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Tyrosine Dephosphorylation and Concurrent Inactivation of Protein Kinase F_A/GSK-3α by Genistein in A431 Cells

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Abstract Modulation 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 become tyrosine-dephosphorylated and inactivated down to less than 30% of control values in a concentration-dependent manner by 50–400 μM genistein (a specific tyrosine kinase inhibitor), as demonstrated by metabolic ^{32}P-labeling of the cells followed by immunoprecipitation and two-dimensional phosphoamino acid analysis and by immunodetection in an antikinase F_A/GSK-3α immunoprecipitate kinase assay. Taken together, the results provide evidence that kinase F_A/GSK-3α may exist in a highly tyrosine-phosphorylated/activated state in resting cells which can be tyrosine-dephosphorylated and inactivated by extracellular stimulus and that tyrosine kinase(s) and/or tyrosine phosphatase(s) may play a role in the modulation of kinase F_A/GSK-3α activity in cells. © 1994 Wiley-Liss, Inc.

Key words: genistein, tyrosine dephosphorylation, inactivation, kinase F_A/GSK-3α, 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], protooncogene 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 functions and pathophysiological functions [reviewed in Yang, 1991; Woodgett, 1991; Yang et al., 1992c, 1993; Hanger et al., 1992].

Recently, the activity of kinase F_A/GSK-3α was shown to be activated by tyrosine phosphorylation [Hughes et al., 1993], representing a new mode of control mechanism for regulating the activity of kinase F_A/GSK-3α. It will be interesting to assess the effects of extracellular stimuli on the phosphotyrosine content and concurrent activity change of kinase F_A/GSK-3α in cells. In this report, modulation of kinase F_A/GSK-3α by tyrosine phosphorylation-dephosphorylation in A431 cells treated with genistein (a tyrosine kinase inhibitor) [Akiyama et al., 1987] was investigated using immunodetection in an antikinase F_A/GSK-3α immunoprecipitate

Abbreviations used: F_A, type-1 protein phosphatase activating factor; GSK-3α, glycogen synthase kinase-3α; MBP, myelin basic protein; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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and metabolic ^{32}P-labeling cells followed by immunoprecipitation and two-dimensional phosphoamino acid analysis and phosphoimage analysis. Here, we report that protein kinase F_A/GSK-3α can be regulated by tyrosine dephosphorylation in cells by extracellular stimuli.

EXPERIMENTAL PROCEDURES

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. 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). Genistein (Gaithersburg, MD), Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), sodium orthovanadate, 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 (San Francisco, CA). Polyvinylidene fluoride (PVDF) membrane was from Millipore (Bedford, MA). 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

Phosphorylase b [Fischer and Krebs, 1958], phosphorylase b kinase [Cohen, 1973], and ^{32}P-phosphorylase a [Krebs et al., 1958] were purified from rabbit skeletal muscle. Protein kinase F_A/GSK-3α [Yang, 1986; Yu and Yang, 1993], ATP.Mg-dependent protein phosphatase [Yang and Fong, 1985], 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, Bedford, MA). 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. Then 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 of conjugated peptide suspended in 500 μl incomplete Freund's adjuvant was used as booster shots. Ten days after the third injection, 40 ml of blood was drawn from marginal ear veins and the antisera were tested for kinase F_A/GSK-3α antibody using immunoblotting (see section on Immunoblots). The rabbits were boosted every 4 weeks with another 500 μg of conjugated peptide in 500 μl of incomplete Freund's adjuvant, and 40 ml of blood was routinely drawn every 10 days after each booster injection. Another New Zealand rabbit was bled without immunization (preimmune 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. Preimmune antibodies were purified from preimmune serum by protein A-Sepharose.

Culture of A431 Cells, Drug 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 experiments, cells ($1–2 \times 10^6$) were plated on 100 mm culture dishes. For drug treatment, aliquots of stock solution of genistein (20 mM dissolved in DMSO) were added directly into the culture medium to make the required final concentrations. After a 2 h incubation at 37°C, 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 seconds 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.

Metabolic ^{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 2 h. Drugs at various concentrations were added to the medium and incubated for another 2 h. 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 the Immunoprecipitates

Before immunoprecipitation, protein concentrations of the cell extracts were first diluted to equal amounts with buffer A. For immunoprecipitation, the cell extracts adjusted to ~1 mg cell protein/ml buffer A were incubated with 2 μl affinity-purified kinase F_A/GSK-3α antibody (10 mg/ml) at 4°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 and 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 immunoprecipitates, 15 μl of immunoprecipitate was incubated with 30 μl of mixture containing 20 mM Tris-HCl at pH 7.0, 0.5 mM dithiothreitol, 0.2 mM [γ-^{32}P]ATP (1 pmol = ~1,000 cpm), 20 mM $MgCl_2$, and 4 mg/ml MBP at 30°C for 10 min. ^{32}P-incorportation into MBP was measured by spotting 30 μl of reaction mixture on phosphocellulose paper (1 × 2 cm) which was washed three times with 75 mM H_3PO_4 and counted in a liquid scintillation counter as described in a previous report [Yang, 1986]. A unit of kinase F_A/GSK-3α as a MBP kinase is that amount of enzyme that catalyzes the incorporation of 1 nmol of phosphate into the protein substrate per minute at 30°C. For phosphatase activating factor (F_A) activity assay, the immunoprecipitates were diluted to appropriate concentrations and assayed as described in a previous report [Yang, 1986]. A unit of F_A as a phosphatase activating factor is that amount of enzyme that catalyzes the formation of one unit of inactive phosphatase per minute at 30°C. A unit of phosphatase is that amount of enzyme that catalyzes the release of 1 nmol of phosphate from the ^{32}P-phosphorylase a per minute at 30°C [Yang and Fong, 1985].

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 three 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 three 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 three 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-indolyl phosphate at room temperature for 5–10 min. The reaction was stopped by 10 mM EDTA, and the nitrocellulose paper was 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

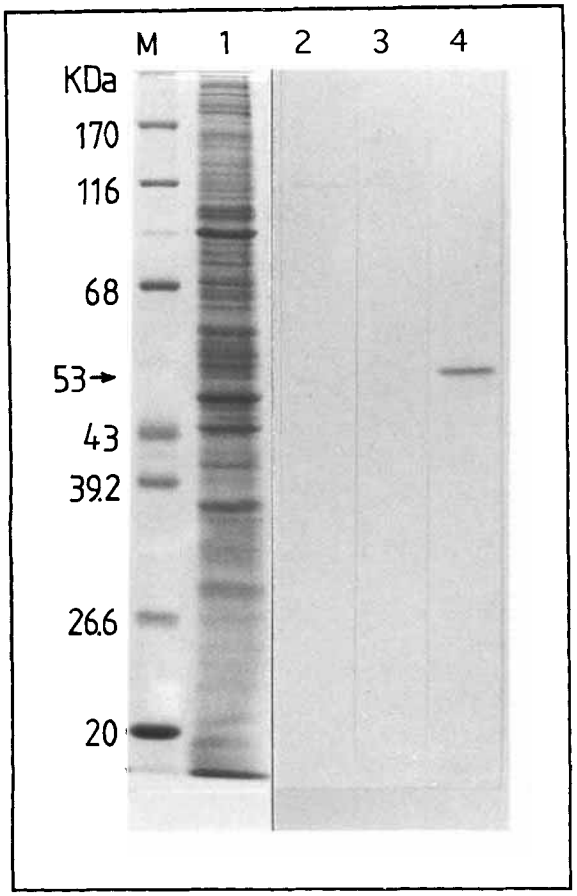


Fig. 1. Immunospecificity of the antikinase F_A/GSK-3α antibody in cell extracts. A431 cell extracts (50 μg protein/well) were subjected to 10% SDS-PAGE, electrotransferred to nitrocellulose membrane, and immunoblotted with 1 μg/ml preimmune antibody (**lane 2**) or with 1 μg/ml antikinase F_A/GSK-3α antibody in the presence (**lane 3**) or absence (**lane 4**) of 2.5 μg/ml C-terminal peptide from amino acids 462–475 of kinase F_A/GSK-3α as described under Experimental Procedures. **Lane M:** Marker proteins. **Lane 1:** Protein staining pattern of the cell extracts.

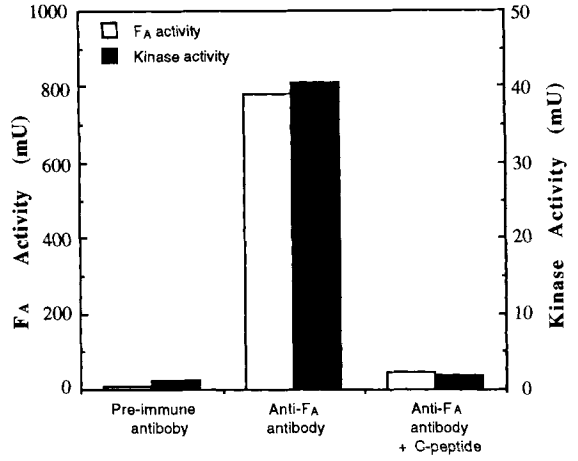
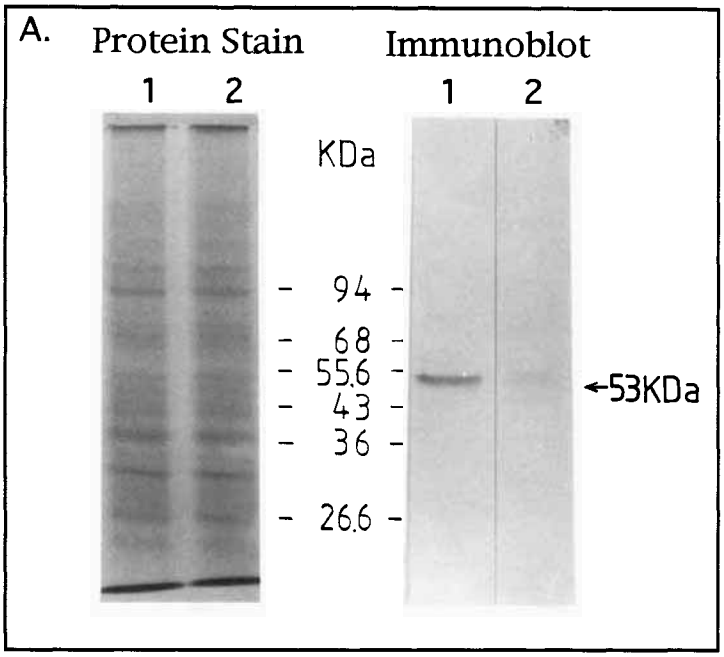


Fig. 2. Immunodetection of kinase F_A/GSK-3α activity in cell extracts. A431 cell extracts adjusted to ~500 μg cell protein in 500 μl buffer A was subjected to immunoprecipitation by 20 μg preimmune antibody or by 20 μg antikinase F_A/GSK-3α antibody in the absence and presence of 50 μg C-terminal peptide from amino acids 462–475 of kinase F_A/GSK-3α. The kinase F_A/GSK-3α in the immunoprecipitates was assayed as an activating factor of type-1 protein phosphatase and as a MBP kinase [Yang, 1986] using inactive phosphatase and MBP as substrates. Detailed conditions for cell extract preparations, immunoprecipitation, and enzyme activity assays were as described under Experimental Procedures.

min. After brief centrifugation to remove the insoluble protein A beads, the samples were subjected to 10% SDS-PAGE, electrotransferred to PVDF membrane, and visualized by staining with Amido Black (0.25% in water). The ^{32}P-labeled proteins localized by autoradiography were excised from the membrane 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, Farmingdale, NY) and subjected to high-voltage electrophoresis in the first dimension on the thin-layer cellulose (TLC) plate in pH 1.9 buffer (formic acid/acetic acid/H_2O = 50/156/1,794) at 1.5 KV at 20°C for 20 min. After being air-dried, the TLC 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. The dried plates were subjected to autoradiography using storage phosphor screen and analyzed by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Analytic Method

Protein concentrations were determined by using BCA protein assay reagents from Pierce (Rockford, IL). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [1970]. Molecular weight markers used are α_2-macroglobulin (170 KDa), β-galatosidase (116.4 KDa), phosphorylase b (94 KDa), fructose-6-phosphate kinase (85.2 KDa), bovine serum albumin (68 KDa), glutamate dehydrogenase (55.6 KDa), ovalbumin (43 KDa), aldolase (39.2 KDa), glyceraldehyde 3-phosphate dehydrogenase (36 KDa), triosephosphate isomerase (26.6 KDa), and trypsin inhibitor (20 KDa). Autoradiography was carried out at −70°C with a Fuji RX X-ray film



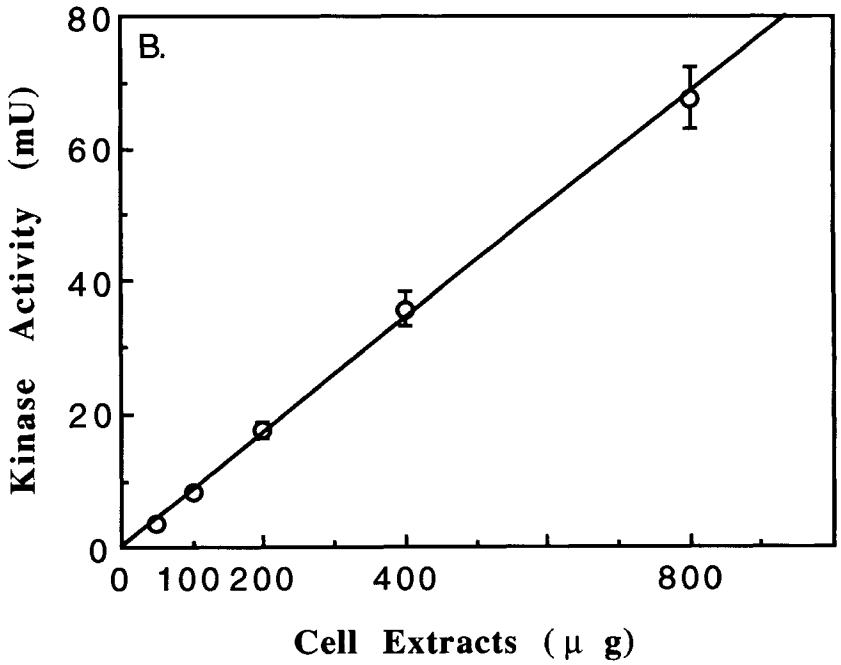


Fig. 3. Efficacy of immunoprecipitation of kinase F_A/GSK-3α from cell extracts. **A:** A431 cell extracts adjusted to ~1 mg cell protein/ml buffer A were divided into two equal parts and incubated without (**lane 1**) or with (**lane 2**) 20 μg antikinase F_A/GSK-3α antibody at 4°C for 1.5 h and then with 100 μl protein A-Sepharose CL-4B (20% v/v in buffer A) at 4°C for another 1.5 h. After centrifugation to spin down protein A beads, equal amounts of the supernatant (40 μl) were subjected either to 10% SDS-PAGE and protein staining or to immunoblotting by antikinase F_A/GSK-3α antibody. **B:** The cell extracts at various concentrations ranging from 50–800 μg of cell protein in 500 μl buffer A were immunoprecipitated by 20 μg antikinase F_A/GSK-3α antibody as described above and then subjected to kinase activity assay in the immunoprecipitates using MBP as substrate as described under Experimental Procedures. Data were the average of three independent experiments and expressed as means ± SD.

using a Kodak X-Omatic cassette with intensifying screens or using a storage phosphor screen and PhosphorImager. The results from autoradiography were quantified by Computing Densitometer (Molecular Dynamics).

RESULTS

Characterization of Antikinase F_A/GSK-3α Antibody

The antikinase F_A/GSK-3α antibody produced and affinity-purified as described in Experimental Procedures was found to be very specific towards immunoblotting kinase F_A/GSK-3α at a molecular weight of 53 KDa from A431 cell extracts (Fig. 1, lanes M,1,4), which could be blocked by the C-terminal peptide from amino acids 462–475 of kinase F_A/GSK-3α (Fig. 1, lane 3), demonstrating the immunospecificity of antikinase F_A/GSK-3α antibody produced here. Preimmune serum was also used as control to probe F_A/GSK-3α from cell extracts, and there was no kinase F_A/GSK-3α detectable in the immunoblot (Fig. 1, lane 2). To test whether this antibody can be used to immunoprecipitate kinase F_A/GSK-3α without blocking the enzyme activity, the antibody was next subjected to immunoprecipitation with kinase F_A/GSK-3α from A431 cell extracts. The enzyme activities of kinase F_A/GSK-3α assayed as an activating factor of type-1 protein phosphatase and as a myelin basic protein (MBP) kinase [Yang, 1986] could be recovered and detected in the immunoprecipitates from cell extracts (Fig. 2, middle columns). In contrast, when antibody was preincubated with C-terminal peptide or preimmune serum was used to replace the antibody, there was almost no detectable activity in the immunoprecipitates (Fig. 2), demonstrating that this antibody can immunoprecipitate kinase F_A/GSK-3α from A431 cells without blocking the enzyme activities. To further test the efficacy of this antibody towards immunoprecipitation of kinase F_A/GSK-3α from A431 cells, various concentrations of cell extracts ranging from 50–800 μg of cell protein in 500 μl buffer A were next subjected to immunoprecipitation with 20 μg antibody. When cell extracts (500 μg of cell protein in 500 μl buffer A) were subjected to preimmunoprecipitation with 20 μg of antibody, almost no kinase F_A/GSK-3α could be detected in the immunoblot (Fig. 3A, lane 2) which is in sharp contrast to the cell extracts without preimmunoprecipitation with antikinase F_A/GSK-3α antibody and immunoblotted under identical conditions (Fig. 3A, lane 1). Furthermore, the enzyme activities immunoprecipitated from cell extracts were found to be proportional to the cell protein concentrations in the extracts (Fig. 3B), demonstrating the efficacy of this antibody towards immunoprecipitation of kinase F_A/GSK-3α from the crude extracts of A431 cells.

Effect of Genistein on the Cellular Activity of Kinase F_A/GSK-3α in A431 Cells

Genistein, a specific tyrosine kinase inhibitor, basically has no direct effect on the activity of purified kinase F_A/GSK-3α (not shown). However, when A431 cells were treated with 50–400 μM genistein at 37°C for 2 h followed by immunoprecipitation of kinase F_A/GSK-3α from cell extracts using the antibody described above, the cellular activity of kinase F_A/GSK-3α could be inhibited down to less than 30% of control values by genistein in a concentration-dependent manner (Fig. 4). In sharp contrast, under identical conditions, genistein caused no change in the immunoblotted protein level (Fig. 5), indicating that genistein did not generate de novo protein systhesis of the enzyme and that a posttranslational modification of preexisting protein was involved in the genistein-mediated inactivation of protein kinase F_A/GSK-3α (Figs. 4, 5).

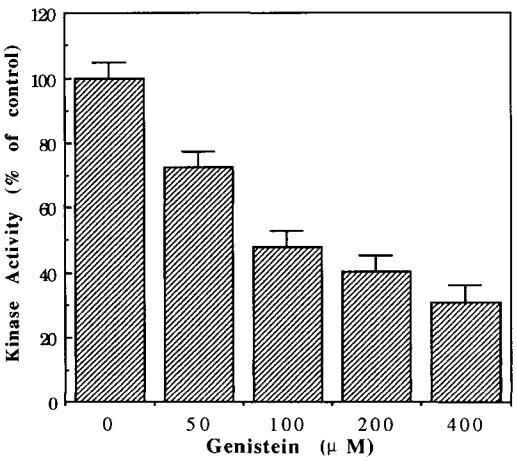


Fig. 4. Effect of genistein on the cellular activity of kinase F_A/GSK-3α in A431 cells. A431 cells (4×10^6 cells) were treated with various concentrations of genistein as indicated at 37°C for 2 h. The cell extracts adjusted to ~0.5 mg cell protein in 500 μl buffer A were immunoprecipitated by 20 μg antikinase F_A/GSK-3α antibody followed by kinase activity assay using MBP as substrate in the immunoprecipites as described under Experimental Procedures. Data were the average of three independent experiments and expressed as means ± SD.

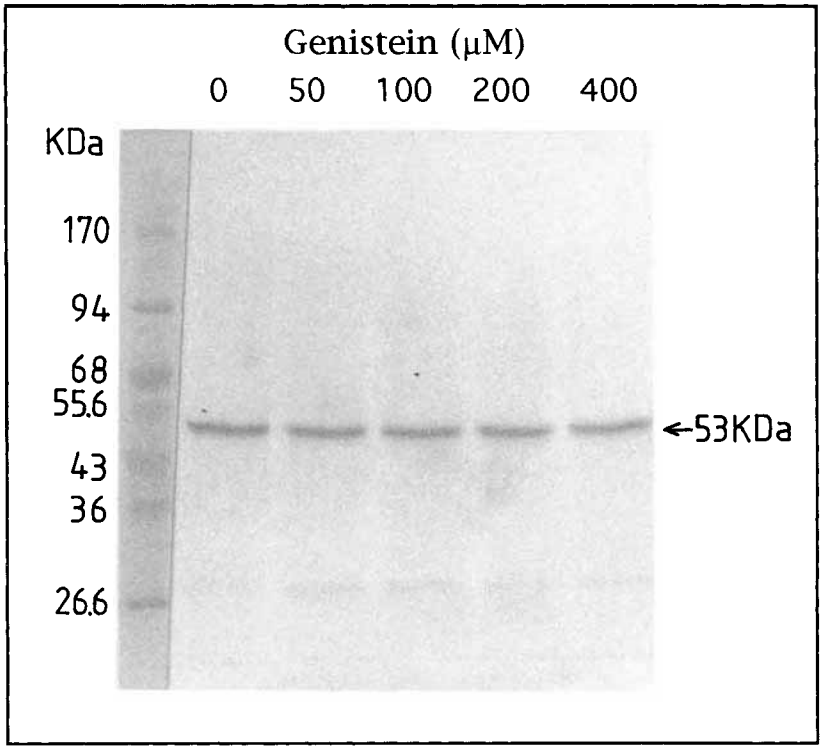


Fig. 5. Immunoblot of kinase F_A/GSK-3α from crude extracts of A431 cells treated with genistein. A431 cells (4 × 10^6 cells) were treated with various concentrations of genistein as indicated at 37°C for 2 h, and 40 μg of the cell extracts were immunoblotted by antikinase F_A/GSK-3α antibody on 10% SDS-PAGE as described under Experimental Procedures.

Effect of Genistein on the In Vivo Phosphorylation of Kinase F_A/GSK-3α in A431 Cells

To investigate the role of protein phosphorylation 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 various concentrations of genistein. This was evaluated by metabolic labeling the cells with [^{32}P]orthophosphate at 37°C for 2 h followed by another 2 h incubation with genistein. Cells were subsequently immunoprecipitated with antikinase F_A/GSK-3α antibody and subjected to SDS-PAGE followed by autoradiography (Fig. 6A). Genistein caused decreased phosphorylation of the 53 KDa kinase F_A/GSK-3α and a coimmunoprecipitated phosphoprotein at a molecular weight of 62 KDa tentatively termed pp62 in a concentration-dependent manner in intact cells. Densitometric quantification of the phosphorylated proteins in the autoradiogram further revealed a decreased phosphorylation pattern (Fig. 6B), which is similar to what was observed in the in vitro kinase assay in the immunoprecipitates from cells treated with genistein and processed under identical conditions (Fig. 4). To explore in greater detail, the ^{32}P-labeled protein bands corresponding to kinase F_A/GSK-3α and pp62 were excised from the autoradiogram and subjected to two-dimensional phosphoamino acid analysis on thin-layer cellulose plates followed by autoradiography using storage phosphor screen and analyzed by PhosphorImager. Kinase F_A/GSK-3α appeared to be highly phosphorylated on both serine and tyrosine residues in the untreated cells (Fig. 7A, panel 1). The tyrosine phosphorylation level was decreased by genistein in a pattern (Fig. 7B), which is similar to what was observed in the in vitro kinase activity assay in the immunoprecipitates obtained from cells treated with genistein and processed under identical conditions (Fig. 4), demonstrating that the enzyme activity was proportional to the content of phosphotyrosine in kinase F_A/GSK-3α. In sharp contrast, the content of phosphoserine in kinase F_A/GSK-3α was not significantly affected by genistein and had no correlation with the kinase activity change (Figs. 4, 7A, panels 1–5). On the other

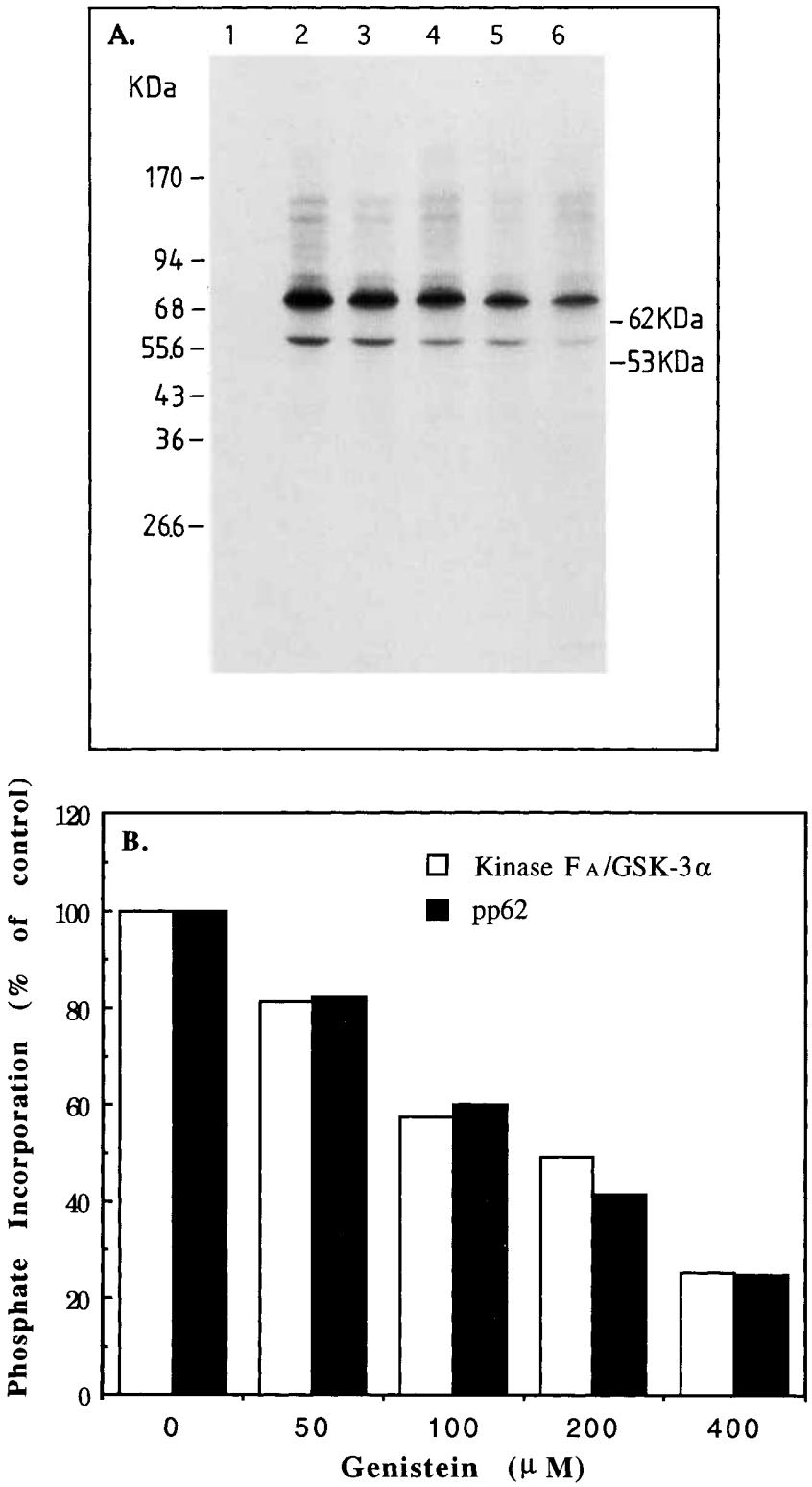


Fig. 6. The in vivo ^{32}P-labeled kinase F_A/GSK-3α immunoprecipated from A431 cells treated with genistein. Metabolic ^{32}P-labeled A431 cells (1×10^7 cells) were treated with various concentrations of genistein as indicated at 37°C for 2 h. The cell protein concentrations were adjusted to equal value (1.5 mg cell protein/ml buffer A), and 750 μl extracts were subjected to immunoprecipitation by 20 μg antikinase F_A/GSK-3α antibody. The immunoprecipitates were analyzed by 10% SDS-PAGE, electrotransferred to PVDF membrane, and autoradiographed. **A:** Autoradiography of the protein-transferred PVDF membrane. **Lane 1:** Immunoprecipitates from untreated cells in the presence of 50 μg C-terminal peptide from amino acids 462–475 of kinase F_A/GSK-3α. **Lanes 2–6:** Immunoprecipitates from cells treated with 0, 50, 100, 200, and 400 μM genistein in the absence of C-terminal peptide. **B:** Densitometric quantification of the phosphorylation levels of kinase F_A/GSK-3α and the coimmunoprecipitated phosphoprotein, pp62 in A. Detailed conditions were as described under Experimental Procedures.

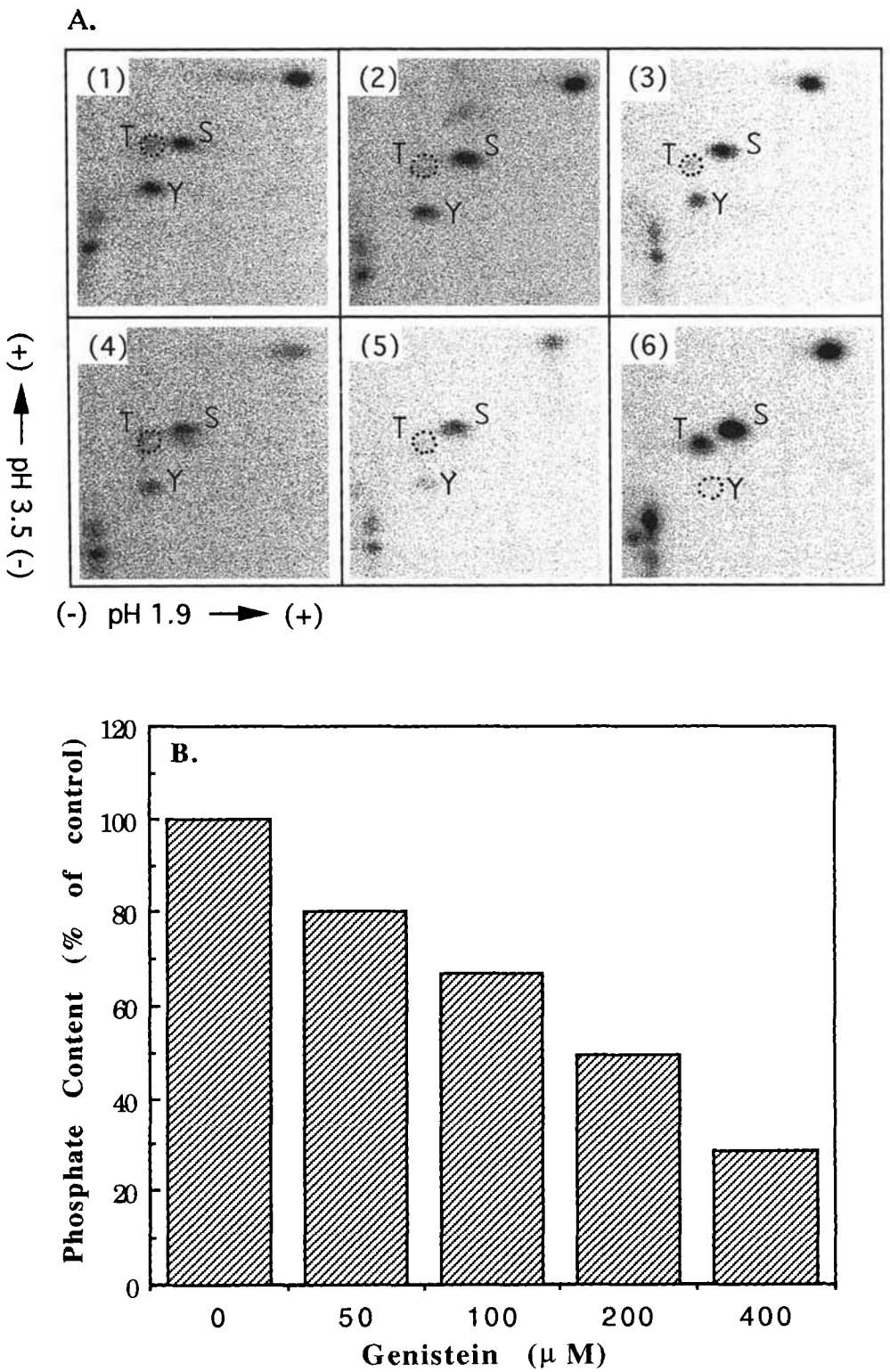


Fig. 7. Phosphoamino acid analysis of the in vivo ^{32}P-labeled kinase F_A/GSK-3α immunoprecipitated from A431 cells treated with genistein. The ^{32}P-labeled protein bands corresponding to kinase F_A/GSK-3α and pp62 as shown in Figure 6 were excised from the membrane, directly hydrolyzed in 5.7 N 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 using a storage phosphor screen and analyzed by PhosphorImager as described under Experimental Procedures **A: Panels 1–5:** Two-dimensional phosphoamino acid analysis of ^{32}P-labeled kinase F_A/GSK-3α immunoprecipitated from cells treated with 0, 50, 100, 200, and 400 μM genistein, respectively. **Panel 6:** Phosphoamino acid analysis of ^{32}P-labeled pp62 coimmunoprecipitated with kinase F_A/GSK-3α from untreated cells. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. **B:** Quantification of the phosphotyrosine content of kinase F_A/GSK-3α from panels 1–5 of A.

hand, pp62 which coimmunoprecipitated with kinase F_A/GSK-3α from A431 cells appeared to have only serine and threonine phosphorylated but without tyrosine phosphorylation (Fig. 7A, panel 6), further supporting the notion that pp62 is an endogenous substrate which coimmunoprecipitated with kinase F_A/GSK-3α from A431 cells since the decreased phosphorylation level of pp62 appeared in good correlation with the decreased tyrosine phosphorylation and kinase activity patterns of kinase F_A/GSK-3α (Figs. 4, 6B, 7B).

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 of kinase F_A/GSK-3α activity in cells treated with genistein, a specific inhibitor of both receptor-type and nonreceptor tyrosine kinase [Akiyama et al., 1987]. We found that genistein could cause dephosphorylation and concurrent inactivation of kinase F_A/GSK-3α 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 as proposed by Hughes et al. [1993]. The regulation of tyrosine kinase and/or tyrosine phosphatase specific for kinase F_A/GSK-3α may therefore play an important role in the signal transduction of kinase F_A/GSK-3α in cells, which obviously presents an intriguing issue deserving further investigation.

During the immunoprecipitation of kinase F_A/GSK-3α from A431 cells, a phosphoprotein at a molecular weight of 62,000 tentatively termed pp62 always coimmunoprecipitated with kinase F_A/GSK-3α and appeared to have both serine and threonine residues phosphorylated but without tyrosine phosphorylation. More interestingly, the phosphate content of pp62 was also decreased by genistein in a concentration-dependent fashion which appeared in good correlation with the decreased tyrosine phosphorylation and concurrently decreased cellular activity patterns of kinase F_A/GSK-3α generated by genistein in cells (cf. Figs. 4, 6, 7). The results taken together with the fact that kinase F_A/GSK-3α is a specific serine/threonine kinase [Yang, 1991; Woodgett, 1991; Yang et al., 1993c] support the notion that the coimmunoprecipitated pp62 may represent a physiologically relevant endogenous protein substrate for kinase F_A/GSK-3α in the cell. Moreover, the coimmunoprecipitation of pp62 with kinase F_A/GSK-3α from A431 cell extracts like kinase F_A/GSK-3α can also be completely blocked by the C-terminal peptide of kinase F_A/GSK-3α (Fig. 6A, lane 1), further supporting the notion that pp62 is an endogenous substrate of kinase F_A/GSK-3α which can be complexed with the immunoprecipitated kinase F_A/GSK-3α during immunoprecipitation by antikinase F_A/GSK-3α antibody from A431 cell extracts. The biological function of pp62 obviously presents an intriguing subject deserving investigation. On the other hand, kinase F_A/GSK-3α also had serine residue phosphorylated in vivo (Fig. 7, panel 1) [Hughes et al., 1993]. However, the phosphoserine content in kinase F_A/GSK-3α appeared to be insignificantly affected by genistein in cells and had no correlation with the kinase activity change (Figs. 4, 6, 7), suggesting that the in vivo serine phosphorylation in kinase F_A/GSK-3α may represent a "silent" phosphorylation unrelated to the regulation of kinase F_A/GSK-3α activity in cells but may play a role maybe related to cell compartmentation and protein interactions, which remains to be established. Nevertheless, the results presented here clearly demonstrate that kinase F_A/GSK-3α is highly tyrosine phosphorylated and is active in resting cells. Following stimulation by genistein (a specific tyrosine kinases inhibitor), kinase F_A/GSK-3α could be inactivated via tyrosine dephosphorylation, representing a new mode of control mechanism for regulating the activity of this multisubstrate/multifunctional independent protein kinase in cells.

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