cases, while in other cases, TPA action is antagonized by vitamin A, vitamin D or steroids. For example, (1) induction of collagenase or ornithine decarboxylase by TPA is inhibited by vitamin A or steroids,[25, 26] (2) vitamin D-mediated regulation of osteocalcin is inhibited by TPA or steroids,[20] (3) tumor-promoting activity of TPA is enhanced by steroids or vitamin D in some cases, and is inhibited by vitamin A

in other cases,[27-29] (4) human promyelocytic leukemia cells HL-60 are induced to differentiate to mature monocytes or granulocytes by TPA or vitamins A and D.[30] These results suggest an intimate relationship of TPA with steroids and vitamins A and D in the molecular mechanism of actions elicited by these substances, and the possible existence of a cross-talk mechanism[20] of these substances. In addition, the mode of biological effects elicited by tumor promoters is rather similar to that of steroid and thyroid hormones, and vitamins A and D, which exert their biological effects by binding to specific nuclear receptor(s) belonging to the erbA-related steroid/thyroid nuclear receptor superfamily.[31, 32]

Under the circumstances mentioned above, the presence of a tumor promoter receptor(s) other than PKC(s) was suspected. Perrella *et al.* reported the presence of specific high-affinity binding sites of TPA (association constant of ca. $5 \times 10^8 \, M^{-1}$) in the isolated nuclei and the nuclear macromolecules in mouse epidermis, and suggested the existence of nuclear receptors for TPA,[33] though the further characterization or the nature of the TPA binding sites has not been reported. Recently, we reported the presence in HL-60 cells of a tumor promoter-specific binding protein which lacks PKC activity.[34] The protein exists in cytosolic fraction but translocates into nuclear fraction on treatment of the cells with TPA, and has been named cytosolic-nuclear tumor promoter-specific binding protein (CN-TPBP).[34] The possibility that CN-TPBP is a tumor promoter receptor, translocating into nuclei as a ligand-receptor complex, which interacts with specific DNA-sites to regulate the gene expression, was suggested in that paper. CN-TPBP also exists in a suspension culture of HeLa cells (sus.HeLa). In this study, we investigated some properties of CN-TPBP; i.e., kinetics of TPA-dependent nuclear translocation in sus.HeLa, ligand binding selectivity, the apparent molecular weight, and complex formation with the 90 kDa heat shock protein (hsp90). We discuss here the possibility of the existence of an additional pathway other than the PKC activation pathway as well as the possibility that CN-TPBP is a member of the steroid/thyroid receptor superfamily or related nuclear receptors.

MATERIALS AND METHODS

Materials [^{3}H]12-O-Tetradecanoylphorbol 13-acetate ([^{3}H]TPA, 12.7 Ci/mmol) was purchased from NEN. Teleocidin B, thapsigargin, debromoaplysiatoxin, and okadaic acid were supplied by Dr. H. Fujiki (National Cancer Center Research Institute). Immunoglobulin G fraction of rabbit antiserum raised against hsp90 (anti-hsp90) was a generous gift from Dr. I. Yahara (The Tokyo Metropolitan Institute of Medical Science). In-

dolactam analogs were prepared as described previously.[35]

Preparations of cytosolic and nuclear fractions sus.-HeLa provided by Ajinomoto Company was maintained in RPMI 1640 medium supplemented with fetal calf serum (5% v/v) under 5% carbon dioxide at 37°C. The cells were collected by centrifugation after incubation with or without TPA (4 nM) for the period indicated in the text. The cells were lysed by hypotonic shock, and the cytosolic and nuclear fractions of the cells were prepared as described previously.[34] The recovery of the intact nuclei was confirmed by microscopic observation after Wright-Giemsa staining. Usually, more than 85% of the particles prepared by this method are intact nuclei.[36]

TPA-specific binding activity The cell extracts prepared as above were separated by high-performance liquid chromatography (HPLC; conditions were as described in the figure legends). The HPLC fractions were assayed for TPA-binding activity by filter binding assay using nitrocellulose membranes as described previously,[34, 37] or by the dextran-coatd charcoal method. Briefly, a portion of each fraction was mixed with 10% volume of DMSO, and incubated with 4 nM [^{3}H]TPA (12.7 Ci/mmol, NEN) in the presence or absence of a 200-fold excess of TPA for 16 h at 4°C. (Incubation with 4 nM [^{3}H]TPA for longer than 9 h was necessary to replace TPA already bound to the binding proteins.) After the incubation, 3/10 volume of a dextran-coated charcoal suspension was added. The mixture was kept at 4°C for 15 min, then centrifuged (10,000 rpm×10 min at 4°C). Then 9/10 volume of the supernatant was mixed with Atomlight (DuPont), and the radioactivity was measured with a liquid scintillation counter.

The dextran-coated charcoal suspension was prepared by sonication (2 h) of a mixture of charcoal (activated, Norit A, Sigma, C-5260, 1 g) and dextran (Sigma, D-9260, 0.1 g) in 100 ml of 0.6 M NaCl-20 mM Tris (pH 8.0)-10% DMSO.

PKC activity PKC activity was measured by use of the protein kinase C enzyme assay system (Amersham) according to the protocol recommended by the supplier.

RESULTS

HPLC analysis of whole-cell extract of sus.HeLa First we investigated TPA-specific binding proteins and PKC activity in sus. HeLa maintained in RPMI 1640 supplemented with fetal calf serum (5% v/v) under 5% carbon dioxide at 37°C. The cells were extracted with 0.6 M KCl-20 mM Tris (pH 8.0) by homogenization and centrifuged ($10^4 g \times 2$ h). The supernatant was desalted and analyzed by HPLC with a Mono Q column (Pharmacia) (Fig. 1). A broad peak of TPA-specific binding activity (T_R=32–60 min, 0.25–0.40 M NaCl) was
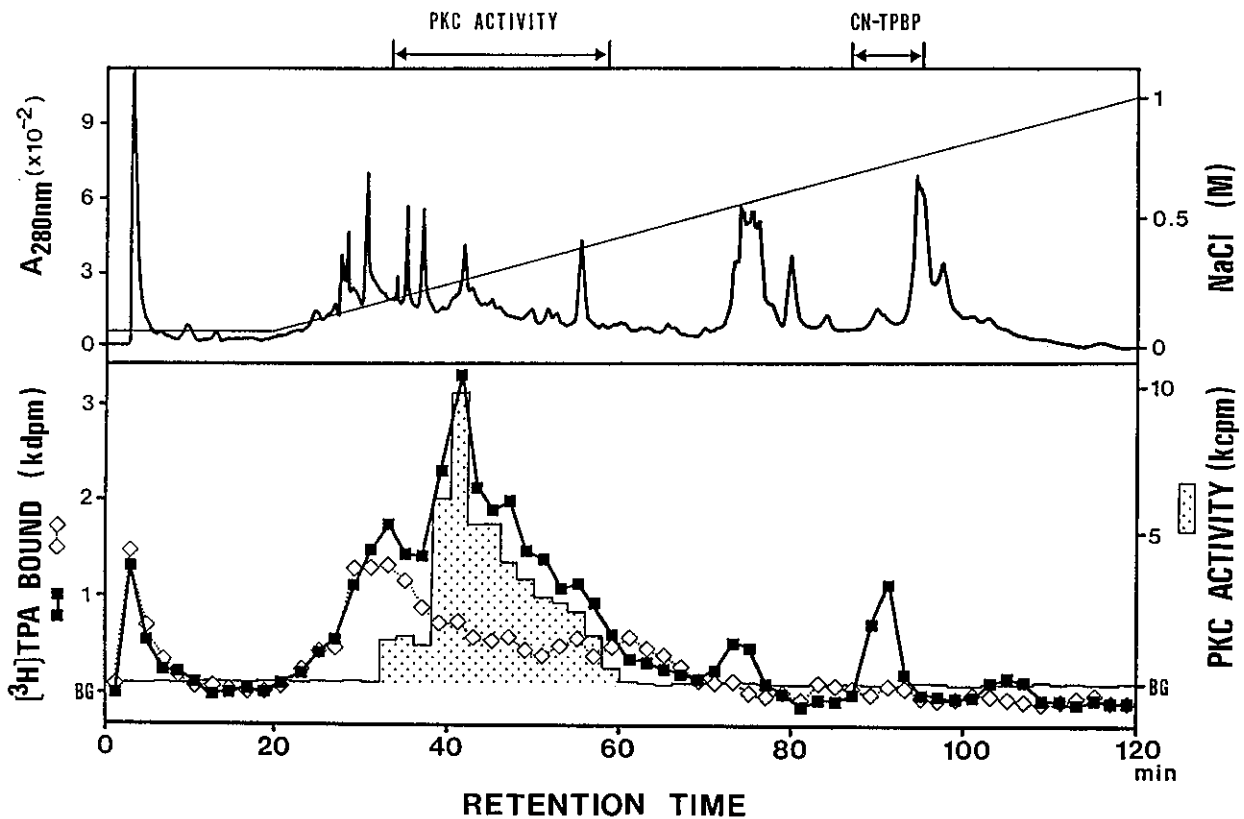
Fig. 1. HPLC analysis of whole-cell extract prepared from sus.HeLa. Column: Mono Q HR5/5 (Pharmacia). Eluent: A linear gradient of 0.05–1.0 M NaCl (9.5 mM NaCl increase/min) in 20 mM Tris (pH 8.0). Flow rate: 0.5 ml/min. Sample: sus.HeLa (2 ×10⁷ cells) was extracted with 0.6 M KCl-20 mM Tris (pH 8.0). The extract was concentrated by ultrafiltration (cut-off molecular weight of 10 kDa) and diluted by addition of 20 volumes of 20 mM Tris (pH 8.0). Horizontal scale: Retention time (T_R, min). Upper: Monitored by measuring the absorbance at 280 nm. NaCl concentration of the eluent is also indicated in the figure. Lower: Monitored by measuring [³H]TPA-binding activity (the vertical scale on the left) and PKC activity (the vertical scale on the right). Each fraction was incubated with [³H]TPA (4 nM) in the absence (■) or in the presence (◇) of excess TPA (800 nM). PKC activity of each fraction was measured as described in "Materials and Methods" and is indicated as the radioactivity (kcpm, shaded rod) of ³²P incorporated into the substrate.

observed. This peak was superimposed on a peak of PKC activity. Ths elution of PKCs from a Mono Q column with 0.25–0.40 M NaCl agrees with the reports of other investigators.[38] Therefore, we regarded this broad peak as PKC fraction. We also reproducibly observed three other peaks of TPA-specific binding activity; T_R=70–78 min (TPA-specific binding protein-1; TBP-1), 88–96 min (TBP-2) and 102–110 min (TBP-3). These three peaks did not show any PKC activity when the fractions were assayed using the Protein kinase C enzyme assay system (Amersham). The TPA-specific binding activity of these three peaks were lost after treatment with trypsin. We compared the chromatogram of sus.HeLa (Fig. 1) with that of HL-60 whole-cell extract (data not shown), in which we have previously established the presence of

CN-TPBP.[34] TBP-2 (the peak of T_R=88–96 min, 0.65–0.75 M NaCl) shown in the figure was tentatively identified as CN-TPBP, judging from the retention time. **TPA-dependent translocation of CN-TPBP from cytosol to nuclei** To investigate the distribution of CN-TPBP in the cells and its ligand-dependent translocation, sus.-HeLa treated with TPA (4 nM) for various periods was fractionated into cytosolic and nuclear fractions, and analyzed by HPLC. As shown in Fig. 2-a, CN-TPBP (T_R=88–96 min) was localized in cytosol of cells not treated with TPA (0 min, Fig. 2-a). No CN-TPBP was detected in the nuclear fraction prepared from cells not treated with TPA. TBP-1 and TBP-3 (T_R=70–78 min and 102–110 min, respectively) were detected in both the cytosolic and nuclear fractions prepared from the cells.

(a)

(b)

Fig. 2. Translocation of CN-TPBP. (a) HPLC analysis (the conditions were the same as Fig. 1). sus.HeLa (2.5×10^7 cells) was incubated with TPA for the period indicated on the top of each panel. Then the cells were fractionated as described in "Materials and Methods," and analyzed by HPLC (Mono Q column). Only the area of the CN-TPBP fraction; i.e., $T_R = 70$–110 min, is shown. Each fraction was incubated with [^{3}H]TPA in the absence (●) or in the presence (○) of excess TPA (800 nM). (b) Time-dependent changes of the amounts of CN-TPBP in the cytosolic and nuclear fractions prepared from sus.HeLa incubated with TPA. Horizontal scale: The period (h) of incubation with TPA. Vertical scale: TPA-specific binding activity measured as the amount of [^{3}H]TPA bound (kdpm) minus the amount of [^{3}H]TPA bound in the presence of excess TPA. ■: Cytosolic fraction. ●: Nuclear fraction.
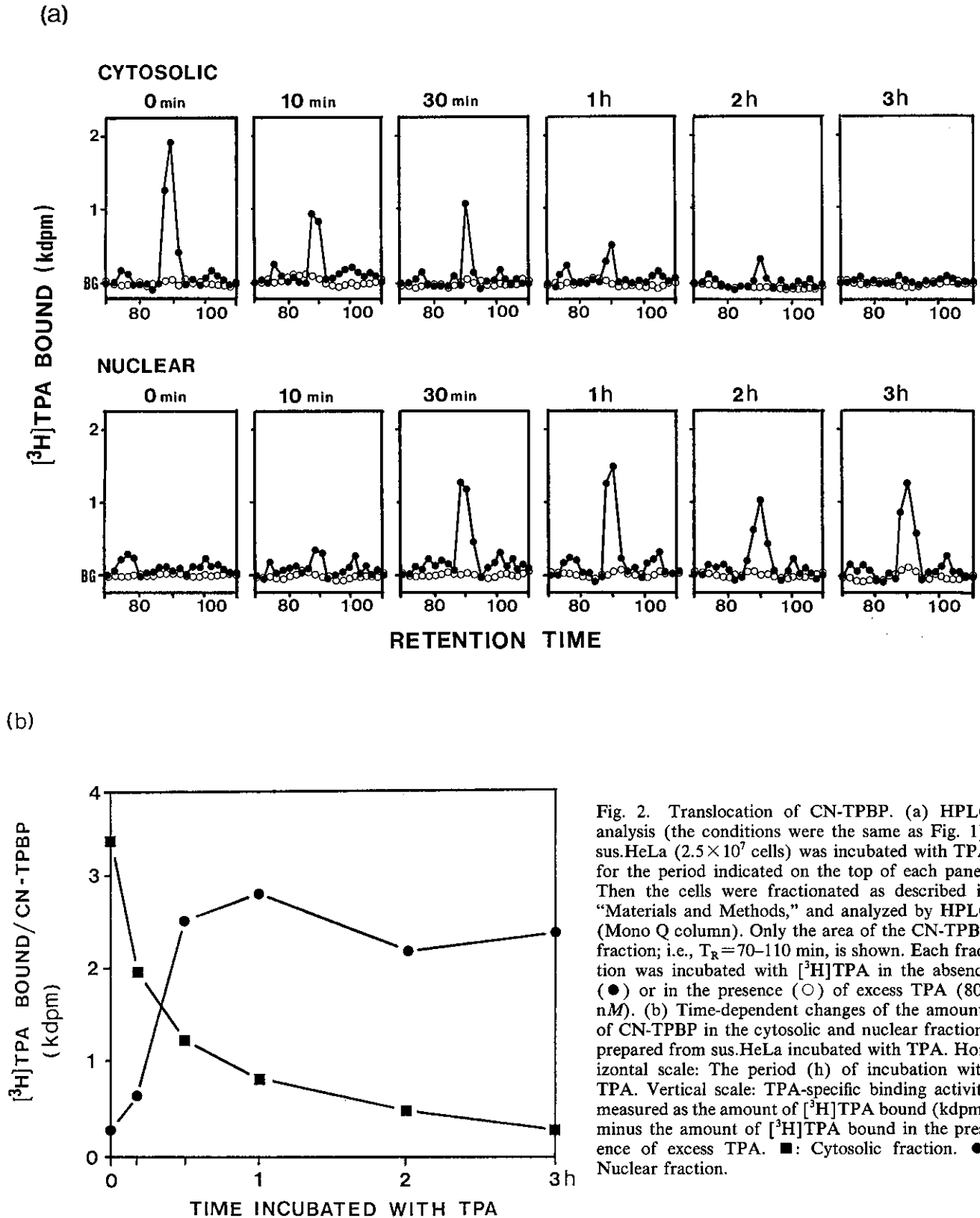
Table I. TPA-Binding and PKC Activities of Mono Q-Separated Fractions Obtained from Cytosolic and Nuclear Soluble Proteins of sus.HeLa[a]

Time treated with TPA	0.25–0.40 M NaCl[b] (PKC fraction)		0.65–0.75 M NaCl[b] (CN-TPBP fraction)	
	TPA-binding ×10² (dpm)	PKC activity (kcpm)[c]	TPA-binding ×10² (dpm)	PKC activity (kcpm)[c]
Cytosolic fraction				
0 min	206.5	825.4	34.4	−0.1
10 min	207.1	868.8	19.7	−0.1
30 min	207.7	836.5	12.2	0.4
1 h	203.1	838.6	8.7	0.3
2 h	198.6	826.1	5.0	0.6
3 h	205.7	835.4	2.6	0.2
Nuclear fraction				
0 min	3.0	3.5	2.5	0.4
10 min	3.9	3.6	6.7	−0.3
30 min	3.4	3.3	26.2	0.3
1 h	3.3	3.5	28.7	−0.1
2 h	3.6	3.2	22.5	0.0
3 h	4.3	3.3	24.5	0.3

a) Values are for 2.5×10^7 cells.
b) CN-TPBP and PKC activity were eluted from the Mono Q column with 0.65–0.75 M and 0.25–0.40 M NaCl, respectively.
c) Background (blank value) was subtracted.

Treatment of the cells with TPA (4 nM) resulted in a decrease of CN-TPBP in the cytosolic fraction, and the increase (appearance) of CN-TPBP in the nuclear fraction. The results indicate that CN-TPBP localized in the cytosol was translocated into nuclei after binding to TPA. This translocation is an early event; i.e., the translocation started within 10 min after the addition of TPA to the cell culture. The content of CN-TPBP in the nuclear fraction seemed to reach a plateau within 30 min (Fig. 2-b). After the treatment with TPA for 3 h, CN-TPBP was detected only in the nuclear fraction and no CN-TPBP was detected in the cytosolic fraction (3 h, Fig. 2-a). Of course, no PKC activity was detected in CN-TPBP fractions prepared from cytosolic or nuclear fraction (Table I).

Both the TPA-binding and PKC activities of the PKC fraction (eluted from Mono Q column with 0.25–0.40 M NaCl) were found only in the cytosolic fraction (Table I). The treatment of the cells with TPA had essentially no effect on the TPA-binding and PKC activities of the PKC fraction (Table I). No down-regulation[39] or translocation into the nuclei of these activities of the PKC fraction was observed in our experiments.

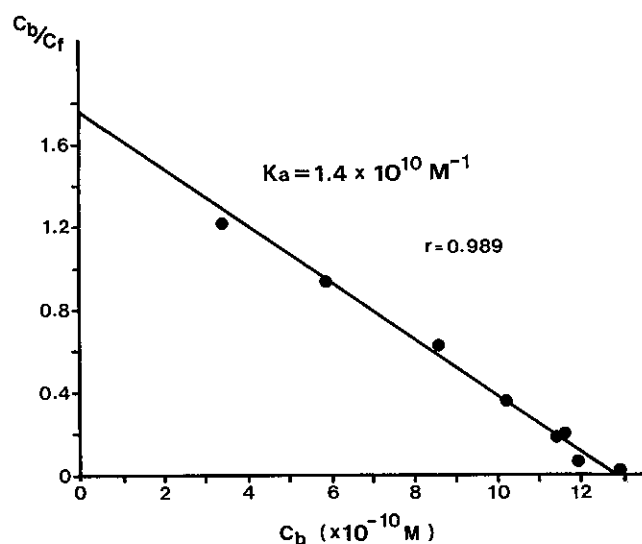The results obtained for sus.HeLa, i.e., nuclear translocation of CN-TPBP, are similar to those obtained for HL-60.[34]

Fig. 3. Scatchard analysis of the [³H]TPA/CN-TPBP complex. C_b: Concentration of bound [³H]TPA determined from the radioactivity by filter binding assay. C_f: Concentration of free [³H]TPA determined from the radioactivity of the filtrate of the filter binding assay. Ka: Association constant calculated from the slope of the regression curve drawn by the least-squares method.
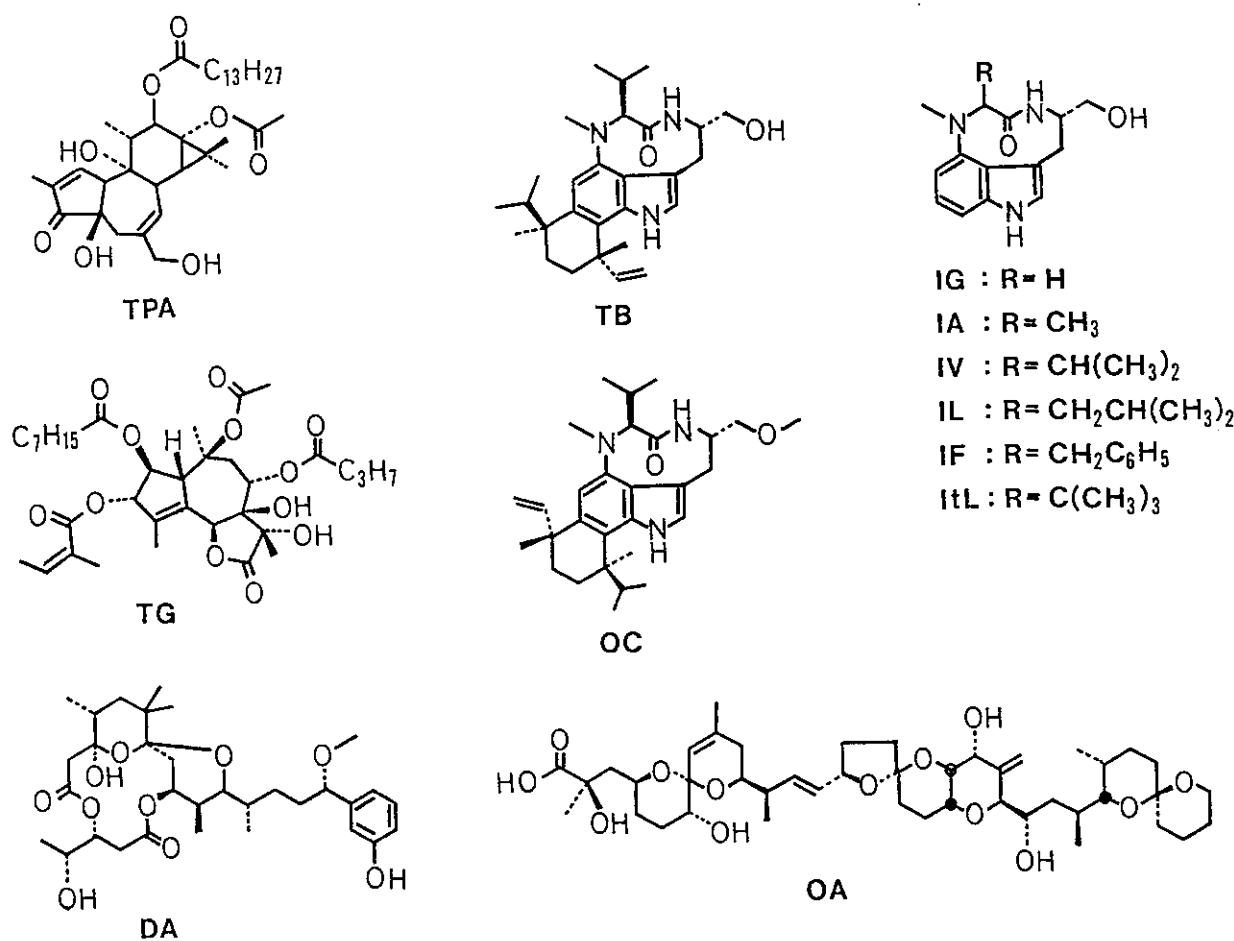
Fig. 4. Structures of tumor promoters and their analogs. TPA: 12-O-Tetradecanoylphorbol 13-acetate. TG: Thapsigargin. DA: Debromoaplysiatoxin. TB: Teleocidin B. OC: Olivoretin C. OA: Okadaic acid. IV: ($\pm$)-Indolactam-V. IG: ($\pm$)-Indolactam-G. IA: ($\pm$)-Indolactam-A. IL: ($\pm$)-Indolactam-L. IF: ($\pm$)-Indolactam-F. ItL: ($\pm$)-Indolactam-tL.

Ligand-binding of CN-TPBP The association constant of CN-TPBP with TPA was estimated by Scatchard analysis (Fig. 3). The CN-TPBP fraction was prepared from sus.HeLa whole-cell extract by successive separation using QAE-Sephadex column and Mono Q HPLC. The partially purified CN-TPBP was incubated with various concentrations of [³H]TPA at 4°C for 2 h, and then their binding was measured. Under the experimental conditions employed, good linearity was obtained by the least-squares method ($r = 0.989$, Fig. 3). The association constant was estimated to be 1.4×10^{10} M^{-1}. From the concentration of the binding sites obtained in the experiments, the amount of CN-TPBP in one cell could be estimated roughly to be 5000–10000 molecules (though the exact recovery and stability of CN-TPBP, and the efficiency of extraction of CN-TPBP were not known).

The binding affinity of CN-TPBP toward other tumor promoters and their analogs (Fig. 4) was investigated by means of binding competition assay (Fig. 5). The partially purified CN-TPBP was incubated with [³H]TPA (8 nM) in the presence or absence of various concentrations of other compounds. As shown in Fig. 5-a, CN-TPBP binds TPA, teleocidin B, thapsigargin, phorbol dibutylate, and debromoaplysiatoxin in a mutually competitive manner. These compounds are all potent tumor promoters.[3, 4] Among them, TPA, teleocidin B, phorbol dibutylate and debromoaplysiatoxin bind and activate PKC, and are classified as TPA-type tumor promoters.[3] It is noteworthy that thapsigargin, which was established to lack ability to bind or activate PKC, and is classified as a non-TPA-type tumor promoter,[3, 4] binds CN-TPBP strongly and competitively with TPA. The competitive binding of these structurally different tumor promoters is
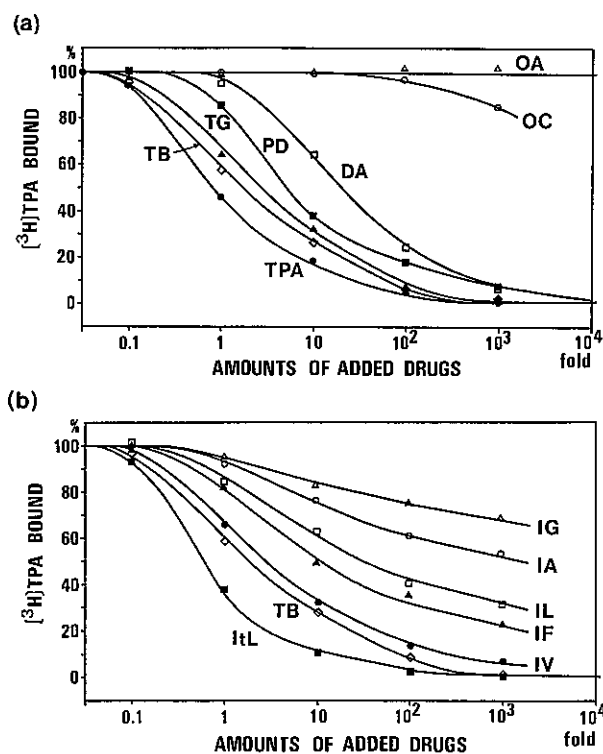
(a)

(b)

Fig. 5. Ligand-binding selectivity of CN-TPBP. The partially purified CN-TPBP was incubated with [^{3}H]TPA in the presence of various amounts of the indicated drugs. TPA-specific binding was determined by subtraction of the amount of [^{3}H]TPA bound in the presence of 1000-fold excess of TPA from the amount of [^{3}H]TPA bound in the absence of other drugs, and the value was defined as 100%. Vertical scale: TPA-specific binding (%). Horizontal scale: Amounts of added drugs (fold against the amount of [^{3}H]TPA). Abbreviations: PD: Phorbol dibutylate. Other abbreviations are the same as those in Fig. 4.

Table II. C_{50} Values for Binding to CN-TPBP and ED_{50} Values for Induction of HL-60 Cell Adhesion

Compound		C_{50} values[a] (M)	ED_{50} values[b] (M)
($\pm$)-Indolactam-G	(IG)	1.0×10^{-3}	3.8×10^{-5}
($\pm$)-Indolactam-A	(IA)	1.9×10^{-5}	3.7×10^{-6}
($\pm$)-Indolactam-V	(IV)	2.5×10^{-7}	5.3×10^{-7}
($\pm$)-Indolactam-L	(IL)	2.3×10^{-8}	9.5×10^{-7}
($\pm$)-Indolactam-F	(IF)	9.5×10^{-8}	7.7×10^{-7}
($\pm$)-Indolactam-tL	(ItL)	4.8×10^{-9}	1.1×10^{-7}

a) The C_{50} value is defined as the concentration of added compound which is needed to inhibit the specific binding of [^{3}H]TPA (8 nM) to CN-TPBP with the efficiency of 50%. The specific binding of [^{3}H]TPA (100%) was defined as the difference of the amounts of [^{3}H]TPA (8 nM) bound to CN-TPBP-fraction (0.25 mg/ml, 0.6 M NaCl-10% DMSO-20 mM Tris (pH 8.0), 4°C) in the presence and in the absence of 8 μM TPA. *b*) The values were taken from Fujiki et al.[3]

not unexpected, because the superposition of these tumor promoters has been established as feasible.[40]

A teleocidin analog, olivoretin C, which possesses no tumor-promoting activity,[35] does not bind to CN-TPBP, though the structure of biologically inactive olivoretin C is very similar to that of biologically active teleocidin B (Fig. 4). Okadaic acid, which is classified as a member of another group of tumor promoters,[3] does not bind to CN-TPBP.

We also investigated the binding affinity of synthetic analogs of teleocidin B, indolactams (Fig. 4).[3, 35] The indolactams possess different potencies of biological activity, and can be used as tools for investigation of the structure-activity relationship of teleocidin-type tumor promoters.[3, 35] From the results of ligand-binding competition assay (Fig. 5-b), we could estimate C_{50} values (the

concentration of added compounds which is needed to inhibit the specific binding of [^{3}H]TPA (8 nM) to CN-TPBP with the efficiency of 50%) as the binding affinity indices (Table II). The order of binding affinity to CN-TPBP of indolactams correlated with the HL-60 cell adhesion-inducing potency of the compounds[3] (Table II).

CN-TPBP does not bind retinoic acid, vitamin D$_3$, dioxin, glucocorticoid, or PKC-inhibitors (staurosporine and H-7) (data not shown).

Estimation of molecular weight of CN-TPBP and complex formation with hsp90 To estimate the apparent molecular weight of CN-TPBP under nondenaturing conditions, the partially purified CN-TPBP was analyzed by size exclusion HPLC (Superose 12, Pharmacia) (Fig. 6-a). A calibration curve of molecular weight versus elution volume/void volume (Ve/Vo) was obtained with molecular weight size markers. As shown in Fig. 6-a, the major peak of TPA-specific binding activity was eluted at the apparent molecular weight of 66 kDa under the experimental conditions employed here (0.6 M KCl-20 mM Tris (pH 8.0)). Though we can not exclude the possibility that the 66 kDa form is a dimer of CN-TPBP, as was the case for retinoid receptors,[37] or a complex of CN-TPBP with some other accessory protein(s) even under the high-salt conditions, we tentatively concluded that the apparent molecular weight of CN-TPBP is 66 kDa.

In Fig. 6-a, a minor peak of TPA-specific binding activity can be seen at the apparent molecular weight of 180 kDa. This 180 kDa form was recognized by anti-hsp90; i.e., addition of anti-hsp90 to CN-TPBP fraction partially purified by Mono Q HPLC resulted in the
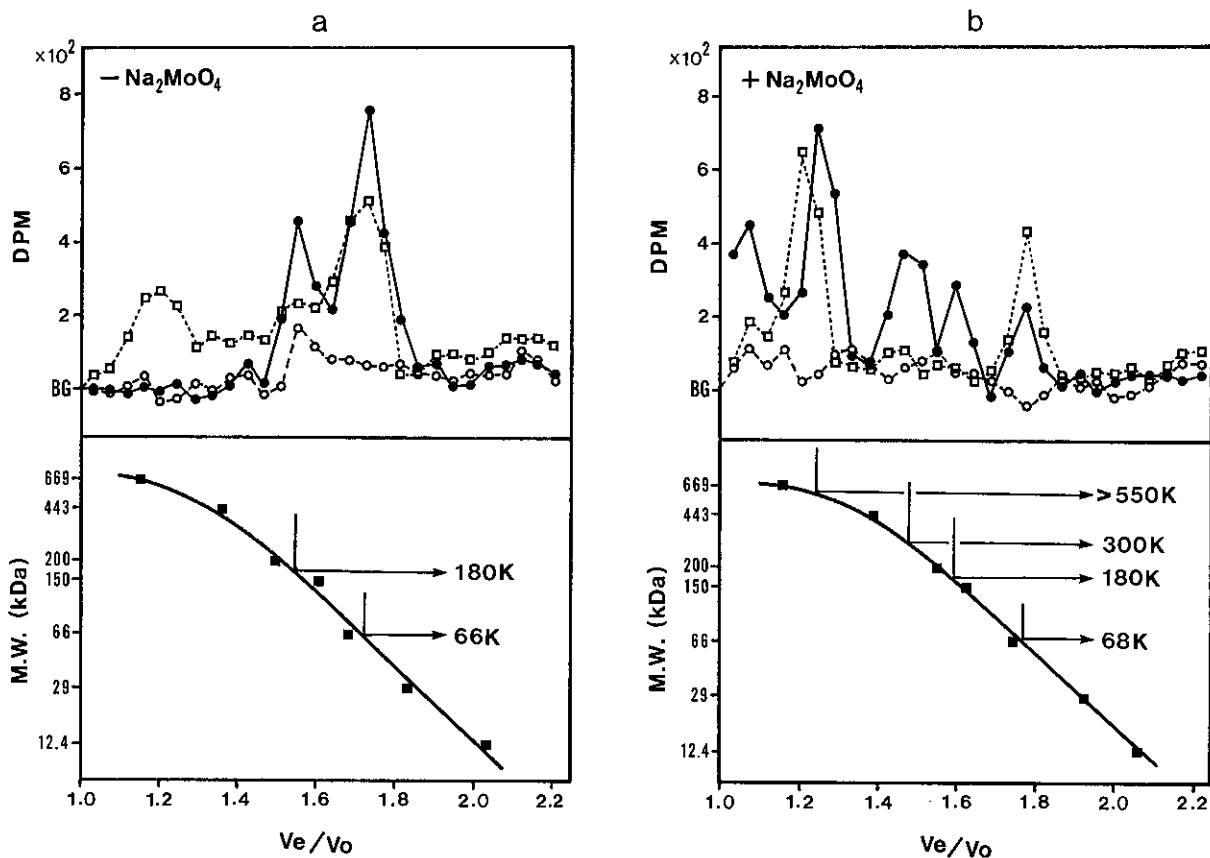
Fig. 6. Estimation of apparent molecular weight of CN-TPBP and its complex formation with hsp90. The partially purified CN-TPBP was analyzed by size exclusion HPLC. CN-TPBP fraction was prepared from sus.HeLa extract by successive separation by QAE-Sephadex and Mono Q HPLC. Column: Superose 12 HR10/30. Eluent: 0.6 M KCl-20 mM Tris (pH 8.0) in the absence (a) or in the presence (b) of 10 mM Na$_2$MoO$_4$. Flow rate: 0.5 ml/min. Horizontal scale: Elution volume (Ve)/void volume (Vo). The void volume was determined by injecting blue dextran. Upper: Monitored by assay of radioactivity of [^{3}H]TPA bound (dpm), measured by the dextran-coated charcoal method described in "Materials and Methods." Each fraction was incubated with [^{3}H]TPA in the absence (●) or in the presence (○) of excess TPA. □: The partially purified CN-TPBP was injected after incubation with anti-hsp90. Lower: The calibration curve for the estimation of apparent molecular weights. The curve was obtained by the least-squares method by injection of molecular weight markers [thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa)].

disappearance of the 180 kDa form on subsequent Superose 12 HPLC analysis. The peak of the 66 kDa form was not affected by the addition of anti-hsp90. Though we can not exclude the possibility that 180 kDa form and 66 kDa form are different TPA binding factors which are eluted together on Mono Q HPLC, the results suggest that 180 kDa form is a complex of 66 kDa CN-TPBP with hsp90 (*vide infra*).

Hsp90 is known to form a complex, which is stabilized by molybdate, with nuclear receptors such as gluco-corticoid and dioxin receptors.[41, 42] Therefore, we examined the effect of molybdate on the Superose 12 profile

of CN-TPBP fraction. As shown in the figure, the HPLC profile in the presence of 10 mM molybdate was different from that in the absence of molybdate (Fig. 6-a and b). In the presence of molybdate (Fig. 6-b), CN-TPBP was detected as a peak of 68 kDa (very close to 66 kDa, probably identical with the 66 kDa form in Fig. 6-a), which was not recognized by anti-hsp90. However, the relative amount of 68 kDa form was lower than that observed in the HPLC profile obtained in the absence of molybdate. The relative amount of 180 kDa form was correspondingly higher in the presence of molybdate. In addition, a 300 kDa form appeared in the presence of

molybdate. Both 180 and 300 kDa forms were recognized by anti-hsp90 (Fig. 6-b). In the cases of glucocorticoid and dioxin receptors, the stoichiometry of the most stable receptor/hsp90 complex is known to be receptor(1):hsp-90(2).[41, 42] Therefore, 180 kDa and 300 kDa forms might be complexes of CN-TPBP(1):hsp90(1) and CN-TPBP(1):hsp90(2), respectively.

In the presence of molybdate, a large complex of CN-TPBP (>550 kDa, at Ve/Vo of around 1.2) was also observed. Because hsp90 is known to associate with other proteins such as actin,[43] the >550 kDa form might be a complex containing CN-TPBP, hsp90 and other proteins, or aggregated oligomer.

We used the CN-TPBP fraction partially purified by MonoQ HPLC for the studies mentioned above; it is possible that contamination with hsp90, actin and some other proteins in the CN-TPBP fraction occurred. Hsp90 and actin are known to be eluted from a Mono Q column with a high concentration of salt.[42] It is also possible that the peak of CN-TPBP detected on Mono Q profile is a mixture of CN-TPBP and complexes of CN-TPBP with other proteins such as hsp90.

DISCUSSION

The protein CN-TPBP described in this paper has unique features which indicate that it could play an essential role in the biological effects elicited by tumor promoters.

CN-TPBP exists in cytosol and translocates into nuclei after binding a tumor promoter. The translocation starts to occur early (within 10 min after the addition of TPA). Some of the effects of TPA on the expression of specific genes such as c-fos are known to occur during 10–30 min after TPA addition.[6, 7] The translocation of CN-TPBP would precede the response of cells to TPA at the mRNA level. Such fast ligand-dependent translocation is well known for glucocorticoid receptor.[31, 32]

CN-TPBP binds TPA with a association constant of the order of 10^{10} M^{-1}, a value which corresponds to the effective concentration of TPA in some fundamental biological assay systems (usually of the order of pM). CN-TPBP binds TPA-type tumor promoters such as TPA, phorbol dibutylate, teleocidin B and aplysiatoxin in a mutually competitive manner. The lack of binding activity of CN-TPBP toward biologically inactive olivoretin C, possessing a quite similar structure to biologically active teleocidin B, suggests the importance of the binding of tumor promoters to CN-TPBP. The correlation between the binding affinity of the synthetic indolactam analogs to CN-TPBP and the HL-60 cell adhesion-inducing potency of the compounds also suggests the importance of the binding to CN-TPBP.

In addition, CN-TPBP binds a non-TPA-type tumor promor, thapsigargin. Though the biological effects such as tumor promotion of thapsigargin are similar to those of TPA-type (PKC-activating) tumor promoters, the effects of thapsigargin cannot be explained in terms of the participation of PKC, because thapsigargin does not bind to or activate PKC. The presence of a binding protein, CN-TPBP, which is common for TPA-type tumor promoters and thapsigargin, raises the possibility of a uniform interpretation of the molecular mechanism for TPA- and at least some non-TPA-type tumor promoters. Because not all of the PKC-activating compounds are tumor promoters, and not all of the PKC inhibitors are inhibitors of tumor promoters, the essential role of PKC in tumor promotion is doubtful. The binding spectrum of CN-TPBP, which is wider than that of PKC, might suggest a possible role of CN-TPBP in the general biological effects elicited by tumor promoters. However, okadaic acid, which is also a potent non-TPA-type tumor promoter, does not bind CN-TPBP. It presumably exerts its tumor-promoting activity via a pathway other than the PKC-participating or the CN-TPBP-participating pathway. The existence of a receptor-like specific binding protein for okadaic acid in cytosolic and membrane fractions has been demonstrated.[44]

CN-TPBP possesses the ability to form complexes with hsp90 which are stabilized by molybdate. This feature is general for the steroid/thyroid receptor superfamily, such as receptors of glucocorticoid and dioxin, as well as the feature of ligand-dependent translocation into nuclei. Such characteristics of CN-TPBP raise the possibility that CN-TPBP is a member of the steroid/thyroid receptor superfamily or related transcription factors. The similarity of the mode of action elicited by tumor promoters with steroids, thyroid hormones, vitamins A and D also suggests the existence of specific receptors which act as ligand-dependent transcription factors. Hsp90 might act as a modulator of the specific DNA binding activity of CN-TPBP, or an inhibitor of the translocation of CN-TPBP into nuclei, as has been found for receptors of glucocorticoid and dioxin.[31, 32, 41, 42] As far as investigated, CN-TPBP fulfills the criteria for the protein to be a receptor of tumor promoters, and it might be a member of the steroid/thyroid receptor superfamily or related nuclear receptors. If this is the case, it would be of great interest to identify the intrinsic (natural, indigenous) ligand(s) of CN-TPBP which functions in the cells under normal conditions.

In conclusion, we have described the existence of CN-TPBP, which has unique features suggesting that the protein may play an essential role in the biological effects elicited by tumor promoters; i.e., (1) ligand-dependent translocation into nuclei, (2) a wide ligand-binding spectrum, (3) a correlation between the ligand-binding

affinity and the biological effects elicited by the ligands, and (4) complex formation with hsp90 and its stabilization by molybdate. By showing the existence of a possible additional pathway other than the pathway of PKC activation, the data presented in this paper require a re-evaluation of the currently prevailing hypothesis that PKC is the tumor promoter receptor.

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