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# Calphostin C Induces Tyrosine Dephosphorylation/Inactivation of Protein Kinase FA/GSK-3α in a Pathway Independent of Tumor Promoter Phorbol Ester-Mediated Down-Regulation of Protein Kinase C

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**Abstract** The signal transduction mechanism of protein kinase FA/GSK-3α by tyrosine phosphorylation in A431 cells was investigated using calphostin C as an inhibitor for protein kinase C (PKC). Kinase FA/GSK-3α could be tyrosine-dephosphorylated and inactivated to ~10% of control in a concentration-dependent manner by 0.1–10 μM calphostin C ($IC_{50}$, ~1 μM), as demonstrated by immunoprecipitation of kinase FA/GSK-3α from cell extracts, followed by phosphoamino acid analysis and by immunodetection in an antikinase FA/GSK-3α immunoprecipitate kinase assay. In sharp contrast, down-regulation of PKC by 0.05 μM calphostin C ($IC_{50}$, ~0.05 μM for inhibiting PKC in cells) or by tumor promoter phorbol ester TPA was found to have stimulatory effect on the cellular activity of kinase FA/GSK-3α, when processed under identical conditions. Furthermore, TPA-mediated down-regulation of PKC was found to have no effect on calphostin C-mediated tyrosine dephosphorylation/inactivation of kinase FA/GSK-3α. Taken together, the results provide initial evidence that the PKC inhibitor calphostin C may induce tyrosine dephosphorylation/inactivation of kinase FA/GSK-3α in a pathway independent of TPA-mediated down-regulation of PKC, representing a new mode of signal transduction for the regulation of this multisubstrate/multifunctional protein kinase by calphostin C in cells. Since kinase FA/GSK-3α is a possible carcinoma dedifferentiation/progression-promoting factor, the results further suggest calphostin C as a potential anticancer drug involved in blocking carcinoma dedifferentiation/progression, possibly via inactivation of protein kinase FA/GSK-3α in tumor cells. 

**Key words:** protein kinase FA/GSK-3α, PKC inhibition, calphostin C, down-regulation, carcinoma dedifferentiation/progression

Protein kinase FA was originally identified as an activating factor of ATP · Mg-dependent type-1 protein phosphatase but has subsequently been demonstrated to be a protein kinase identical to glycogen synthase kinase-3α (GSK-3α) [Yang et al., 1980; Vandenheede et al., 1980; Hemmings et al., 1981; Woodgett, 1990]. Kinase FA/GSK-3α was further identified as a multisubstrate protein kinase capable of acting on many substrates, including R subunit of cAMP-dependent protein kinase [Hemmings et al., 1982], myelin basic protein (MBP) [Yang, 1986], nerve growth factor receptor [Taniuchi et al., 1986], G-subunit of phosphatase-1 [Fiol et al., 1988; Dent et al., 1989], neuronal cell adhesion molecule [Mackie et al., 1989], neurofilament [Guan et al., 1991], proto-oncogene products such as c-myc and c-jun [Boyle et al., 1991], synapsin I [Yang et al., 1992a,b], and brain microtubule-associated proteins such as MAP-2 [Yang et al., 1991, 1993a] and τ [Yang et al., 1991, 1993b,c, 1994a; Mandelkow et al., 1992; Hanger et al., 1992] and brain clathrin-coated vesicles [Yu and Yang, 1993a]. By its dual role as a multisubstrate protein kinase and as a multisubstrate protein phosphatase activator, kinase FA/GSK-3α may modulate phosphorylation and de-

Abbreviations used: kinase FA, protein phosphatase activating factor; GSK-3α, glycogen synthase kinase-3α; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; MBP, myelin basic protein; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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phosphorylation of many key proteins involved in the regulation of diverse cell and pathophysiological functions [Yang, 1991; Yang et al., 1992c, 1994a; Yu and Yang, 1993a; Woodgett, 1991; Mandelkow et al., 1992; Hanger et al., 1992].

The activity of kinase FA/GSK-3α was recently shown to be regulated by tyrosine phosphorylation and dephosphorylation [Hughes et al., 1993; Yu and Yang, 1994a], representing a new mode of control mechanism for regulating the activity of kinase FA/GSK-3α in cells. However, the signal transduction pathway for regulating tyrosine phosphorylation/dephosphorylation of kinase FA/GSK-3α in cells remains to be established. In previous reports, we have identified okadaic acid (a specific inhibitor for protein serine/threonine phosphatases 1 and 2A) [Bialojan and Takai, 1988; Cohen et al., 1990] and tumor promoter phorbol ester TPA (a specific modulator for PKC) [Nishizuka, 1986] as two potent extracellular mitogens that could reversibly modulate tyrosine phosphorylation/activation of kinase FA/GSK-3α in A431 cells [Yu and Yang, 1994b; Yang et al., 1994b]. TPA-mediated activation of PKC or okadaic acid could induce tyrosine dephosphorylation/inactivation of kinase FA/GSK-3α, whereas the TPA-mediated down-regulation of PKC could induce tyrosine phosphorylation/activation of kinase FA/GSK-3α, suggesting an involvement of phosphatases 1 and 2A and PKC in the regulation of phosphotyrosine contents and cellular activity of kinase FA/GSK-3α in cells [Yu and Yang, 1994b; Yang et al., 1994b]. In this report, modulation of kinase FA/GSK-3α by tyrosine phosphorylation/dephosphorylation in A431 cells treated with calphostin C (a specific PKC inhibitor) [Tamaoki, 1991] was further investigated using immunodetection in an antikinase FA/GSK-3α immunoprecipitate kinase assay and phosphoamino acid analysis of the immunoprecipitates from cell extracts on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by chemiluminence quantification. Here, we report that the specific PKC inhibitor calphostin C can differentially modulate the activity of kinase FA/GSK-3α in cells. At a concentration of 0.05 μM, calphostin C acting like TPA [Yang et al., 1994b] can induce the activation of kinase FA/GSK-3α via inhibition of PKC, but at concentrations of 0.1–10 μM, calphostin C can reversely induce tyrosine dephosphorylation and concurrent inactivation of protein kinase FA/GSK-3α in a PKC-independent pathway, and possibly via inhibition of a tyrosine kinase specific for kinase FA/GSK-3α, representing a new mode of signal transduction process for the regulation of protein kinase FA/GSK-3α by calphostin C in cells. Since kinase FA/GSK-3α is a possible carcinoma dedifferentiation/progression-promoting agent [Lee et al., 1995; Yang et al., 1995], the results further suggest calphostin C as a potential anticancer drug involved in blocking carcinoma dedifferentiation/progression possibly via inactivation of protein kinase FA/GSK-3α in tumor cells.

## EXPERIMENTAL PROCEDURES

### Materials and Methods

[$\gamma$-$^{32}$P]ATP was purchased from Amersham (Buckinghamshire, UK). Human epidermoid carcinoma A431 cells were obtained from American Type Culture Collection (Maryland). Plastic wares for cell culture were from Falcon (New Jersey). Antibiotics, fetal bovine sera, and complete and incomplete Freund's adjuvants were from Gibco (Rockville, MD). Calphostin C, 12-O-tetradecanoylphorbol-13-acetate (TPA), Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin (BSA), gelatin, dimethyl sulfoxide (DMSO), sodium pyrophosphate, Tween 20, poly(Glu,Tyr) 4:1 polypeptide, monoclonal antiphosphoserine mouse ascites fluid clone PSR-45, monoclonal antiphosphothreonine mouse ascites fluid clone PTR-8, monoclonal antiphosphotyrosine mouse ascites fluid clone PT-66, and goat antirabbit IgG antibody conjugated with alkaline phosphatase were from Sigma (St. Louis, MO). Goat antimouse IgG antibody conjugated with alkaline phosphatase was from BioRad (California). Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1$^{3,7}$]decan}-4-yl)phenyl phosphate (CSPD) was from Boehringer Mannheim (Mannheim, Germany). Phenylmethanesulfonyl fluoride (PMSF), benzamidine, aprotinin, Triton X-100, NaF, and glutaraldehyde were from Merck (Darmstadt, Germany). Polyvinylidene fluoride (PVDF) membrane was from Millipore (Bedford, MA). The alkaline phosphatase-conjugated substrate kit was from BioRad. Protein A–Sepharose CL-6B and CNBr-activated Sepharose 4B were from Pharmacia (Uppsala, Sweden).

### Protein Purification

Protein kinase FA/GSK-3α and MBP were purified to homogeneity from porcine brain fol-

lowing the purification procedures as described in previous reports [Yang, 1986; Yu and Yang, 1993b, 1994c]. When analyzed by gel electrophoresis in the presence of SDS and Coomassie Blue staining, the purified kinase FA gave a single major protein band at a $M_r$ of 53,000. Analysis of the radioactively autophosphorylated kinase FA on the autoradiogram also revealed a single major phosphorylated protein band at $M_r$ 53,000. The enzyme preparations were also identified by using specific antibody against GSK-3α prepared as described in previous reports [Yu and Yang, 1993b, 1994a,b]. The antibody could not cross-react with GSK-3β but could specifically immunoblot and immunoprecipitate only GSK-3α from brain extracts. The kinase FA preparations used in this report therefore belong to the category of GSK-3α, according to the definition proposed by Woodgett [1990, 1991].

### Production of Anti-kinase FA/GSK-3α Antibody

The peptide TETQTGQDWQAPDA, corresponding to the C-terminal regions from amino acids 462–475 of the sequence of kinase FA/GSK-3α [Woodgett, 1990] was synthesized by peptide synthesizer (model 9050, Milligen (Bedford, MA)). The cysteine residue was added to the N-termimus, in order to facilitate coupling of the peptide to BSA according to the procedure described by Reichlin [1980], using glutaraldehyde as the cross-linker. The detailed procedure for production of anti-kinase FA/GSK-3α antibody was as described in previous reports [Yu and Yang, 1994a,b]. The antisera (20 ml) were affinity-purified by adsorption to 3.5 ml of Sepharose 4B covalently coupled with 10 mg of C-terminal peptide of kinase FA/GSK-3α, as described above. After adsorption, the column was washed with 50 ml of solution A (100 mM Tris at pH 7.5) and then with 50 ml of solution A containing 0.5 M NaCl. The antibody was eluted with 35 ml of 100 mM glycine at pH 2.5. The eluted fractions were collected in tubes containing 3.5 ml of 1 M Tris–HCl at pH 8.0 and mixed immediately. The purified antibody was concentrated by dialyzing against 30% polyethylene glycol (PEG), suspended in 20 mM Tris–HCl at pH 7.0, 50% glycerol and 0.02% $NaN_3$, stored at −20°C and used as antikinase FA/GSK-3α antibody. Preimmuned antibidies were purified from preimmuned serum by protein A–Sepharose. The antikinase FA/GSK-3α antibody produced here can specifically immunoblot kinase FA/GSK-3α and efficiently immunoprecipitate kinase FA/GSK-3α from the cell extracts without blocking enzymatic activity (see Results section).

### Culture of A431 Cells, Treatment of Drugs, and Preparation of Cell Extracts

A431 cells were cultured at 37°C in a 92.5% air/7.5% $CO_2$ and water-saturated atmosphere in 75-$cm^2$ flasks in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 25 U/ml penicillin, and 25 μM/ml streptomycin. For drug treatment, aliquots of stock solution of calphostin C (2 mM dissolved in DMSO) or TPA (1 mM dissolved in DMSO) were added directly into the culture medium to make the required final concentrations. After incubation, A431 cells ($4 \times 10^6$ cells/100-mm culture dishes) were washed twice with ice-cold phosphate-buffered saline (PBS), scraped in PBS, and collected by centrifugation at 800*g* for 10 min at 4°C. The collected cells were homogenized in 500 μl of solution B (10 mM Tris–HCl at pH 7.4, 2 mM EDTA, 1 mM EGTA, 50 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1 mM benzamidine, 0.5 mg/ml aprotinin, 50 mM NaF, and 0.2 mM sodium orthovanadate) on ice by Sonic Dismembrator (model 150, Fisher) for 3 × 10 sec at 40% power output. Cell extracts were then ultracentrifuged at 160,000*g* for 30 min at 4°C, and the supernatants were used as the cell extracts.

### Immunoprecipitation and Kinase FA/GSK-3α Activity Assay in the Immunoprecipitate

Before immunoprecipitation, protein concentrations of the cell extracts were first diluted to equal amounts with solution B. For immunoprecipitation, 500-μl cell extracts ( ~ 1.0 mg/ml protein) were incubated with 1 μl of affinity-purified kinase FA/GSK-3α antibody (20 mg/ml pure IgG) at 4°C for 1.5 h and then with 100 μl of protein A–Sepharose CL-4B (20% v/v in solution A) for another 1.5 h with shaking. The immunoprecipitates were collected by centrifugation, washed three times with 0.5 M NaCl, once with 1 ml of solution C (20 mM Tris–HCl at pH 7.0, 0.5 mM dithiothreitol. 1 mM PMSF, 1 mM benzamidine, 0.5 mg/ml aprotinin), and resuspended in 60 μl of solution C. For kinase FA/GSK-3α activity assay in the immunoprecitates, 10 μl immunoprecitate was incubated with a 15-μl mixture containing 20 mM Tris–HCl at pH 7.0, 0.5 mM dithiothreitol, 0.2 mM [$\gamma$-$^{32}$P]ATP, 20 mM $MgCl_2$, and 4 mg/ml MBP at 30°C for 10 min. $^{32}$P-incorporation into MBP was measured by spotting a 20-μl reaction mix-

ture on phosphocellulose paper (1 × 2 cm), washing three times with 75 mM $H_3PO_4$ and counting in liquid scintillation counter as described in a previous report [Yang, 1986].

### Immunoblots

Proteins were transferred from unstained SDS gels to PVDF membrane in a Mighty Small Transphor (Hoffer) at 350 mA in transfer buffer (10 mM 3-[cyclohexylamino]-1-propane-sulfonic acid (CAPS) at pH 10.0, and 20% methanol) at 4°C for 2 h. The detailed procedure for immunoblotting analysis of kinase FA/GSK-3α from A431 cells was as described in previous reports [Yu and Yang, 1994b; Yang et al., 1994b].

### Phosphoamino Acid Analysis

The samples obtained from immunoprecipitation were mixed with equal volume of 2× Laemmli sample buffer and boiled at 100°C for 5 min. After brief centrifugation to remove the insoluble protein A beads, samples were subjected to 10% SDS–PAGE, followed by electrotransfer to PVDF membrane. The membrane was incubated in solution D (20 mM Tris–HCl at pH 7.4, 0.5 M NaCl, 0.05% Tween 20, and 1% gelatin) at 25°C for 1 h to block the free protein-binding sites. The phosphorylated protein bands were identified by immunoblotting analysis with antiphosphoserine (1:500 in solution D), antiphosphothreonine (1:2,000 in solution D) and antiphosphotyrosine (1:2,000 in solution D) mouse ascites fluid, respectively, at 25°C for 4 h and then incubated with goat antimouse IgG antibody conjugated with alkaline phosphatase (1:2,000 in solution D) at 25°C for 1 h, followed by autoradiography at 25°C for 0.5–2 min using CSPD (0.25 mM in 0.1 M Tris–HCl at pH 9.5, 0.1 M NaCl and 50 mM $MgCl_2$) as alkaline phosphatase substrate at 37°C for 15 min for chemiluminence detection [Gillespie and Hudspeth, 1991] and quantified by computing densitometer (Molecular Dynamics, Sunnyvale, CA).

### Analytic Methods

Protein concentrations were determined using BCA protein assay reagent from Pierce (Rockford, IL). SDS–PAGE was performed according to Laemmli [Laemmli, 1970], using 10% gels. Molecular-weight markers used are phosphorylase b (94 kDa), BSA (68 kDa), glutamate dehydrogenase (55.6 kDa), and glyceraldehyde 3-phosphate dehydrogenase (36 kDa).

### Protein Tyrosine Kinase Activity Assay in Cell Extracts

Protein tyrosine kinase activity in cell extracts was assayed according to Braun et al. [1984] as follows: 10 μl of A431 cell extracts at appropriate concentrations were incubated with a 15-μl reaction mixture containing 10 mM Tris–HCl at pH 7.4, 0.2 mM EDTA, 0.1 mM EGTA, 50 mM NaCl, 0.03% Triton X-100, 1 mM PMSF, 1 mM benzamidine, 0.5 mg/ml aprotinin, 50 mM NaF, 0.2 mM sodium orthovanadate, 1 mg/ml poly(Glu, Tyr) 4:1 polypeptide, 20 mM $MgCl_2$, and 0.2 mM [$\gamma$-$^{32}$P]ATP (~1,000 cpm/pmol) at 30°C for 15 min. $^{32}$P-incorporation into polypeptide substrate was measured by spotting a 20-μl reaction mixture on Whatman 3MM paper (1 × 2 cm), washing three times with 75 mM $H_3PO_4$, and counting in a liquid scintillation counter as described above.

## RESULTS

The antikinase FA/GSK-3α antibody produced and affinity-purified as described under Experimental Procedures was found to be very specific and potent toward immunoblotting kinase FA/GSK-3α at a molecular weight of 53 kDa from A431 cell extracts (Fig. 1, lane 1), demonstrating the immunospecificity of the antikinase FA/GSK-3α antibody produced here. Furthermore, when A431 cells were treated with 0.1–10 μM calphostin C (a specific PKC inhibitor) at 37°C for 1 h, followed by immunoblotting of kinase FA/GSK-3α from cell extracts using antikinase FA/GSK-3α antibody, there was also only one single protein band at a molecular weight of 53 kDa detectable in the immunoblot (Fig. 1, lanes 2–5). Furthermore, calphostin C basically caused no significant change in the immunoblotted protein level of kinase FA/GSK-3α (Fig. 1, lanes 1–5), indicating that calphostin C did not generate de novo protein synthesis of kinase FA/GSK-3α in cells. Moreover, calphostin C at concentrations of up to 20 μM was found to have no direct effect on the activity of the purified kinase FA/GSK-3α in vitro (Fig. 2). However, when A431 cells were treated with 0.05–10 μM calphostin C at 37°C for 1 h, followed by immunoprecipitation of kinase FA/GSK-3α from cell extracts using antikinase FA/GSK-3α antibody as described above, the cellular activity of kinase FA/GSK-3α, which is detectable in the immunoprecipitates from the cell extracts, was found to be differentially regu-

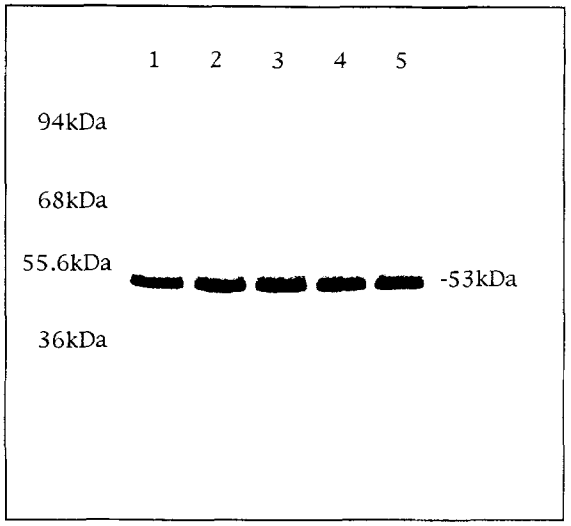


**Fig. 1.** Immunoblot of kinase FA/GSK-3α from crude extracts of A431 cells treated with calphostin C. A431 cells were treated with 0.1–10 μM calphostin C at 37°C for 60 min, and 40 μg of the cell extracts was immunoblotted by antikinase FA/GSK-3α antibody on 10% SDS–PAGE, as described under Experimental Procedures. *Lane 1,* immunoblot of extracts of A431 cells treated without calphostin C; *lanes 2–5,* with 0.1, 1, 5, and 10 μM calphostin C, respectively.

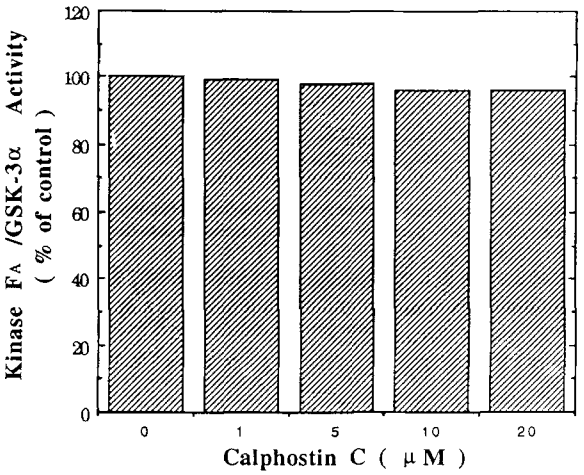


**Fig. 2.** Effect of calphostin C on the purified kinase FA/GSK-3α activity in vitro. The purified kinase FA/GSK-3α was treated with 1–20 μM calphostin C as indicated at 30°C for 60 min in the presence of light, followed by kinase FA/GSK-3α activity assay, as described under Experimental Procedures. Data were average of three independent experiments.

lated. When cells were treated with 0.05 μM calphostin C, the cellular activity of kinase FA/GSK-3α could be activated up to ~130% of control value (Fig. 3), in agreement with the previous report [Yang et al., 1994b] that down-regulation of PKC by tumor promoter phorbol ester TPA could cause activation of kinase FA/GSK-3α in A431 cells, since calphostin C inhibited PKC activity with an $IC_{50}$ value of ~0.05 μM [Kobayashi et al., 1989]. In sharp contrast, when cells were treated with 0.1–10 μM calphostin C, the cellular activity of kinase FA/GSK-3α was found to be inhibited down to less than 10% of control values by calphostin C in a concentration-dependent manner (Fig. 3), indicating that a post-translational modification of kinase FA/GSK-3α independent of PKC could possibly be involved in the calphostin C-mediated inactivation of protein kinase FA/GSK-3α (Figs. 1–3). Moreover, modulation of kinase FA/GSK-3α by calphostin C was found to be light dependent (Fig. 3).

Since calphostin C is a polycyclic hydrocarbon with strong absorbance in the visible and ultraviolet ranges, and its drug actions on cytotoxicity, phorbol ester binding, and kinase activity all required light [Bruns et al., 1991], the results further demonstrate that the modulation process on kinase FA/GSK-3α is indeed due to calphostin C itself [Bruns et al., 1991]. Since calphostin C was identified as a potent and specific PKC inhibitor [Kobayashi et al., 1989], we further tested whether the calphostin C-mediated inactivation of kinase FA/GSK-3α in A431 cells could possibly be due to inhibition of PKC as signal transduction pathway. The cells were first treated with 1 μM tumor promoter phobol ester TPA for 24 h, to down-regulate PKC activity in cells, and then treated with 10 μM calphostin C at 37°C for 1 h. As shown in Figure 4, in contrast to the TPA-mediated down-regulation of PKC in cells, which could cause activation of kinase FA/GSK-3α, calphostin C was found to generate the opposite function and could knock off more than 90% of the total cellular activity (including the TPA-mediated activation) of kinase FA/GSK-3α in a PKC-independent manner, indicating that the calphostin C-mediated inactivation of kinase FA/GSK-3α may not be due to inhibition of PKC in cells. Since the cellular activity of kinase FA/GSK-3α could be regulated by tyrosine phosphorylation in cells [Hughes et al., 1993; Yu and Yang, 1994a,b; Yang et al., 1994b], as well as by serine phosphorylation in vitro [Sutherland and Cohen, 1994], we then subjected the immunoprecipitates of the cells treated with and without 10 μM calphostin C to phosphoamino acid analysis, using antiphosphotyrosine, -phosphoserine, and -phosphothreonine antibodies followed by chemiluminescent detection, which revealed that the calphostin C-mediated

inactivation of kinase FA/GSK-3α could be due to tyrosine-dephosphorylation of the enzyme (Fig. 5, PY). In sharp contrast, calphostin C appeared to have no effect on the phosphoserine and phosphothreonine contents of kinase FA/GSK-3α in cells (Fig. 5, PS and PT).

Taken together, the results demonstrate that calphostin C may modulate phosphotyrosine contents and cellular activities of kinase FA/GSK-3α in two differential pathways. At a low concentration of 0.05 μM, calphostin C could specifically inhibit the cellular activity of PKC to mediate activation of kinase FA/GSK-3α in A431 cells, in agreement with the previous report that down-regulation of PKC could induce activation of kinase FA/GSK-3α in A431 cells [Yang et al., 1994b]. In sharp contrast, at higher concentrations of 0.1–10 μM, calphostin C could generate an opposite function to mediate tyrosine-dephosphorylation/inactivation of kinase FA/GSK-3α in a pathway independent of TPA-mediated down-regulation of PKC in A431 cells (Figs. 3–5).

Since the calphostin C-mediated inactivation of kinase FA/GSK-3α could be due to tyrosine-dephosphorylation of the kinase independent of TPA-mediated down-regulation of PKC as described in Figures 3–5, it is highly possible that calphostin C may cause an inhibition of a tyrosine kinase specific for activation of kinase FA/GSK-3α in cells. To confirm this point, we further tested the cellular activity of tyrosine kinase using poly(Glu, Tyr) polypeptide as substrate and found that calphostin C at concentrations of 0.1–10 μM could inhibit the cellular activity of tyrosine kinase down to less than 10% of control values, even when the cells were pretreated with 1 μM TPA for 24 h to down-regulate PKC (Fig. 6), in a similar pattern to inhibiting the cellular activity of kinase FA/GSK-3α as described in Figures 3 and 4. Taken together, the results further support the notion that calphostin C may induce tyrosine dephosphorylation and concurrent inactivation of kinase FA/GSK-3α in a pathway independent of TPA-mediated down-regulation of PKC and possibly via inhibition of a tyrosine kinase specific for activating kinase FA/GSK-3α in cells.

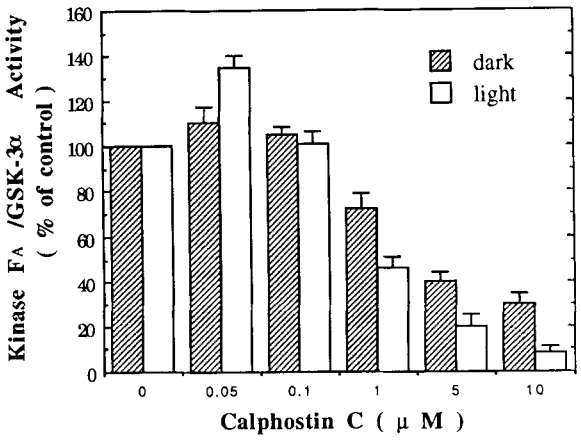


Fig. 3. Effect of calphostin C on the cellular activity of kinase FA/GSK-3α in A431 cells in the presence and absence of light. A431 cells were treated with 0.05–10 μM calphostin C in the presence and absence of light at 37°C for 60 min. Cell extracts (1 mg cell protein in 500 μl of solution B) were immunoprecipitated by 20 μg antikinase FA/GSK-3α antibody, followed by kinase FA/GSK-3α activity assay in the immunoprecipites, as described under Experimental Procedures. Data were the average of four independent experiments and expressed as means ± SD.

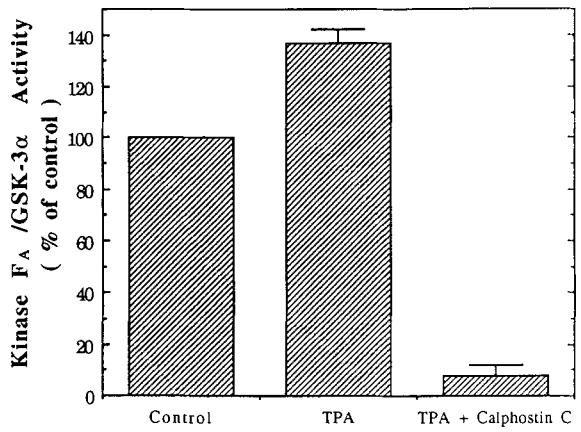


Fig. 4. Effect of calphostin C on the cellular activity of kinase FA/GSK-3α in A431 cells pretreated with TPA. A431 cells were pretreated with or without 1 μM TPA at 37°C for 24 h and then treated with or without 10 μM calphostin C for another 1 h, followed by immunodetection of the cellular activity of kinase FA/GSK-3α in an antikinase FA/GSK-3α immunoprecipitate kinase assay, as described under Experimental Procedures. Data were the average of four independent experiments and expressed as means ± SD.

## DISCUSSION

In this report, we have produced an affinity-purified antikinase FA/GSK-3α antibody that can efficiently and specifically immunoblot and immunoprecipitate kinase FA/GSK-3α activity in cells treated with calphostin C, a specific inhibitor of PKC [Kobayashi et al., 1989]. To our surprise, we found that calphostin C could differentially modulate the cellular activities of kinase FA/GSK-3α in a reversible manner possibly involving both PKC-dependent and -indepen-

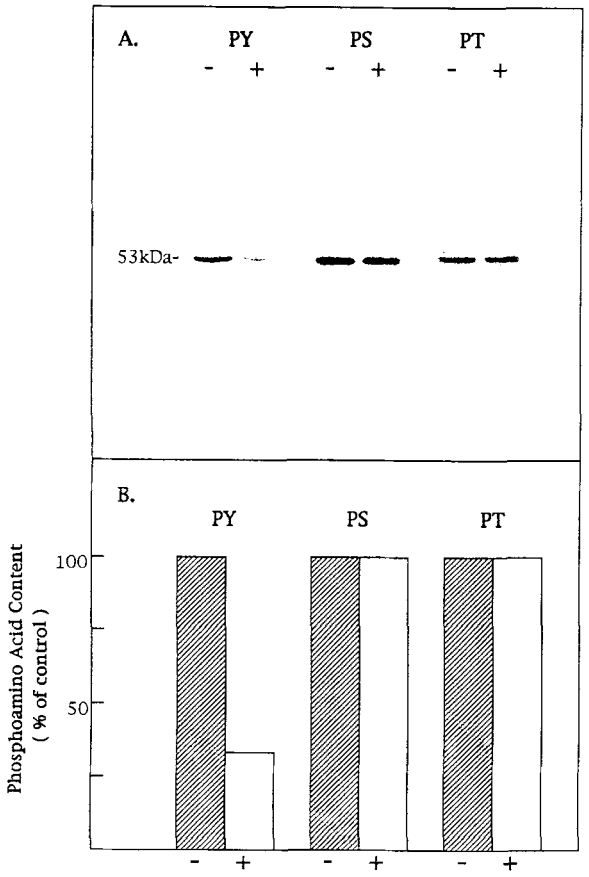


Fig. 5. Phosphoamino acid analysis of kinase FA/GSK-3α immunoprecipitated from A431 cells treated with calphostin C. The immunoprecipitates obtained from A431 cells treated without (−) or with (+) 10 μM calphostin C at 37°C for 60 min were subjected to 10% SDS–PAGE, and then electrotransferred to PVDF membrane. The phosphoamino acid contents of kinase FA/GSK-3α were identified using antiphosphotyrosine (PY), antiphosphoserine (PS), and antiphosphothreonine (PT) antibodies as indicated, followed by chemiluminence visualization and quantification as described under Experimental Procedures. **A:** Autoradiogram. **B:** Quantification of the phosphoamino acid content of kinase FA/GSK-3α from A.

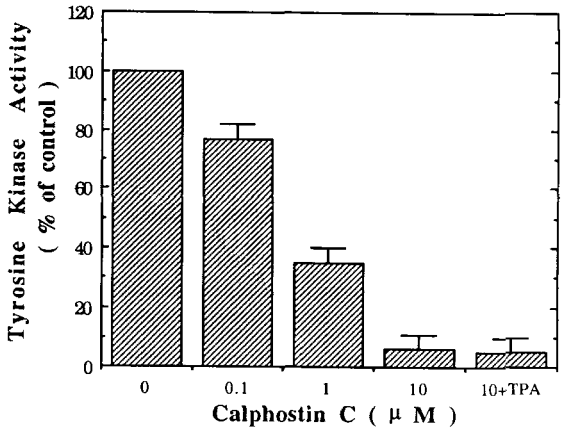


Fig. 6. Effect of calphostin C and TPA on the cellular tyrosine kinase activities in A431 cells. A431 cells were pretreated with or without 1 μM TPA at 37°C for 24 h and then treated with 0–10 μM calphostin C as indicated at 37°C for another 1 h, followed by tyrosine kinase activity assay in the cell extracts, using poly(Glu,Tyr) polypeptide as substrate as described under Experimental Procedures. Data were the average of four independent experiments and expressed as means ± SD.

dent pathways. At a concentration of 0.05 μM, calphostin C could induce activation of kinase FA/GSK-3α via inhibition of PKC as a signal transduction pathway, in agreement with the previous reports that calphostin C inhibits PKC with an $IC_{50}$ value of ~0.05 μM, and down-regulation of cellular PKC by TPA could induce activation of kinase FA/GSK-3α in A431 cells [Yang et al., 1994b; Kobayashi et al., 1989]. Conversely, at concentrations of 0.1–10 μM, calphostin C could generate an opposite function to induce tyrosine dephosphorylation and concurrent inactivation of kinase FA/GSK-3α, even when cells were pretreated with 1 μM TPA for 24 h to down-regulate PKC, indicating that calphostin C could induce inactivation of kinase FA/GSK-3α in a signal transduction pathway independent of TPA-mediated down-regulation of PKC. When using poly(Glu, Tyr) polypeptide as substrate to assay tyrosine kinase activities in A431 cells, we found that calphostin C at concentrations of 0.1–10 μM could potently inhibit the cellular activities of tyrosine kinase in a pattern similar to inhibiting the cellular activities of kinase FA/GSK-3α (Figs. 3, 4, 6). Taken together with all the results presented in this report and in a previous report [Yang et al., 1994b], we concluded that calphostin C at concentrations of $<0.05$ μM may function as a specific PKC inhibitor [Tamaoki, 1991; Kobayashi et al., 1989] to induce activation of kinase FA/GSK-3α in similarity with TPA-mediated down-regulation of PKC to induce activation of kinase FA/GSK-3α as previously reported [Yang et al., 1994b]. In sharp contrast, calphostin C at concentrations of $>0.1$ μM may generate an additional function to induce inactivation of kinase FA/GSK-3α in a pathway independent of TPA-mediated down-regulation of PKC, as presented in this report, representing a new mode of signal transduction pathway for calphostin C in terms of the regulation of a multisubstrate/multifunctional protein kinase FA/GSK-3α in cells. Calphostin C, a newly isolated compound of potential use as an antitumor drug, may therefore possess three distinct drug actions. It

inhibited PKC activity with an $IC_{50}$ value of ~0.05 μM and inhibited cAMP-dependent protein kinase and $p60^{v\text{-}src}$ tyrosine kinase with an $IC_{50}$ value of more than 50 μM, as reported by Kobayashi et al. [1989], and Tamaoki [1991], but it could also inhibit kinase FA/GSK-3α with an $IC_{50}$ value of ~1 μM, as reported here. When manipulating or exploiting calphostin C as a specific and potent PKC inhibitor to study signal transduction mechanism in cells, these three distinct drug actions of calphostin C should be taken into consideration. For instance, calphostin C at concentration of 0.1 μM as reported here appeared to generate two opposite functions: it could inhibit PKC to induce activation of kinase FA/GSK-3α, but it could also simultaneously induce inactivation of kinase FA/GSK-3α. Calphostin C may therefore not represent a very specific PKC inhibitor as previously conceived [Tamaoki, 1991; Kobayashi et al., 1989]. The tyrosine kinase, which is very sensitive to the inhibition by calphostin C and specific for activating kinase FA/GSK-3α in cells, obviously presents an intriguing issue deserving further investigation. Nevertheless, the present study clearly demonstrates that the phosphotyrosine content and cellular activity of protein kinase FA/GSK-3α can be modulated by calphostin C in a pathway independent of tumor promoter phorbol ester-mediated down-regulation of PKC, representing a new mode of drug action for calphostin C involved in the signal transduction in A431 cells. By contrast, calphostin C showed potent cytotoxic effects against HeLa S3 cells and human breast cancer cell MCF-7 and showed antitumor activity against murine lymphocytic leukemia P388 in vivo [Kobayashi et al., 1989]. Since protein kinase FA/GSK-3α has recently been identified as a possible carcinoma dedifferentiation/progression-promoting agent involved in human thyroid tumor cell dedifferentiation [Lee et al., 1995] and in human cervical carcinoma dedifferentiation/progression [Yang et al., 1995], it is highly possible that the cytotoxic and antitumor activity of calphostin C is partly due to the potent inhibition of kinase FA/GSK-3α. This obviously presents an intriguing issue that deserves further investigation.

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