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Tumor Promoter Phorbol Ester Reversibly Modulates Tyrosine Dephosphorylation/Inactivation of Protein Kinase F_A/GSK-3α in A431 Cells

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Abstract The signal transduction mechanism of protein kinase F_A/GSK-3α by tyrosine phosphorylation in A431 cells was investigated. Kinase F_A/GSK-3α was found to exist in a highly tyrosine-phosphorylated/activated state in resting cells but could be tyrosine-dephosphorylated and inactivated to ~60% of the control level when cells were acutely treated with 1 μM tumor promoter phorbol ester (TPA) at 37°C for 30 min, as demonstrated by metabolic ^{32}P-labeling the cells, followed by immunoprecipitation and two-dimensional phosphoamino acid analysis and by immunodetection in an antikinase F_A/GSK-3α immunoprecipitate kinase assay. Conversely, when cells were chronically treated with 1 μM TPA at 37°C for 24 h and processed under identical conditions, kinase F_A/GSK-3α was found to be rephosphorylated on tyrosine residue and reactivated to ~130% of the original control level. Taken together, the results provide initial evidence that the phosphotyrosine content and cellular activity of kinase F_A/GSK-3α can be modulated in a reversible manner by short-term and long-term exposure of A431 cells to TPA. Since acute exposure of cells to TPA causes up-regulation of cellular protein kinase C (PKC) activity and prolonged exposure to TPA causes down-regulation of PKC, the results further suggest that the TPA-mediated modulation of PKC may play a role in the regulation of tyrosine phosphorylation and concurrent activation of kinase F_A/GSK-3α in cells, representing a new mode of signal transduction pathway for the regulation of this multisubstrate/multifunctional protein kinase in cells.

Key words: PKC, TPA, up-regulation, down-regulation, A431 cells

Protein kinase F_A was originally identified as an activating factor of ATP·Mg-dependent type-1 protein phosphatase but has subsequently been demonstrated as 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 F_A/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 [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 molecules [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], ATP-citrate lyase and acetyl-CoA carboxylase [Hughes et al., 1992], and brain microtubule-associated proteins such as MAP-2 [Yang et al., 1991, 1993a] and tau [Yang et al., 1991, 1993b,c; Mandelkow et al., 1992; Hanger et al., 1992]. By its dual role as a multisubstrate protein phosphatase activating factor and as a multisubstrate protein kinase, kinase F_A/GSK-3α may modulate phosphorylation and dephosphorylation states of many key regulatory proteins possibly involved in the regulation of diverse cell and pathophysiological functions [reviewed in Yang, 1991; Woodgett, 1991; Yang et al., 1992c; 1993c; Hanger et al., 1992].

Recently, the activity of kinase F_A/GSK-3α was shown to be activated by tyrosine phosphorylation in vitro [Hughes et al., 1993], suggesting a new mode of control mechanism for regulating the activity of kinase F_A/GSK-3α. However, the

Abbreviations used: F_A, type-1 protein phosphatase activating factor; GSK-3, glycogen synthase kinase-3α; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MBP, myelin basic protein; TPA, tumor promoter phorbol ester 12-O-tetradecanoylphorbol 13-acetate.

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extracellular stimuli that may modulate the tyrosine phosphorylation of kinase F_A/GSK-3α and the signal transduction pathway for regulating the tyrosine phosphorylation–dephosphorylation of kinase F_A/GSK-3α in cells remain to be established. In this report, modulation of kinase F_A/GSK-3α by tyrosine phosphorylation-dephosphorylation in A431 cells treated with tumor promoter phorbol ester (TPA), a specific modulator for protein kinase C [Nishizuka, 1986] was investigated by immunodetection in an antikinase F_A/GSK-3α immunoprecipitates and by metabolic ^{32}P-labeling the cells, followed by immunoprecipitation and two-dimensional phosphoamino acid analysis. Here, we report that the cellular activity and tyrosine content of protein kinase F_A/GSK-3α can be reversibly modulated by TPA, suggesting a possible involvement of the TPA-mediated modulation of protein kinase C in the regulation of tyrosine phosphorylation and concurrent activation of kinase F_A/GSK-3α, representing a new mode of signal transduction pathway linking protein kinase C and kinase F_A/GSK-3α in cells.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP and [^{32}P]orthophosphate (carrier-free) were purchased from Amersham (Buckinghamshire, UK). Human epidermoid carcinoma A431 cells were obtained from the American Type Culture Collection (Maryland). Plastic wares for cell culture were from Falcon (New Jersey). Antibiotics, fetal bovine sera, complete and incomplete Freund's adjuvants were from Gibco (Ontario, Canada). 12-*O*-tetradecanoylphorbol-13-acetate (TPA), Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin (BSA), dimethylsulfoxide (DMSO), sodium pyrophosphate, Tween 20, and goat antirabbit IgG antibody conjugated with alkaline phosphatase were from Sigma (St. Louis, MO). Phenylmethanesulfonyl fluoride (PMSF), benzamidine, aprotinin, Triton X-100, NaF, glutaraldehyde, and cellulose-coated TLC plates were from Merck (Darmstadt, Germany). Nitrocellulose membrane was from Hoffer. Polyvinylidene fluoride (PVDF) membrane was from Millipore. Alkaline phosphatase conjugate substrate kit was from Bio-Rad (California). Protein A–Sepharose CL-6B and CNBr-activated Sepharose 4B were from Pharmacia (Uppsala, Sweden).

Protein Purification

Protein kinase F_A/GSK-3α [Yang, 1986; Yu and Yang, 1993], the catalytic subunit of protein phosphatase 2A [Yang et al., 1986; Yu and Yang, 1989], and myelin basic protein (MBP) [Yang et al., 1987] were purified from porcine brain.

Production of Antikinase F_A/GSK-3α Antibody

The peptide TETQTGQDWQAPDA, corresponding to the carboxyl terminal regions from amino acids 462–475 of the sequence of kinase F_A/GSK-3α [Woodgett, 1990] was synthesized by peptide synthesizer (model 9050, Milligen). The cysteine residue was added to the NH_2-terminus in order to facilitate coupling of the peptide to bovine serum albumin (BSA) according to the procedure described by Reichlin [1980] using glutaraldehyde as the cross-linker; 500 μg of the BSA-conjugated peptide of kinase F_A/GSK-3α as described above suspended in 500 μl complete Freund's adjuvant was injected subcutaneously into two New Zealand white rabbits. After 4 and 8 weeks, another 500-μg-conjugated peptide suspended in 500 μl incomplete Freund's adjuvant was used as a booster shot. Ten days after the third injection, 40 ml blood was drawn from marginal ear veins and the antisera were tested for kinase F_A/GSK-3α antibody using immunoblotting (see below). The rabbits were boosted every 4 weeks with another 500-μg conjugated peptide in 500 μl incomplete Freund's adjuvant, and 40 ml blood was routinely drawn every 10 days after each booster injection. Another New Zealand rabbit was bled without immunization (preimmunized serum).

The antisera (10 ml) were affinity-purified by adsorption to 3.5 ml Sepharose 4B covalently coupled with 10 mg C-terminal peptide of kinase F_A/GSK-3α as described above. After adsorption, the column was washed with 50 ml buffer B (100 mM Tris at pH 7.5) and then with 50 ml buffer B containing 0.5 M NaCl. The antibody was eluted with 35 ml 100 mM glycine at pH 2.5. The eluted fractions were collected in tubes containing 3.5 ml 1 M Tris–HCl at pH 8.0 and mixed immediately. The purified antibody was concentrated by dialyzing against 30% polyethylene glycol, 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 F_A/GSK-3α antibody. Preimmuned antibodies were purified from pre-immuned serum by protein A–Sepharose.

The antikinase F_A/GSK-3α antibody produced here can specifically immunoblot kinase F_A/GSK-3α and efficiently immunoprecipitate kinase F_A/GSK-3α from the cell extracts without blocking the enzyme activity (see Results section).

Culture of A431 Cells, TPA Treatment, 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 μg/ml streptomycin. One day before the experiments, cells ($1–2 \times 10^6$) were plated on 100-mm culture dishes. For drug treatment, aliquots of stock solution of TPA (100 μM dissolved in DMSO) was added directly into the culture medium to make the required final concentrations. After incubation for various time periods as indicated, cells were washed twice with ice-cold phosphate buffer saline (PBS), scraped in PBS and collected by centrifugation at 800*g* for 10 min at 4°C. The collected cells were homogenized in 600 μl buffer A (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 μg/ml aprotinin, 50 mM NaF, 20 mM sodium pyrophosphate, 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 cell extracts.

^{32}P-Labeling of A431 Cells

A431 cells (~1×10^7 cells) in 150-mm petri dishes were washed twice with DMEM without phosphate and incubated in 6 ml DMEM containing 2 mCi [^{32}P]orthophosphate (~0.33 mCi/ml) at 37°C for 3 h and then incubated with 1 μM TPA for another 30 min. For long-term treatment with TPA, A431 cells were first treated with 1 μM TPA at 37°C for 20.5 h, followed by another 3.5-h incubation with [^{32}P]orthophosphate. Cells were then washed twice with ice-cold PBS, disrupted in 800 μl buffer A, adjusted to identical protein concentrations and subjected to immunoprecipitation by antikinase F_A/GSK-3α antibody (see below).

Immunoprecipitation and Kinase F_A/GSK-3α Activity Assays in Immunoprecipitates

Before immunoprecipitation, protein concentrations of the cell extracts were first diluted to equal amounts with buffer A. For immunoprecipitation, 500–750-μl cell extracts (0.8–1.5 mg/ml protein) was incubated with 2 μl affinity-purified kinase F_A/GSK-3α antibody (10 mg/ml pure IgG) at 4[g4]°C for 1.5 h and then with 100 μl protein A–Sepharose CL-4B (20% v/v in buffer A) for another 1.5 h with shaking. The immunoprecipitates were collected by centrifugation, washed three times with 1 ml 0.5 M NaCl, once with 1 ml buffer C (20 mM Tris–HCl at pH 7.0, 0.5 mM dithiothreitol, 1 mM PMSF, 1 mM benzamidine, 0.5 μg/ml aprotinin), and resuspended in 100 μl buffer C. For kinase F_A/GSK-3α activity assay in the immunoprecipitate, 15 μl immunoprecipitate at serial appropriate dilutions to make sure a linear assay was incubated with 30 μl mixture containing 20 mM Tris–HCl, pH 7.0, 0.5 mM dithiothreitol, 0.2 mM [γ-^{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 30-μl reaction mixture on phosphocellulose paper (1 × 2 cm), washing 3 times with 75 mM H_3PO_4 and counting in liquid scintillation counter as described in a previous report [Yang, 1986]. Each assay in the immunoprecipitate was performed in at least three serial dilutions, and each dilution point was performed in duplicate. The cellular activity of kinase F_A/GSK-3α in the immunoprecipitate was calculated from such a linear assay curve. It is important to note that more than 90% of the total kinase F_A/GSK-3α could be immunoprecipitated from A431 cell extracts under this condition, as demonstrated by the immunoblotting analysis (not shown).

Immunoblots

Proteins were transferred from unstained SDS gels to nitrocellulose paper 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 nitrocellulose paper was incubated in TTBS buffer (20 mM Tris–HCl at pH 7.4, 0.5 M NaCl and 0.05% Tween 20) containing 5% nonfat dried milk at room temperature for 1 h to block the free protein-binding sites. After washing 3 times with TTBS buffer, the paper was incubated with 1 μg/ml antikinase

F_A/GSK-3α antibody in TTBS buffer containing 3% nonfat dried milk at room temperature for 6 h, washed 3 times in TTBS buffer, and then incubated with secondary goat antirabbit IgG antibody conjugated with alkaline phosphatase diluted at 1:2,000 in TTBS buffer containing 3% nonfat dried milk at room temperature for 40 min and washed 3 times in TTBS buffer. The kinase F_A/GSK-3α protein was visualized by immersing the paper in 100 mM Tris–HCl at pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.32 mg/ml nitroblue tetrazolium, and 0.16 mg/ml 5-bromo-4-chloro-3-indolylphosphate at room temperature for 5–10 min. The reaction was stopped by 10 mM EDTA. The paper was then dried on filter paper.

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% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by electrotransfer to PVDF membrane. Proteins electrotransferred onto the membrane were visualized by staining with Amido Black (0.25% in water). The ^{32}P-labeled protein bands were localized by autoradiography and corresponding to kinase F_A/GSK-3α were excised and directly hydrolyzed in 5.7 N HCl under N_2 at 110°C for 1 h [Kamps and Sefton, 1989]. The hydrolysate was dried by Speed-Vac (Savant) and subjected to high-voltage electrophoresis in the first dimension on cellulose-coated TLC plate in pH 1.9 buffer (formic acid/acetic acid/H_2O = 50/156/1794) at 1.5 kV at 20°C for 20 min. After air-drying, the plate was subjected to high-voltage electrophoresis in the second dimension in pH 3.5 buffer (acetic acid/pyridine/water = 10/1/189) at 1.3 kV for 16 min. The positions of phosphoamino acids in plates were localized by ninhydrin-stain of standards and by autoradiography.

Analytic Methods

Protein concentrations were determined by using BCA protein assay reagent from Pierce (Illinois). SDS–PAGE was performed by the method of Laemmli (1970) using 10% gels. Molecular-weight markers used are α_2-macroglobulin (170 kDa), phosphorylase b (94 kDa), bovine serum albumin (68 kDa), glutamate dehydrogenase (55.6 kDa), ovalbumin (43 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), and triosephosphate isomerase (26.6 kDa). Autoradiography was carried out at −70°C with an Fuji RX X-ray film using Kodak X-Omatic cassette with intensifying screens. The results from autoradiography were further quantified by Computing Densitometer (Molecular Dynamics, California).

RESULTS

The antikinase F_A/GSK-3α antibody produced and affinity-purified as described in Materials and Methods was found to be very specific and potent toward immunoblotting kinase F_A/GSK-3α at a molecular weight of 53 kDa from A431 cell extracts (Fig. 1, lane 1), demonstrating the immunospecificity of antikinase F_A/GSK-3α antibody produced here. Furthermore, when A431 cells were acutely and chronically treated with 1 μM TPA at 37°C for various time points as indicated, followed by immunoblotting of kinase F_A/GSK-3α from cell extracts using the same antikinase F_A/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 and 3). Furthermore, TPA basically caused no significant change in the immunoblotted protein level of kinase F_A/GSK-3α (Fig. 1), indicating that TPA did not generate de novo protein synthesis of kinase F_A/GSK-3α in cells. Moreover, TPA at concentrations of 1–100 μM were found to have no direct effect on the activity of purified kinase F_A/GSK-3α in vitro (not shown). However, when A431 cells were acutely treated with 1 μM TPA at 37°C for 30 min, followed by immunoprecipitation of kinase F_A/GSK-3α from cell extracts using the antikinase F_A/GSK-3α antibody as described above, the cellular activity of kinase F_A/GSK-3α, which is detectable in the immunoprecipitates from the cell extracts was found to be inactivated down to approximately 60% of control value (Fig. 2), indicating that a post-translational modification of pre-existing protein could possibly be involved in the TPA-mediated inactivation of protein kinase F_A/GSK-3α (Figs. 1, 2). In sharp contrast, when A431 cells were chronically treated with 1 μM TPA at 37°C for 24 h and processed under identical conditions, the cellular activity of kinase F_A/GSK-3α in

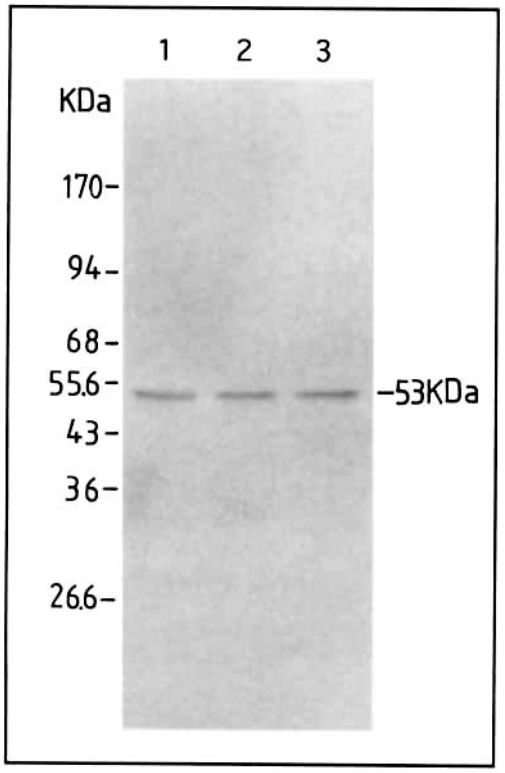

Fig. 1. Immunoblot of kinase F_A/GSK-3α from crude extracts of A431 cells treated with TPA. A431 cells were treated with 1 μM TPA at 37°C for 30 min and 24 h; 40 μg of the cell extracts was immunoblotted by antikinase F_A/GSK-3α antibody on 10% SDS–PAGE as described under Materials and Methods. Lanes 1–3, immunoblot of extracts of A431 cells treated with 1 μM TPA at 37°C for 0, 30 min, and 24 h, respectively.

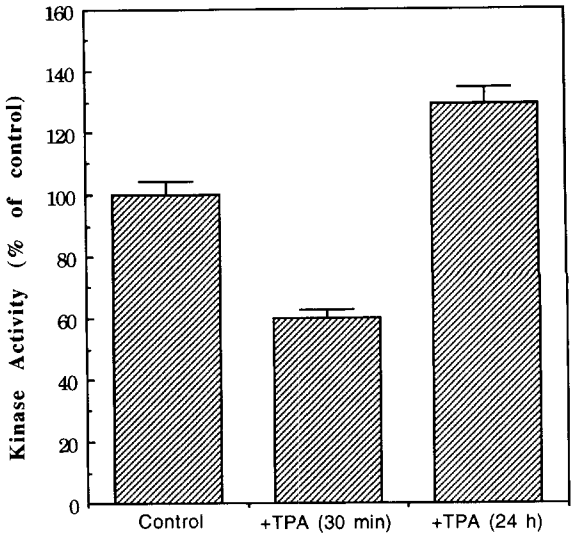

Fig. 2. Effect of TPA on the activity of kinase F_A/GSK-3α in A431 cells. A431 cells were treated with 1 μM TPA at 37°C for 30 min and 24 h. The cell extracts (0.5 mg cell protein in 500 μl buffer A) were immunoprecipitated by 20 μg antikinase F_A/GSK-3α antibody, followed by kinase activity assay in the immunoprecipites as described under Materials and Methods. Data were the average of four independent experiments and expressed as means ± SD.

A431 cells was found to be reactivated up to about 130% of the original control level (Fig. 2).

To investigate the action mechanism of TPA in modulating the activity of kinase F_A/GSK-3α in cells, we have analyzed the phosphorylation states of kinase F_A/GSK-3α in the immunoprecipitates from A431 cells treated with and without 1 μM TPA at 37°C for 30 min and 24 h, respectively. This was evaluated by metabolic labeling the cells with [^{32}P]orthophosphate at 37°C for 3 h, followed by incubation with 1 μM TPA for another 30 min or the cells were first incubated with 1 μM TPA at 37°C for 20.5 h followed by incubation with [^{32}P]orthophosphate at 37°C for 3.5 h. The cell extracts were subsequently immunoprecipitated with antikinase F_A/GSK-3α antibody, the immunoprecipitates were then subjected to SDS–PAGE and electrotransferred to PVDF membrane, followed by autoradiography (Fig. 3A). The short-term treatment of cells with TPA caused decreased phosphorylation of the 53-kDa kinase F_A/GSK-3α and a co-immunoprecipitated phosphoprotein at a molecular weight of 62 kDa, tentatively termed pp62 (Fig. 3A, lanes 2 and 3), in sharp contrast to the long-term treatment of cells with TPA for 24 h, which caused increased phosphorylation of kinase F_A/GSK-3α and pp62 (Fig. 3A, lanes 2–4). Immunoprecipitation of kinase F_A/GSK-3α and pp62 from A431 cell extracts by anti-kinase F_A/GSK-3α antibody could be completely blocked by the C-terminal peptide from amino acids 462–475 of kinase F_A/GSK-3α (Fig. 3A, lane 1), further demonstrating the immunospecificity of this antibody against kinase F_A/GSK-3α. Densitometric quantification of the phosphorylated proteins in the autoradiogram, as shown in Figure 3A, further revealed that acute exposure of cells to TPA for 30 min decreased ~30% of the phosphate content of kinase F_A/GSK-3α in the untreated cells, whereas chronic exposure to TPA for 24 h incresed up to ~120% of the phosphate content of kinase F_A/GSK-3α in the untreated cells (Fig. 3B). Similar decreased and increased phosphorylation levels by the short and long term exposure of cells to TPA could also be observed in the

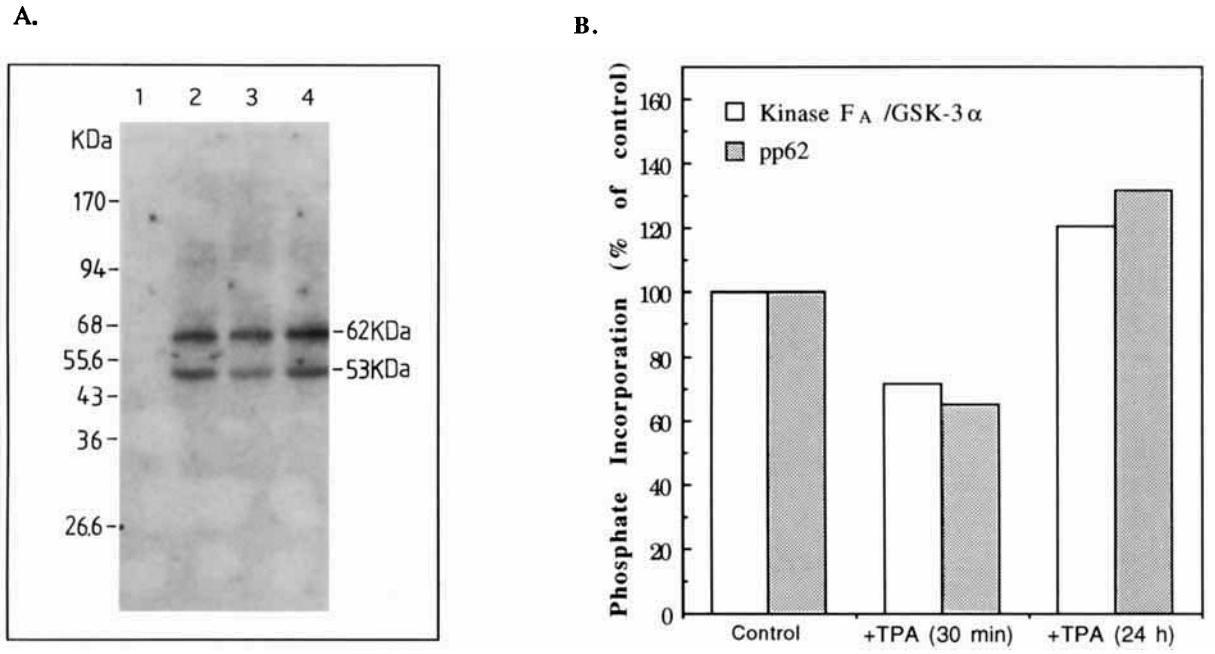

Fig. 3. In vivo ^{32}P-labeled kinase F_A/GSK-3α immunoprecipitated from A431 cells treated with TPA. A431 cells were incubated with ~0.33 mCi/ml [^{32}P]orthophosphate at 37°C of 3 h, followed by incubation with and without 1 μM TPA for 30 min, or cells were first incubated with 1 μM TPA for 20.5 h, followed by incubation with [^{32}P]orthophosphate for 3.5 h. The cell extracts were subsequently immunoprecipitated with 20 μg antikinase F_A/GSK-3α antibody, and the immunoprecipitates were subjected to 10% SDS–PAGE, electrotransferred to PVDF membrane, and autoradiographed as described under Materials and Methods. **A:** Autoradiogram. Lane 1, immunoprecipitation in the presence of 50 μg C-terminal peptide from amino acids 462–475 of kinase F_A/GSK-3α from untreated cells; lanes 2–4, immunoprecipitation without C-peptide from untreated cells (lane 2) or from cells treated with TPA for 30 min (lane 3) and 24 h (lane 4), respectively. **B:** Densitometric quantification of the phosphorylation level of 53 kDa and 62 kDa in A.

co-immunoprecipitated pp62 (Fig. 3B). To explore in greater detail, the ^{32}P-labeled protein bands corresponding to kinase F_A/GSK-3α at a molecular weight of 53 kDa and pp62 at a molecular weight of 62 kDa as shown in Figure 3A were excised from the autoradiogram and further subjected to two-dimensional phosphoamino acid analysis on thin-layer cellulose plates, followed by autoradiography and analyzed by computing densitometer (Fig. 4). Kinase F_A/GSK-3α appeared to be highly phosphorylated on tyrosine residue in the untreated cells (Fig. 4A, panel 1) in contrast to the tyrosine phosphorylation level of kinase F_A/GSK-3α in the TPA-treated cells at 37°C for 30 min, which was significantly decreased (Fig. 4A, panel 2). Conversely, the phosphotyrosine content of the immunoprecipitated kinase F_A/GSK-3α from the TPA-treated cells at 37°C for 24 h appeared to be increased to a even higher level than that of the untreated cells (Fig. 4A, panel 3). Densitometric quantification of the autoradiogram, as shown in Figure 4A, further revealed that approximately 40% of the total phosphotyrosine in kinase F_A/GSK-3α from untreated cells was decreased by the acute treatment with TPA (Fig. 4B), which is in good correlation with the cellular activity decrease of kinase F_A/GSK-3α in the immunoprecipitates obtained from the cells treated with 1 μM TPA at 37°C for 30 min and processed under identical conditions (Fig. 2). Conversely, chronic treatment of the cells with TPA was found to increase the phosphotyrosine content of kinase F_A/GSK-3α up to ~130% of the original control level in the untreated cells (Fig. 4B), which is in agreement with the cellular activity increase of kinase F_A/GSK-3α in the immunoprecipitates from cells treated with 1 μM TPA at 37°C for 24 h and processed under identical conditions (Fig. 2). In sharp contrast, the phosphoserine contents in kinase F_A/GSK-3α immunoprecipitated from both TPA-treated and -untreated cells were found to be almost identical (Fig. 4B), and treatment of the immunoprecipitated kinase F_A/GSK-3α from both treated and untreated cells with protein phosphatase 2A could cause serine dephosphorylation, but without modifying the enzyme activity (not shown),

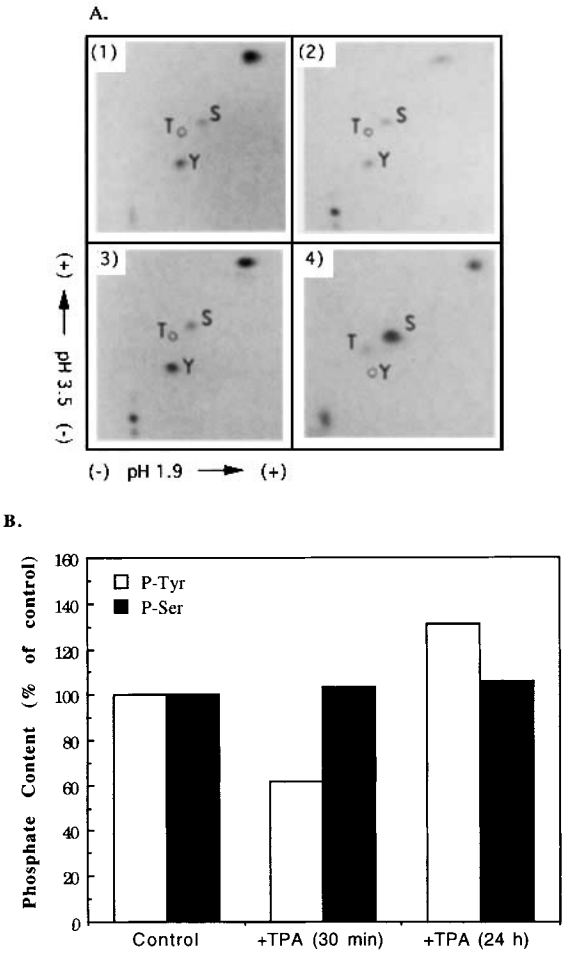

Fig. 4. Phosphoamino acid analysis and densitometric quantification of the in vivo ^{32}P-labeled kinase F_A/GSK-3α immunoprecipitated from A431 cells treated with TPA. The immunoprecipitates obtained from ^{32}P-labeled A431 cells treated with 1 μM TPA at 37°C for 30 min and 24 h were subjected to 10% SDS–PAGE, electrotransferred to PVDF membrane, and autoradiographed as described in the legend to Figure 3. The ^{32}P-labeled protein bands in the autoradiogram corresponding to kinase F_A/GSK-3α at a molecular weight of 53 kDa and pp62 at a molecular weight of 62 kDa were excised from the membrane, directly hydrolyzed in 5.7 N HCl under N_2 at 110°C for 1 h and subjected to two-dimensional phosphoamino acid analysis on thin-layer cellulose plates, followed by autoradiography as described under Materials and Methods. **A:** Autoradiogram. Panels 1–3, two-dimensional phosphoamino acid analysis of ^{32}P-labeled kinase F_A/GSK-3α immunoprecipitated from untreated cells (1) or from cells treated with 1 μM TPA at 37°C for 30 min (2), or from cells treated with 1 μM TPA at 37°C for 24 h (3). Panel 4, phosphoamino acid analysis of ^{32}P-labeled pp62 co-immunoprecipitated with kinase F_A/GSK-3α from untreated cells. The positions of phosphoamino acids were localized by ninhydrin stain of standards. Symbols used are S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. **B:** Densitometric quantification of the phosphotyrosine and phosphoserine contents of kinase F_A/GSK-3α from A.

indicating that serine phosphorylation in kinase F_A/GSK-3α is unrelated to the kinase activity and is unaffected by TPA. This further supports the correlation of tyrosine phosphorylation–dephosphorylation and concurrent activation–inactivation of kinase F_A/GSK-3α in cells (Figs. 2, 4), which is in agreement with the in vitro results reported by Hughes et al. [1993]. On the other hand, the phosphorylation level of pp62, which co-immunoprecipitated with kinase F_A/GSK-3α from A431 cells, appeared to be phosphorylated mainly on serine residue and without tyrosine phosphorylation (Fig. 4A, panel 4). The decreased serine phosphorylation level appeared to be in good correlation with the decreased tyrosine phosphorylation and concurrently decreased cellular activity of kinase F_A/GSK-3α in cells (Figs. 2–4), suggesting that pp62 is an endogenous substrate for kinase F_A/GSK-3α in cells which co-sendimented with kinase F_A/GSK-3α during immunoprecipitation.

DISCUSSION

In this report, we have produced and affinity-purified antikinase F_A/GSK-3α antibody that can efficiently and specifically immunoblot and immunoprecipitate kinase F_A/GSK-3α from A431 cell extracts for immunodetection kinase F_A/GSK-3α activity in cells treated with tumor promoter phorbol ester TPA, a specific modulator of protein kinase C [Nishizuka, 1986]. We found that acute treatment of the cells with 1 μM TPA at 37°C for 30 min could cause tyrosine dephosphorylation and concurrent inactivation of kinase F_A/GSK-3α down to ~60% of control value in A431 cells, demonstrating that extracellular stimuli may regulate the phosphotyrosine content in kinase F_A/GSK-3α and thereby modulate the cellular activity of this kinase in cells. In sharp contrast, when cells were chronically treated with 1 μM TPA at 37°C for 24 h and processed under identical conditions, the cellular activity and phosphotyrosine content of kinase F_A/GSK-3α was found to be increased up to about 130% of control level, indicating that tumor promoter phorbol ester can reversibly modulate the tyrosine phosphorylation state and cellular activity of kinase F_A/GSK-3α in cells. Since acute exposure of cells to TPA causes up-regulation of cellular protein kinase C activity [Nishizuka, 1986], whereas prolonged exposure of cells to TPA causes down-regulation of protein kinase C [Rozengurt et al., 1983; Blackshear et al.,

1985; Chida et al., 1986; Woodgett and Hunter, 1987; Murray et al., 1987; Kazlauskas and Cooper, 1988; Ohmichi et al., 1992], the results further suggest that the TPA-mediated tyrosine phosphorylation–dephosphorylation and concurrent activation-inactivation of protein kinase F_A/GSK-3α could possibly be in part due to the modulation of protein kinase C, representing a new mode of signal transduction pathway for the regulation of this multisubstrate/multifunctional protein kinase in cells [Yang, 1991; Woodgett, 1991; Hughes et al., 1993]. The unidentified tyrosine kinase and/or phosphatase specific for kinase F_A/GSK-3α that can possibly be regulated by protein kinase C obviously presents an intriguing issue deserving further investigation. Nevertheless, the present study clearly demonstrates that the phosphotyrosine content and cellular activity of protein kinase F_A/GSK-3α can be modulated in a reversible manner by phorbol ester tumor promoter in cells.

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