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Phorbol Ester-induced G1 Arrest in BALB/MK-2 Mouse Keratinocytes Is Mediated by δ and η Isoforms of Protein Kinase C

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We investigated the possible negative regulation of the cell cycle by protein kinase C (PKC) isoforms in synchronously grown BALB/MK-2 mouse keratinocytes, in which PKC isoforms were overexpressed by using the adenovirus vector Ax. Cells at the G1/S boundary of the cell cycle were the most sensitive to the inhibitory effect of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a PKC agonist, resulting in G1 arrest. TPA-induced inhibition of DNA synthesis was augmented by overexpression of the η and δ isoforms, but rescued by the dominant-negative and antisense η isoforms. In contrast, the α and ζ isoforms showed no effect on DNA synthesis with or without TPA treatment. Immunoblotting indicated cell cycle-dependent expression of the η isoform, being highest in cells at the G1/S boundary. The present study provides evidence that the η and δ isoforms of PKC are involved in negative regulation of cell cycle at the G1/S boundary in mouse keratinocytes.

Key words: PKC — G1 arrest — Mouse keratinocytes

PKC has been implicated as a key enzyme in the regulation of signal transduction pathways of cell growth and differentiation.[1,2] Agonists of PKC such as TPA stimulate or inhibit DNA synthesis depending on the cell type and the conditions. In epithelial or endothelial cells, PKC agonists inhibit DNA synthesis and cell growth,[3–8] suggesting that under certain conditions PKC may mediate inhibitory signaling of cell cycle progression.

PKC is a complex family of 10 isoforms, which can be divided into three groups based on their structures and activation mechanisms; (a) Ca^{2+}, PS and DG-dependent conventional PKC (α, βI, βII, and γ isoforms); (b) Ca^{2+}-independent novel PKC (δ, ε, η, and θ isoforms); (c) Ca^{2+} and DG-independent atypical PKC (ζ and λ/ι isoforms). We have cloned the η and θ isoforms from a cDNA library of mouse skin.[9,10] Unlike other members of the PKC family, the η isoform is expressed predominantly in epithelial tissues, such as the skin, tongue, esophagus, stomach, intestine, trachea and bronchus. *In situ* hybridization and immunohistochemical studies of various epithelial tissues showed that the η isoform is localized in differentiated or differentiating epithelial cells rather than in the proliferating basal cells.[11] In the skin, it is localized exclusively in the granular layer, which is composed of terminally differentiated keratinocytes with keratohyalin granules.

In a previous study, we found that the η isoform is activated to a much greater extent by cholesterol sulfate than by PS plus phorbol ester.[12] In the presence of cholesterol sulfate, phorbol ester only marginally activated the η isoform, suggesting that cholesterol sulfate itself acts as a second messenger for the η isoform. We also demonstrated that cholesterol sulfate is a transcriptional activator of transglutaminase 1[13], a key enzyme for keratinization that crosslinks precursor proteins of the cornified envelope.[14] Cholesterol sulfate inhibits tumor promotion when applied topically at the tumor promotion phase in the two-stage carcinogenesis of mouse skin.[15]

In the present study, we investigated the possible involvement of PKC isoforms in TPA-induced growth inhibition of synchronously growing BALB/MK-2 keratinocytes, into which the PKC-isoform genes were introduced using an adenovirus vector. The results provide evidence that the cell cycle machinery in mouse keratinocytes is regulated negatively by the η and δ isoforms of PKC.

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The abbreviations used are: ATP, adenosine 5-triphosphate; DG, diacylglycerol; DMSO, dimethylsulfoxide; EGF, epidermal growth factor; EGTA, ethyleneglycol bis(2-aminoethylether)tetracetic acid; FCS, fetal calf serum; PKC, protein kinase C; PS, phosphatidylserine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Cell culture and synchronization BALB/MK-2, a cell line derived from mouse keratinocytes,[16] was grown at 37°C in Eagle's minimum essential medium (Gibco BRL Life Technologies, Inc., Grand Island, NY) containing a low concentration of calcium (0.05 m*M*), 8% dialyzed FCS and 4 ng/ml of EGF. BALB/MK-2 cells were synchronized in the G0/G1 phase by EGF depletion for 3 days and subsequent stimulation of cell growth by the addition of EGF at 4 ng/ml.[17] The cells were grown in 12-well culture plates, and were sampled at the indicated time-points for determination of DNA synthesis, sensitivity to TPA and expression of the PKC isoforms.

DNA synthesis and flow cytometry DNA synthesis was determined by measuring the incorporation of [methyl-^{3}H]thymidine (1 μCi/ml)(Amersham International, Buckinghamshire, UK) for 2 h. After having been washed three times with phosphate-buffered saline, the cells were solubilized with 0.5 *N* NaOH and neutralized with 1 *N* HCl. Trichloroacetic acid was added at a final concentration of 10%, followed by filtration through glass-fiber filters (Whatman GF/C, Whatman International Ltd., Maidstone, UK) and measurement in a liquid scintillation counter. For cell cycle analysis, the cells were suspended in citrate buffer (250 m*M* sucrose, 40 m*M* sodium citrate and 5% DMSO); 0.003% trypsin and 0.1% Nonidet P-40 were added and the suspension was rotated at room temperature for 10 min. After addition of 0.5 μg/ml trypsin inhibitor and 100 μg/ml ribonuclease A, the cells were stained with propidium iodide (416 ng/ml) for 20 min in ice in the dark, followed by analysis by flow cytometry using a Becton-Dickinson FACScan and the SFIT program (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Antibodies Polyclonal antibody against the η isoform was raised in rabbits using a synthetic peptide of its D4 region having the amino acid sequence I^{666}NQDEFRNFSYVSPELQL^{683}.[11] Anti-α and -ζ antibodies and anti-cdk2 antibody were purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-δ antibody was purchased from Seikagaku Kogyo (Tokyo).

cdk2 kinase activity Immunoprecipitates by anti-cdk2 antibody were subjected to measurement of kinase activity using H1 histone as a substrate. In brief, 50 μg of cellular protein was immunoprecipitated with 1 μg of the anti-cdk2 antibody in 200 μl of a lysis buffer containing 50 m*M* Tris-HCl (pH 7.4), 120 m*M* NaCl, 0.5% Nonidet P-40, 100 m*M* NaF, 2 m*M* EGTA, 50 μg/ml leupeptin, 50 μg/ml aprotinin, 0.5 m*M* phenylmethylsulfonyl fluoride. Immunocomplexes bound to protein A-Sepharose beads were resuspended in 40 μl of reaction buffer containing 50 m*M* Tris-HCl (pH 7.4), 10 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 1 μ*M* histone H1 (Boehringer Mannheim, Mannheim, Germany), 1 μ*M* ATP and 5 μCi [γ-^{32}P]ATP, and incubated for 10 min at 25°C, followed by SDS-PAGE on 12% gel and autoradiography.

Immunoblotting Cells were solubilized in standard SDS lysis buffer and boiled at 95°C. Protein extracts were subjected to SDS-PAGE at 8% and blotted onto nitrocellulose membrane filters. After having been blocked with 3% gelatin, the membranes were probed with the respective antibodies, followed by treatment with horseradish peroxidase conjugated with goat anti-rabbit IgG antibody. Enhanced chemiluminescence (Du Pont NEN, Boston, MA) was used for detection.

Autophosphorylation Cells were solubilized in 0.5% Triton-X 100 in an extraction buffer containing 250 m*M* sucrose, 2 m*M* EDTA, 0.5 m*M* EGTA, 50 m*M* 2-mercaptoethanol, 100 μg/ml leupeptin and 2 m*M* phenylmethylsulfonyl fluoride. The η isoform was immunoprecipitated with anti-η antibody and protein A-Sepharose in the extraction buffer without 2-mercaptoethanol at 4°C. The immunoprecipitate was incubated in 50 μl of an autophosphorylation reaction mixture containing 20 m*M* Tris-HCl (pH 7.5), 1 μCi of [γ-^{32}P]ATP (6000 Ci/mmol), 5 m*M* magnesium acetate, 1 m*M* EGTA, 50 μg/ml PS, 50 ng/ml TPA, 100 mg/ml leupeptin, 2 m*M* ortho-sodium vanadate and 20 μ*M* ATP, in ice for 20 min, followed by SDS-PAGE at 8% and autoradiography.

Adenovirus constructs The recombinant adenovirus vector pAxCAwt was derived from human adenovirus 5 lacking the E1 and E3 regions.[18] It contained a composite CAG promoter consisting of cytomegalovirus immediate early enhancer, chicken β-actin promoter, a rabbit β-globin polyadenylation signal which evokes strong induction of any inserted cDNA.[19] PKC cDNAs encoding the rabbit α isoform,[20] mouse δ isoform,[21] mouse η isoform,[9] dominant-negative η isoform and mouse ζ isoform[11] were blunted by Klenow fragment and inserted into the *Swa*I site of pAxCAwt.[24] These cosmids were co-transfected with Ad5DIXDNA-TPC by the calcium phosphate method into 293 cells, in which recombinant adenovirus vectors were generated through homologous recombination.[22] The control *lacZ* gene construct (AxCA-lacZ) carrying *β-galactosidase* gene was a gift from Dr. I. Saito (Inst. of Medical Science, Univ. of Tokyo, Tokyo). Cells were harvested 48 h after infection and lysed by repeated freezing and thawing. Virus titers were determined in terms of the plaque-forming activity in 293 cells.[23] For each vector, a single batch of the adenovirus stock with a high titer was used.

Construction of a dominant-negative η isoform of PKC A dominant-negative mutant of the η isoform was generated by substitution of the lysine residue with alanine at the ATP-binding site (384 codon) in the catalytic domain.[24]

RESULTS

Synchronization of cell cycle In order to examine the involvement of the PKC isoforms in cell cycle progression, BALB/MK-2 cells were arrested at the G0/G1 phase by EGF depletion for 3 days, and subsequently the cell cycle was allowed to progress synchronously by the addition of 4 ng/ml EGF (time 0). As shown in Fig. 1A, DNA synthesis increased after a lag time of 9 h, reaching a peak at 21 h. Flow cytometric analysis indicated pro-

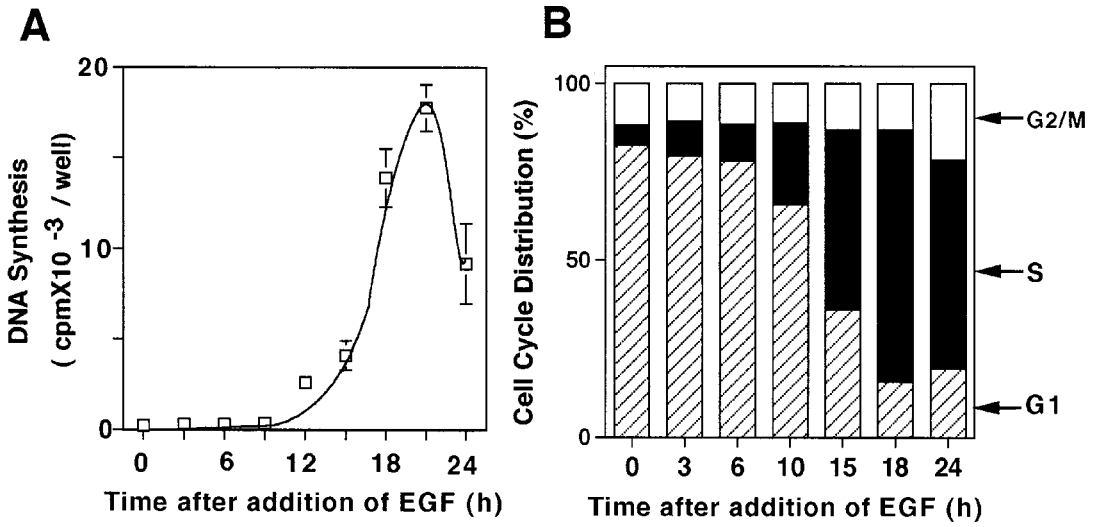

Fig. 1. Synchronous culture of BALB/MK-2 cells in terms of DNA synthesis (A) and cell cycle analysis (B). A. BALB/MK-2 cells were arrested at the G0/G1 phase by EGF depletion for 3 days, followed by the addition of 4 ng/ml EGF to allow synchronous progression of the cell cycle. DNA synthesis was measured by [^{3}H]thymidine pulse-labeling for 2 h. Vertical bars indicate SDs (n=3). B. Analysis of cell cycle progression using flow cytometry.

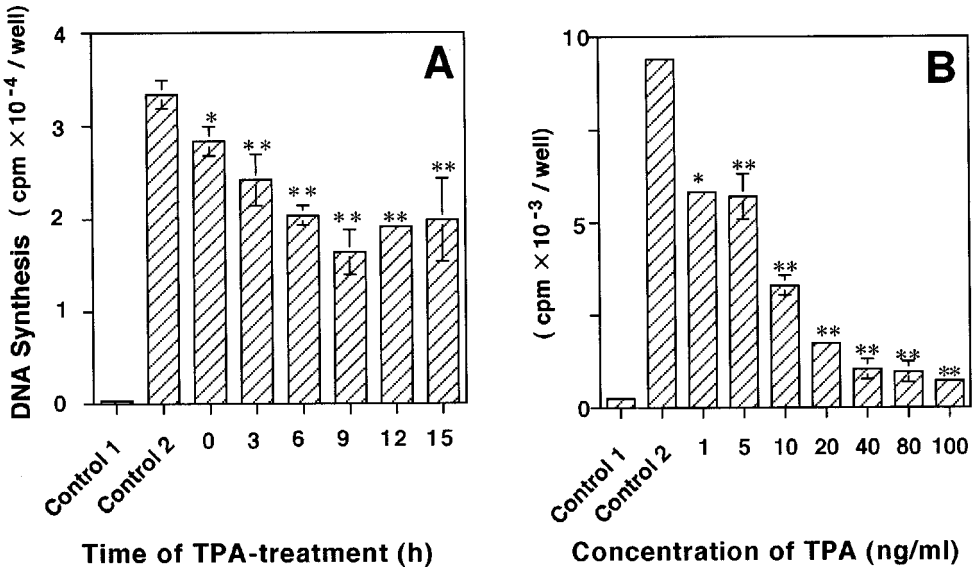

Fig. 2. Cell cycle-dependent (A) and dose-dependent (B) inhibition of DNA synthesis by TPA in synchronously growing BALB/MK-2 cells. A. TPA at 20 ng/ml was added for 2 h at the indicated time points to synchronously growing BALB/MK-2 cells. [^{3}H]Thymidine incorporation into DNA was determined at 19–21 h. B. Synchronous cultures were treated at 9 h with TPA at concentrations in the range of 1–100 ng/ml, followed by measurement of DNA synthesis at 19–21 h. Control 1, negative control without EGF addition or TPA treatment; control 2, positive control with EGF addition at 0 h but without TPA treatment. *, ** Significant difference from the control 2 at P<0.05 or P<0.01, respectively.

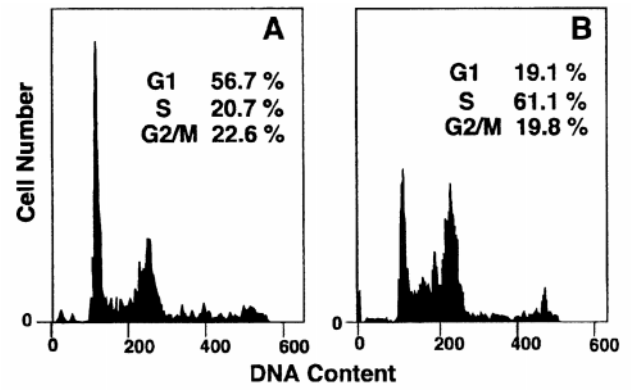

Fig. 3. G1 arrest of cells treated with TPA(20 ng/ml) at 10–12 h after the addition of EGF (A), in comparison with cells treated with TPA at 0–2 h (B).

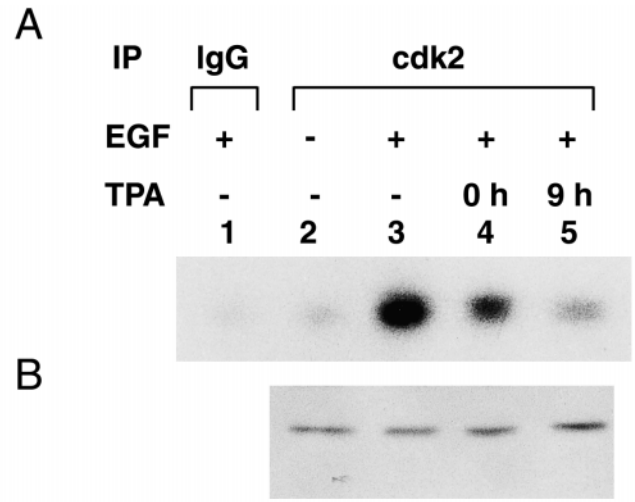

Fig. 4. Cell cycle-dependent inhibition of cdk2 kinase activity (A) without change in the protein expression level (B). A. Lane 1, control IgG; lane 2, negative control without EGF addition or TPA treatment corresponding to control 1 of Fig. 2; lane 3, positive control with EGF addition corresponding to control 2 of Fig. 2; lane 4, treated with 10 ng/ml TPA at 0–2 h after the addition of EGF; lane 5, treated with TPA at 9–11 h.

gression of the cell cycle from the G1 to the S phase: cells in S phase constituted 6% of the total cell population at time 0, and the percentage increased with time, with 70% of the population being in the S phase at 18 h (Fig. 1B).

Cell cycle-dependent inhibition of DNA synthesis by TPA Phorbol esters inhibit the growth of normal human keratinocytes and mouse BALB/MK-2 keratinocytes, but not of fibroblasts (data not shown). To examine the possible association of the cell cycle with phorbol ester-

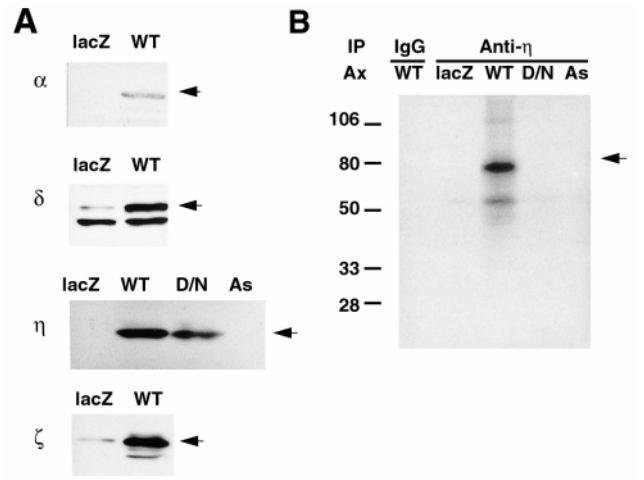

Fig. 5. Expression (A) and kinase activity (B) of PKC isoforms introduced by Ax, an adenovirus vector, into BALB/MK-2 cells. A. Ax vectors carrying the PKC isoform cDNAs were infected at a multiplicity of infection (m.o.i.) of 6 into BALB/MK-2 cells. After 24 h, total cell lysates were subjected to SDS-PAGE on 8% gel and probed with the antibodies against the PKC isoforms. WT, wild-type; D/N, dominant-negative; As, antisense. Ax-*lacZ* was used as the control. B. BALB/MK-2 cells were infected by Ax carrying the η isoform and its mutants. The immunoprecipitated η isoform from Triton X-100-soluble fraction was incubated for autophosphorylation in the presence of 50 μg/ml PS, 50 ng/ml TPA and [γ-^{32}P]ATP, then subjected to SDS-PAGE on 8% gel and autoradiography.

induced growth arrest, a synchronous culture of BALB/MK-2 cells was pulse-exposed to 20 ng/ml of TPA for 2 h at the indicated time-points after the addition of EGF, followed by measurement of DNA synthesis at 19–21 h by assessing [^{3}H]thymidine incorporation after incubation for 2 h.

As shown in Fig. 2A, the inhibitory effect of TPA was cell cycle-dependent, being most evident at 9 h after the addition of EGF, i.e., on cells at the G1/S boundary. Inhibition of DNA synthesis by TPA at this phase was dose-dependent within a dose range of 1–100 ng/ml (Fig. 2B). Flow cytometric analysis demonstrated that TPA treatment at 10 h led to accumulation of cells at the G1 phase (Fig. 3). This inhibition was accompanied by a decrease in cdk2 kinase activity, while the level of expression of the protein remained unchanged (Fig. 4).

These results indicate that cells at the G1/S boundary of the cell cycle are the most sensitive to TPA treatment, resulting in G1 arrest.

Involvement of the δ and η isoforms in TPA-induced arrest of cell cycle To examine the possibility that a PKC isoform(s) mediates the cell cycle arrest induced by TPA, we used the adenovirus vector Ax, which allows strong expression of the inserted PKC isoforms and their

mutants at an efficiency of 100% under the control of the composite CAG promoter in BALB/MK-2 cells. As shown in Fig. 5A, the α, δ, η and ζ isoforms of PKC were strongly expressed in cells infected with the Ax containing the respective isoforms, compared to Ax-*lacZ*-infected control cells. Immunoblotting detected expression

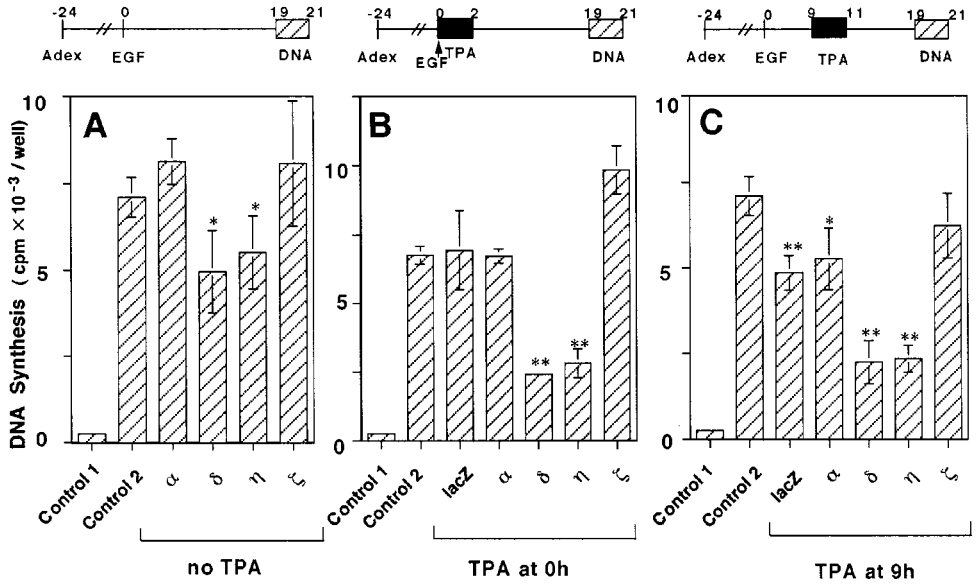

Fig. 6. Cell cycle-dependent, TPA-induced inhibition of DNA synthesis in synchronously growing BALB/MK-2 cells that overexpress the α, δ, η and ζ isoforms of PKC. A, Without TPA treatment; B, TPA treatment at 0–2 h (G0/G1 phase); C, TPA treatment at 9–11 h (G1/S boundary). BALB/MK-2 cells were infected with Ax-*lacZ* (lacZ) or Ax-PKC isoform cDNAs (α, δ, η and ζ) 24 h before the addition of EGF. These cultures were followed by no treatment (A), TPA (10 ng/ml) treatment at 0–2 h (G0/G1 phase) (B), or TPA (10 ng/ml) treatment at 9–11 h (G1/S boundary) (C). DNA synthesis was measured at 19–21 h. These experimental protocols are schematically illustrated on top of the panels. Control 1, a negative control without EGF or TPA treatment; control 2, a positive control with EGF addition at 0 h but without TPA treatment. Vertical bars, SDs of 3 samples. *, ** Significant difference from the control 2 at $P<0.05$ or $P<0.01$, respectively.

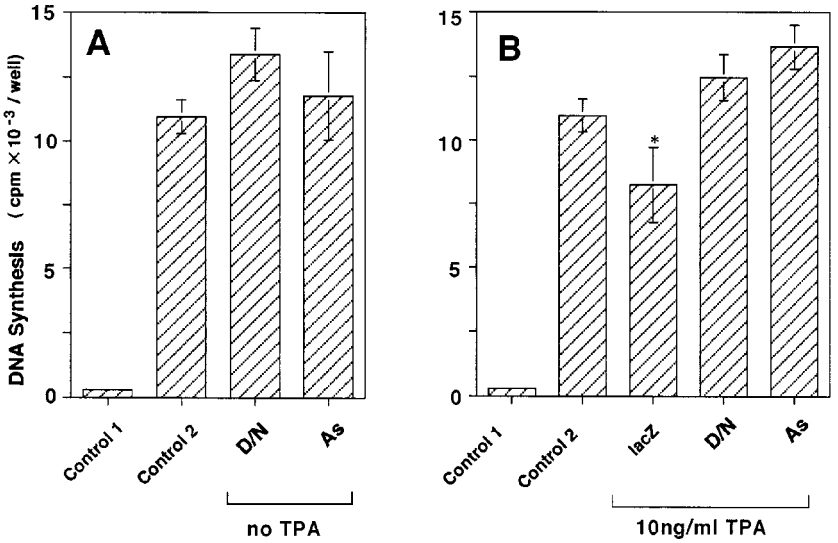

Fig. 7. Dominant-negative η and antisense η rescued the TPA-induced inhibition of DNA synthesis. BALB/MK-2 cells were infected with Ax-*lacZ*, dominant-negative η (D/N) or antisense η (As) 24 h before the addition of EGF. These cultures were treated without (A) or with 10 ng/ml of TPA at 9–11 h (B). Vertical bars indicate SDs (n=3). Controls 1 and 2, see the legend to Fig. 6.

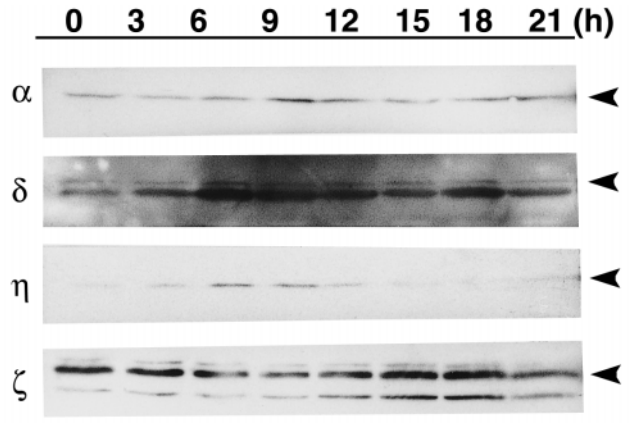

Fig. 8. Cell cycle-dependent expression of PKC isoforms in synchronously growing BALB/MK-2 cells. Expression of the α, δ, η and ζ isoforms was examined by immunoblotting at the indicated time points. The locations of these isoforms were determined by a parallel run of Ax-infected cells overexpressing the isoforms.

of the dominant-negative η isoform, but not of the antisense η isoform. Kinase activity was demonstrated by autophosphorylation of the immunoprecipitated η isoform (Fig. 5B).

When synchronous cultures of BALB/MK-2 cells were infected with the Ax-PKC 24 h before the addition of EGF, the δ and η isoforms were found significantly to suppress DNA synthesis at 19–21 h, whereas no inhibition was observed with the α and ζ isoforms (Fig. 6A). This result indicates that overexpression of the δ or η isoform is inhibitory to DNA synthesis.

In the experiments shown in Fig. 6, B and C, Ax-infected synchronous cultures were treated with TPA for 2 h, at 0 h (G0/G1 phase) and 9 h (G1/S boundary), followed by measurement of DNA synthesis at 19–21 h. DNA synthesis was inhibited by TPA treatment at 9 h, but not at 0 h (control 2 versus lacZ in Fig. 6C; also see Fig. 2A), indicating that cells at the G1/S boundary are sensitive to the inhibitory effect of TPA. In the cells overexpressing the δ or η isoform, DNA synthesis was remarkably inhibited by TPA treatment at both 0 h and 9 h (Fig. 6, B and C, respectively). Overexpression of the δ and η isoforms thus augmented TPA-induced inhibition at the G1/S boundary. In contrast, no TPA-induced inhibition was observed in cells overexpressing the α and ζ isoforms. These data suggest that the δ and η isoforms mediate the inhibitory effect of TPA.

Further evidence of the involvement of the η isoform was obtained from experiments in which the dominant-negative and antisense η isoforms were applied. The dominant-negative mutant was generated by site-directed mutagenesis at the ATP-binding site in the catalytic domain of the η isoform. As shown in Fig. 7, the dominant-negative η and antisense η were found to rescue the inhibitory effect of TPA at 9 h (Fig. 7B), while in the absence of TPA treatment, the dominant-negative and antisense η showed no effect on DNA synthesis (Fig. 7A). These results indicate that the η isoform is involved in TPA-induced inhibition at the G1/S boundary.

Expression of PKC isoforms during cell cycle Possible cell cycle-dependent expression of the α, δ, η and ζ isoforms was examined using synchronous cultures of BALB/MK-2 cells. As seen in Fig. 8, immunoblotting of the η isoform increased 6–9 h after EGF addition, i.e., at the G1/S boundary, while that of the ζ isoform decreased. The expression levels of the α and δ isoforms remained unchanged with progression of the cell cycle. The expression pattern of the η isoform coincides with the sensitivity to the inhibitory effect of TPA.

DISCUSSION

In the present study, we demonstrated negative regulation of the cell cycle by the η and δ isoforms of PKC in BALB/MK-2 keratinocytes, based mainly on the following findings; a) cells at the G1/S boundary are the most sensitive to TPA-induced inhibition of DNA synthesis, resulting in G1 arrest; b) overexpression of the η and δ isoforms using adenovirus vectors inhibits DNA synthesis; c) TPA-induced inhibition of DNA synthesis is augmented by the η and δ isoforms, but rescued by the dominant-negative and antisense η isoform; d) the α and ζ isoforms have no such effect on DNA synthesis; e) the η isoform is expressed in cells at the G1/S boundary at higher levels than in the other phases of the cell cycle.

We used a synchronous culture system of BALB/MK-2 cells, in which the cells were arrested at the G0/G1 phase by EGF depletion and then stimulated to grow synchronously by the addition of EGF.[17] Results of pulse treatment with TPA suggest that TPA arrests cell growth before entering the S phase, resulting in accumulation of cells at the G1 phase. However, once the cells had entered the S phase, no inhibition of DNA synthesis was induced by TPA treatment.

Expression of the η isoform was found to be associated with the cell cycle, being at a high level at the G1/S boundary, i.e., at the phase most sensitive to TPA-induced inhibition of DNA synthesis. To our knowledge, no report has been published previously on cell cycle-associated expression of PKC isoforms. The higher level of expression of the η isoform in cells at the G1/S boundary may be linked to TPA sensitivity.

One of the standard strategies used for elucidating the possible function of a gene of interest, is to generate cell lines that express it at a higher level. However, epithelial cells possess a drawback, in that it is difficult to introduce

foreign genes into these cells using conventional transfection methods. We overcame this problem by using an adenovirus vector,[18, 19] which allows introduction of foreign genes into keratinocytes with 100% efficiency and expression of these genes at a high level for at least 72 h.[24] Using this method, we found that the introduction of the η and δ isoforms by itself resulted in inhibition of DNA synthesis in synchronously growing BALB/MK-2 cells. Further, TPA-induced inhibition was counteracted by introduction of the dominant-negative and the antisense η isoforms. Although no further experiments were done with the dominant-negative or antisense δ isoform, these observations strongly suggest that the η and δ isoforms are involved in TPA-induced inhibition of DNA synthesis in BALB/MK-2 cells.

Several reports have been published on the involvement of PKC isoforms in the regulation of the cell cycle. Livneh *et al.*[25] demonstrated that ectopic expression of the η isoform in NIH3T3 cells resulted in inhibition of cell cycle progression and also induction of adipocyte differentiation. Although the η isoform is expressed at a high level in epithelial cells in close association with differentiation,[11] this experiment suggests that the η isoform acts as a regulator of cell cycle and an inducer of cell differentiation under ectopic situations as well as under physiological conditions.

Watanabe et al.[26] demonstrated that TPA treatment of CHO cells overexpressing the δ isoform blocked cell cycle progression. This inhibition was specific to the δ isoform: the growth of CHO cells overexpressing the α, βII or ζ isoforms was not inhibited. It was also specific to the G2/M phase, based on the results of flow cytometric and morphological analysis. Harrington *et al.*[8] reported that DNA synthesis in capillary endothelial cells was markedly inhibited by TPA treatment when the δ isoform was overexpressed, whereas it was enhanced when the α isoform was overexpressed. Cell-cycle analysis suggested that the δ isoform-overexpressing cells enter the S phase inappropriately and are delayed in passage through the S phase. In vascular smooth muscle cells, phorbol ester and DG potently inhibited serum-stimulated DNA synthesis and transition from the G1/S boundary.[4–6] However, the inhibitory effect was not observed in TPA-pretreated cells in which at least the α and ε isoforms were down-regulated. Frey *et al.*[7] demonstrated that treatment with PKC agonists resulted in cell cycle arrest in the G1 phase, in parallel with the activation and subsequent down-regulation of the α, δ and ε isoforms. In rat renal proximal epithelial cells, treatment with phorbol ester caused stimulation of cell growth and down-regulation of the α isoform.[27] These studies suggest the apparent association of phorbol ester-induced inhibition/stimulation of DNA synthesis with down-regulation of a particular isoform. But such an association in itself does not imply that the observed effect is mediated by the down-regulated isoform.

The specific effects of individual PKC isoforms on DNA synthesis and cell proliferation vary according to cell type and cellular conditions. These specificities may be mediated by substrates or effectors with tissue or cell-type specificity. A full understanding of the mechanism by which PKC isoforms mediate cell-type specific regulation will require identification of their downstream pathways.

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