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ARTICLES

Heat Stress Induces Tyrosine Phosphorylation/Activation of Kinase FA/GSK-3α (a Human Carcinoma Dedifferentiation Modulator) in A431 Cells

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Abstract Exposure of A431 cells to a rapid temperature increase from 37° to 46°C could induce an increased expression (~200% of control) and tyrosine phosphorylation/activation (~300% of control) of protein kinase FA/glycogen synthase kinase-3α (kinase FA/GSK-3α) in a time-dependent manner, as demonstrated by an anti-kinase FA/GSK-3α immunoprecipitate kinase assay and by immunoblotting analysis with anti-kinase FA/GSK-3α and anti-phosphotyrosine antibodies. The heat induction on the increased expression of kinase FA/GSK-3α could be blocked by actinomycin D but not by genistein. In contrast, the heat induction on tyrosine phosphorylation/activation of kinase FA/GSK-3α could be blocked by genistein or protein tyrosine phosphatase, indicating that heat stress induces a dual control mechanism, namely, protein expression and subsequent tyrosine phosphorylation to cause cellular activation of kinase FA/GSK-3α. Taken together, the results provide initial evidence that kinase FA/GSK-3α represents a newly described heat stress–inducible protein subjected to tyrosine phosphorylation/activation, representing a new mode of signal transduction for the regulation of this human carcinoma dedifferentiation modulator and a new mode of heat induction on cascade activation of a protein kinase. J. Cell. Biochem. 66:16–26, 1997.

Key words: heat stress; kinase FA/GSK-3α; tyrosine phosphorylation/activation; cascade activation; protein expression

Protein kinase FA was originally identified as an activating factor of Mg.ATP-dependent type-1 protein phosphatase [Yang et al., 1980; Vandenheede et al., 1980] but has subsequently been demonstrated as a protein kinase identical to glycogen synthase kinase-3 (GSK-3) [Embi et al., 1980; Hemmings et al., 1981]. Two classes of rat brain cDNA for GSK-3 have been cloned and isolated: GSK-3α and GSK-3β [Woodgett, 1990]. GSK-3α represents the enzyme originally purified from tissues identical to kinase FA [Yang, 1991], whereas GSK-3β is a novel newly described 47 kDa protein with 85% amino acid identity to GSK-3α [Woodgett, 1990]. The two types of cDNA are the products of distinct genes as determined by genomic organization and nucleic acid sequence analysis [Woodgett, 1990]. Although GSK-3β has 85% amino acid identity and catalytic properties similar to GSK-α [Hughes et al., 1992], they are distinctly different in terms of substrate specificity, biological activity, and signal transduction. Kinase FA/GSK-3α appeared to have higher activity as a protein phosphatase activator and lower activity as a protein kinase towards the other protein substrates, whereas GSK-3β appeared to have much lower activity as a protein phosphatase activator and higher activity as a protein kinase towards the other protein substrates [Wang et al., 1994a; Song and Yang, 1995], and the phosphorylation sites of tau protein by GSK-3α and GSK-3β are distinctly different [Yang et al., 1994a; Song and Yang, 1995]. More importantly, GSK-3β, but not GSK-3α, can be directly phosphorylated/inactivated by certain isotypes of protein kinase C in vitro [Goode et al., 1992]. Most importantly, GSK-3β, but not

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

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GSK-3α, is able to restore wild-type phenotype in zeste-white 3/shappy mutants [Ruel et al., 1993] and recombinant GSK-3β autophosphorylated on Ser, Thr, and Tyr residues. Phosphorylation at different residues differentially controls GSK-3β activity, Ser/Thr phosphorylation causing inactivation and Tyr phosphorylation resulting in activation [Wang et al., 1994b]. In sharp contrast, kinase FA/GSK-3α underwent autophosphorylation only on Ser and Thr residues but not on Tyr residue. Moreover, kinase FA/GSK-3α behaved differently from GSK-3β in autophosphorylation. Besides the lack of Tyr phosphorylation, the autophosphorylation of FA/GSK-3α did not affect its activity [Wang et al., 1994b]. However, recent reports revealed that kinase FA/GSK-3α could be serine-phosphorylated/inactivated to ~50% by insulin/epidermal growth factor and insulin-like growth factor-1 possibly via mitogen-activated protein kinase/p90 ribosomal protein S6 kinase signaling pathway [Ramakrishna and Benjamin, 1988; Hughes et al., 1992; Sutherland and Cohen, 1994; Cross et al., 1994; Saito et al., 1994; Eldar-Finkelman et al., 1995]. Moreover, the activity of kinase FA/GSK-3α was also shown to be regulated by tyrosine phosphorylation and dephosphorylation in cells [Hughes et al., 1993; Yu and Yang, 1994a; Yang et al., 1994b], representing a new mode of control mechanism for regulating the activity of this kinase. However, the signal transduction for regulating tyrosine phosphorylation and concurrent activation of kinase FA/GSK-3α in cells remains to be established.

In addition to Mg.ATP-dependent protein phosphatase and glycogen synthase as its substrates, kinase FA/GSK-3α was further identified as a multisubstrate protein kinase possibly involved in the regulation of diverse cell functions [for reviews, see Yang, 1991; Woodgett, 1991; Plyte et al., 1992; Yang, 1994]. Sites phosphorylated by kinase FA/GSK-3α can be divided into two classes. For some substrates, prior phosphorylation of the substrate, to form the motif -S-$(X)_3$-S(P)-, is a requirement, whereas in other substrates no previous phosphorylation is needed [Roach, 1991; Wang et al., 1994a]. In either case, many of the sites have Pro residues close to the modified Ser/Thr, demonstrating that kinase FA/GSK-3α is a member of the so-called proline-directed protein kinase (PDPK) family [Hemmings et al., 1982; Hemmings and Cohen, 1983; Hunter et al., 1988; Dent et al., 1989; Vulliet et al., 1989; Fiol et al., 1990; Ramakrishna et al., 1990; Boyle et al., 1991; Plyte et al., 1992; Mandelkow et al., 1992; Yang et al., 1993, 1995a; Yu and Yang, 1994c; Singh et al., 1995]. Based on computer-assisted sequence analysis of transcriptional factors and viral oncoproteins [Suzuki, 1989], as well as analysis of site-specific protein phosphorylation both in vitro and in vivo [Moreno and Nurse, 1990; Pines and Hunter, 1990; Lin et al., 1991], it appears that proline-directed protein phosphorylation sites represent a unique structural motif that has been conserved and canalized as a major regulatory theme [Hall and Vulliet, 1991; Williams et al., 1992; Warburton et al., 1993]. It has been proposed that overexpression, dysregulation, or viral subversion of some certain PDPKs could be associated with neoplastic transformation and tumorigenesis. Consistent with this notion, clinical studies confirmed that overexpression of kinase FA/GSK-3α (a member of PDPK family) indeed appeared to be closely correlated with the states of dedifferentiation/progression of human thyroid tumor cells [Lee et al., 1995] and human cervical carcinoma [Yang et al., 1995b].

In this report, kinase FA/GSK-3α has further been identified as a newly described heat stress–inducible protein kinase subjected to tyrosine phosphorylation/activation, representing a new mode of signal transduction mechanism for the regulation of this multisubstrate/multifunctional protein kinase in cells and a new mode of heat induction process on cascade activation of a protein kinase during heat stress. Since kinase FA/GSK-3α is a possible human carcinoma dedifferentiation/progression modulator, the association of the elevated expression of this PDPK activity with human carcinoma dedifferentiation/progression during severe heat stress is therefore discussed.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP was purchased from Amersham (Buckinghamshire, UK). Human epidermoid carcinoma A431 cells were obtained from American Type Culture Collection (Rockville, MD). These cells are one of a series of human tumor-derived cell lines and have been used for studies of hormone actions and tumor promoter effects. A431 cells within passages 15–30 were used for all the experiments in this text. Plastic wares for cell culture were from Falcon (Ox-

nard, CA). Antibiotics, fetal bovine sera, complete and incomplete Freund's adjuvants were from Gibco (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin, dimethyl sulfoxide (DMSO), sodium orthovanadate, Tween 20, genistein, actinomycin D, 3-[cyclohexylamino]-1-propane-sulfonic acid (CAPS), and goat anti-rabbit IgG antibody conjugated with alkaline phosphatase were from Sigma (St. Louis, MO). Disodium 3-(4-methoxyspiro [1,2-dioxetane-3,2′-(5′-chloro) tricyclo [3.3.1.1^{3,7}] decan]-4-yl) phenyl phosphate (CSPD), protein tyrosine phosphatase, and streptavidin conjugated with alkaline phosphatase were 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). Protein A-Sepharose CL-6B and CNBr-activated Sepharose 4B were from Pharmacia (Uppsala, Sweden). Biotinylated anti-phosphotyrosine antibody (PY-20) was from Transduction Lab (Lexington, KY). X-ray film was from Fuji (Tokyo, Japan).

Protein Purification and Analytic Methods

Myelin basic protein (MBP) and protein kinase FA/GSK-3α were purified to homogeneity from porcine brain following the purification procedures as described in previous reports [Yang, 1986; Yu and Yang, 1993, 1994c]. Protein concentrations were determined by using BCA protein assay reagent from Pierce (Rockford, IL). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (Laemmli, 1970) using 10% gels. Molecular weight markers used were: phosphorylase b (94 kDa), bovine serum albumin (68 kDa), glutamate dehydrogenase (55.6 kDa), and glyceraldehyde 3-phosphate dehydrogenase (36 kDa).

Production of Anti-Kinase FA/GSK-3α Antibody

The peptide TETQTGQDWQAPDA, corresponding to the carboxyl terminal region from amino acids 462 to 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 NH_2 terminus in order to facilitate coupling of the peptide to bovine serum albumin according to the procedure described by Reichlin [1980], using glutaraldehyde as the cross-linker. The detailed procedure for production and affinity-purification of anti-kinase FA/GSK-3α antibody was as described in previous reports [Yu and Yang, 1994a,b]. 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 anti-kinase FA/GSK-3α antibody. The anti-kinase FA/GSK-3α antibody produced here can specifically immunoblot kinase FA/GSK-3α and efficiently immunoprecipitate all of the kinase FA/GSK-3α from A431 cell extracts without blocking the enzyme activity essentially as described in previous reports [Yang et al., 1994b; Yu and Yang 1993, 1994a,b].

Culture of A431 Cells, Heat and 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 DMEM supplemented with 10% heat-inactivated fetal bovine serum, 25 U/ml penicillin, and 25 μg/ml streptomycin. The exponentially growing A431 cells were freshly plated 2 days before use. The culture medium was changed at night before each experiment. A431 cells were subjected to a sudden and rapid increase in a water-bath at 46°C in a humidified incubator. For drug treatment, aliquots of stock solution of genistein (20 mM dissolved in DMSO) or actinomycin D (5 mg/ml dissolved in 95% ethanol) were added directly into the culture medium to make the required final concentrations. After incubation, A431 cells (4×10^6 cells/100-mm culture dishes) were washed twice with ice-cold phosphate buffer saline (PBS), scraped in PBS, and collected by centrifugation at 800*g* at 4°C for 10 min. The collected cells were homogenized in 500 μl solution A (10 mM Tris-HCl at pH 7.4, 50 mM NaCl, 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 1 mM sodium orthovanadate) on ice by Sonic Dismembrator (model 150, Fisher, Farmingdale, NY) at 40% power output for 3×10 s. The homogenates were then ultracentrifuged at 160,000*g* at 4°C for 30 min and the supernatants were used as the cell extracts.

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

Before immunoprecipitation, protein concentrations of the cell extracts were first diluted to equal amounts with solution A. For immunoprecipitation, 500 µl cell extract (~1.0 mg/ml protein) was incubated with 1 µl affinity-purified anti-kinase FA/GSK-3α antibody (20 mg/ml pure IgG) at 4°C for 1.5 h and then with 100 µl 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 solution B (20 mM Tris-HCl at pH 7.0, 0.5 mM dithiothreitol, 1 mM PMSF, 1 mM benzamidine, and 0.5 mg/ml aprotinin), and resuspended in 60 µl solution B. For kinase FA/GSK-3α activity assay in the immunoprecipitate, 10 µl immunoprecipitate at appropriate dilution was incubated with 15 µl 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-incorporation into MBP was measured by spotting 20 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 previous reports [Yang, 1986; Yu and Yang, 1994a,b].

Immunoblotting and Phosphotyrosine Analysis

For immunoblotting analysis of kinase FA/GSK-3α from A431 cells, the cell extracts containing ~40 µg of cell protein were subjected to 10% SDS-PAGE, electrotransferred to PVDF membrane, and then immunoblotted with 1 µg/ml of anti-kinase FA/GSK-3α antibody and goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (1:2,000) essentially as described in previous reports [Yang et al., 1994b; Yu and Yang, 1994a,b]. For phosphotyrosine analysis of kinase FA/GSK-3α, the anti-kinase FA/GSK-3α immunoprecipitates were subjected to 10% SDS-PAGE, electrotransferred to PVDF membrane, immunoblotted with biotinylated anti-phosphotyrosine antibody (PY-20) (1:2,000 in solution C containing 20 mM Tris-HCl at pH 7.4, 0.5 M NaCl, 0.05% Tween 20, and 3% nonfat dried milk) at 25°C for 4 h and then incubated with streptavidin conjugated with alkaline phosphatase (1:2,000 in solution C) at 25°C for 40 min. Both immunoblots were developed with the CSPD system using 0.25 mM CSPD 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 chemiluminescence detection [Gillespie and Hudspeth, 1991]. The luminescent light emission was recorded on X-ray film and quantified by computing densitometer (Molecular Dynamics, Sunnyvale, CA).

RESULTS

To determine whether heat stress affects the cellular activity of kinase FA/GSK-3α, A431 cells were exposed to a rapid temperature increase from 37° to 46°C. The heat stress was found to induce an increase in cellular activity of kinase FA/GSK-3α to ~300% of control level in a time-dependent manner as demonstrated by immunodetection in an anti-kinase FA/GSK-3α immunoprecipitate kinase assay from the cell extracts (Fig. 1A). Employing immunoblotting analysis of the same cell extracts with anti-kinase FA/GSK-3α antibody, we further identified that the same heat stress could also induce an increase in the protein level of kinase FA/GSK-3α to ~200% of control level in a time-dependent manner (Fig. 1B). Furthermore, when A431 cells were first heat-shocked at 46°C for 15 min and then recovered at 37°C for 0–24 h, the cellular activity of kinase FA/GSK-3α could also be activated to ~300% of control level within 4 h recovery (Fig. 2). Taken together, the results provide initial evidence that kinase FA/GSK-3α may represent a newly described heat stress-inducible protein subject to heat induction of cellular activation.

To further study the molecular action mechanism for cellular activation of kinase FA/GSK-3α in heat-stressed A431 cells, we pretreated the cells with 0.5 µg/ml actinomycin D at 37°C for 30 min or with 400 µM genistein at 37°C for 2 h followed by heat shock at 46°C for another 45 min. The cells were then subjected to the immunoprecipitate kinase assay and immunoblotting analysis with anti-kinase FA/GSK-3α antibody. As shown in Figure 3, actinomycin D could completely block the increased expression of kinase FA/GSK-3α, suggesting that heat stress induces an increased expression of kinase FA/GSK-3α on a transcriptional level. In contrast, genistein could completely block the heat stress-induced cellular activation of kinase FA/GSK-3α (Fig. 3A) but could not block the increased expression of the kinase (Fig. 3B).

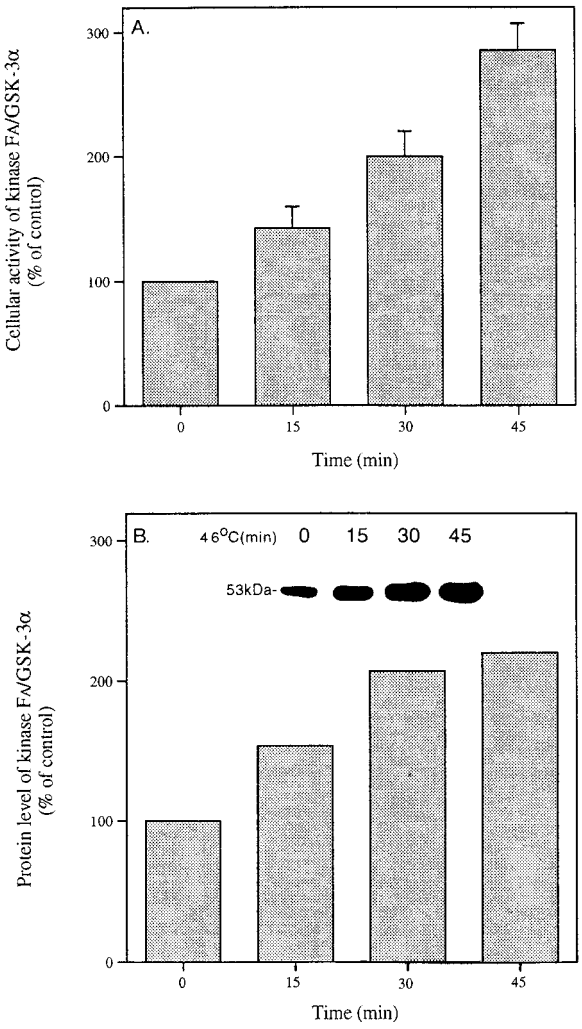

Fig. 1. Heat stress on cellular activity and protein level of kinase FA/GSK-3α in A431 cells. **A:** A431 cells were heat-shocked at 46°C for various time points as indicated. The cell extracts were first adjusted to identical protein concentrations (500 μg of cell protein in 0.5 ml of solution A) and then immunoprecipitated by 20 μg anti-kinase FA/GSK-3α antibody followed by kinase activity assay in the immunoprecipitates. Data were the average of four independent experiments and expressed as means ± S.D. **B:** The same cell extracts containing ~40 μg of cell protein were subjected to immunoblot analysis with anti-kinase FA/GSK-3α antibody. The immunoblotts were developed with the CSPD system and quantified by computing densitometer. Detailed conditions were as described under Materials and Methods.

The results suggest an involvement of post-modification in the cellular activation of kinase FA/GSK-3α in heat-stressed cells.

Since genistein is a potent and specific tyrosine kinase inhibitor capable of inducing tyrosine dephosphorylation and concurrent inactivation of kinase FA/GSK-3α in A431 cells [Yu and Yang, 1994a], we next subjected the anti-kinase FA/GSK-3α immunoprecipitates obtained from heat-stress/genistein-treated and -untreated A431 cells to phosphotyrosine analysis using anti-phosphotyrosine antibody followed by chemiluminescence detection and quantification. In agreement with cellular activation levels (Fig. 3A), the heat stress could induce tyrosine phosphorylation of kinase FA/GSK-3α to ~300% of control level (Fig. 4, lane 2), which could be inhibited to ~40% of control by genistein (Fig. 4, lane 4). The results further indicate that the heat stress may induce tyrosine phosphorylation to cause cellular activation of kinase FA/GSK-3α (Figs. 3 and 4). For final demonstration that the heat stress–induced tyrosine phosphorylation is indeed correlated with the cellular activation of kinase FA/GSK-3α, we subjected the anti-kinase FA/GSK-3α immunoprecipitates obtained from heat-stressed A431 cells to the treatment with protein tyrosine phosphatase in the presence and absence of 1 mM sodium orthovanadate (a potent and specific protein tyrosine phosphatase inhibitor) [Gordon, 1991]. As shown in Figure 5, the heat-induced activation of kinase FA/GSK-3α could be inhibited by protein tyrosine phosphatase. Moreover, the inhibitory effect of protein tyrosine phosphatase on cellular activation of kinase FA/GSK-3α could be blocked by orthovanadate (Fig. 5), demonstrating an association of tyrosine phosphorylation with cellular activation of kinase FA/GSK-3α in heat-stressed A431 cells (Figs. 4 and 5). Taken together, the results provide initial evidence that kinase FA/GSK-3α represents a newly described heat-inducible protein subjected to tyrosine phosphorylation/activation during the heat induction process (Figs. 1–5), representing a new control mechanism for regulating the cellular activity of kinase FA/GSK-3α in heat-stressed cells.

DISCUSSION

In this report, we demonstrate that the multisubstrate protein kinase FA/GSK-3α is a newly described heat-inducible protein kinase subjected to tyrosine phosphorylation/activation in heat-stressed A431 cells. Similar to the well-established inducible/constitutive heat shock proteins 72/73 kDa (HSP 72/73) [Schlesinger, 1990; Welch, 1992], kinase FA/GSK-3α was constitutively expressed in normal growth condition but its activity could be enhanced several-fold during heat induction (see Fig. 1). Although

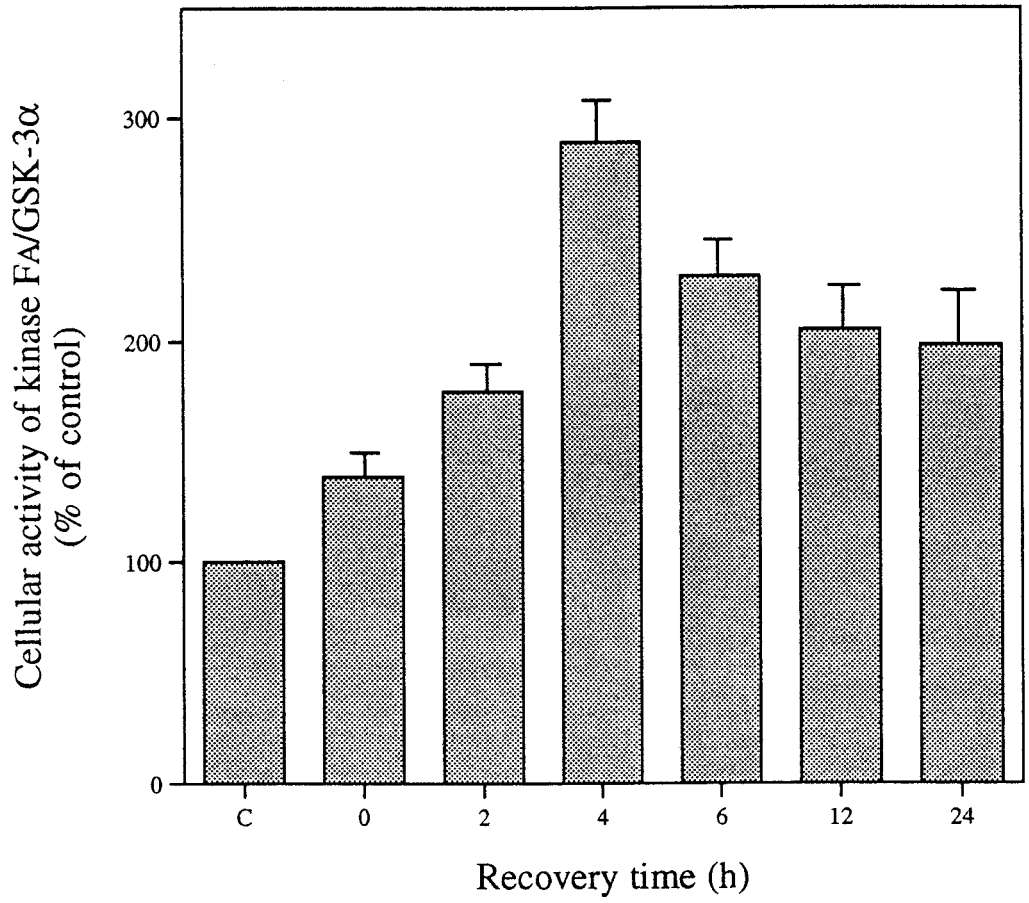

Fig. 2. Effect of recovery from heat stress on the cellular activity of kinase FA/GSK-3α in A431 cells. A431 cells were heat-shocked at 46°C for 15 min and then recovered at 37°C for various time points as indicated. The cell extracts were then subjected to immunoprecipitation by anti-kinase FA/GSK-3α antibody followed by kinase activity assay in the immunoprecipitates as described in the legend to Figure 1. Data were the average of three independent experiments and expressed as means ± S.D.

phosphorylation has been proposed as a key step to activate the constitutively expressed heat shock transcription factors to regulate the expression of heat shock genes following heat shock [Sorger and Pelham, 1988; Sorger, 1991; Morimoto, 1993; Sarge et al., 1993], very little is known about the signal transduction pathway leading to the activation of heat shock transcription factors [Mivechi et al. 1994]. Since kinase FA/GSK-3α has been identified as a possible regulator of transcription factors/proto-oncogenes [Boyle et al., 1991; de Groot et al., 1992, 1993; Plyte et al., 1992; Nikolakaki et al., 1993], it is highly possible that the heat stress–activated kinase FA/GSK-3α may function as a modulator of some certain heat shock transcription factors to modulate the expression of heat shock genes and heat shock proteins during heat stress. This obviously presents an intriguing issue which deserves further investigation.

In comparisons with the other reported heat-inducible protein kinases such as mitogen-activated protein kinases Erks-½ and stress-activated protein kinases (SAPKs) [Kyriakis et al., 1994b], kinase FA/GSK-3α appeared to be a rather distinct stress-inducible kinase. While Erks-½ are strongly activated by mitogens and less sensitive to heat stress and SAPKs are largely refractory to mitogens and instead are strongly activated by heat stress [Kyriakis et al., 1994b], kinase FA/GSK-3α appeared to be moderately modulated by both mitogens and heat stress as presented in this study and in previous reports [Cross et al., 1994; Welsh et al., 1994; Yu and Yang, 1994a,b; Yang et al., 1994b; Saito et al., 1994; Eldar-Finkelman et al., 1995]. Most interestingly, in contrast to SAPKs and Erks-½, which require both tyrosine and serine/threonine phosphorylation for activity [Kyriakis et al., 1994a; Anderson et al.

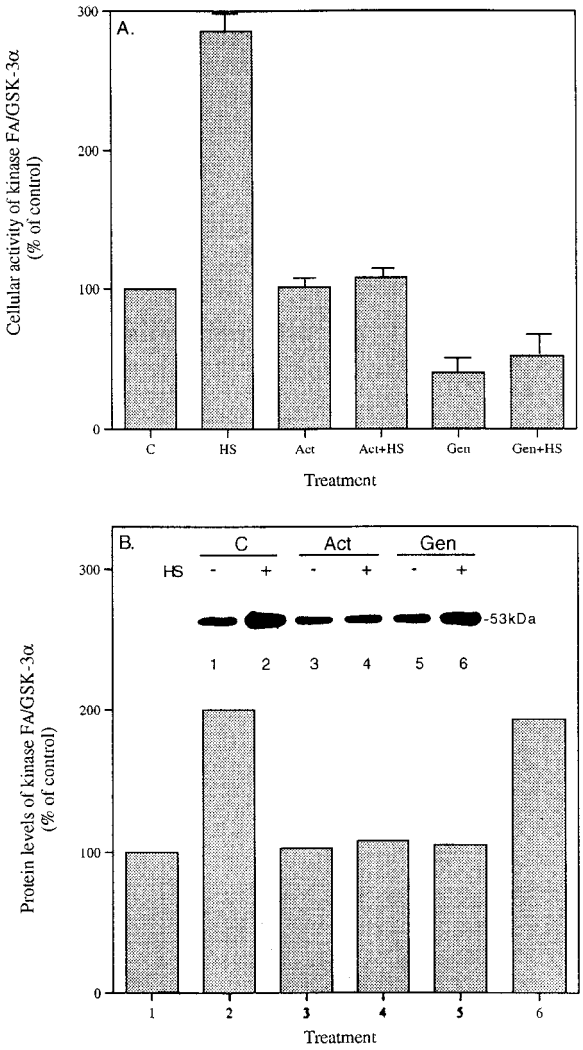

Fig. 3. Effects of actinomycin D and genistein on cellular activity and protein level of kinase FA/GSK-3α in heat-stressed A431 cells. A431 cells were first treated with 0.5 μg/ml actinomycin D at 37°C for 30 min or with 400 μM genistein at 37°C for 2 h followed by heat shock at 46°C for another 45 min. The cellular activity **(A)** and protein level **(B)** of kinase FA/GSK-3α in the cell extracts were determined by immunoblot analysis and kinase activity assay in the immunoprecipitate of kinase FA/GSK-3α as described in the legend to Figure 1. Data were the averages of four independent experiments and expressed as means ± S.D. C, control without any treatment; HS, Heat-shock; Act, treated with actinomycin D but without heat-shock; Act+HS, pretreated with actinomycin D followed by heat-shock; Gen, treated with genistein but without heat-shock; Gen+HS, pretreated with genistein followed by heat-shock. Data in B were the representative results of four independent experiments.

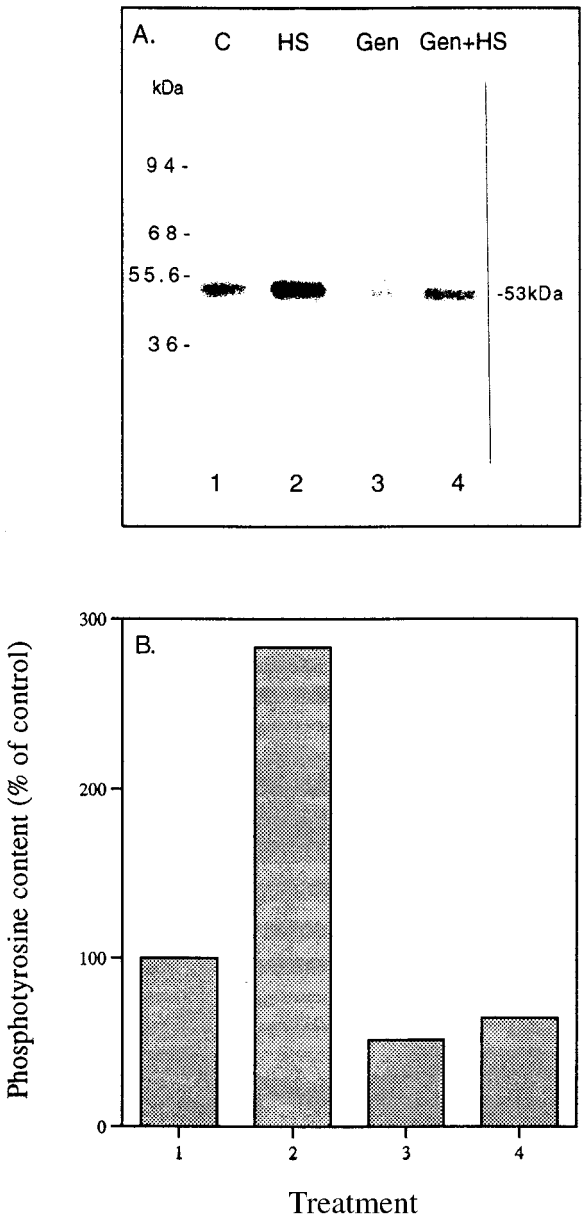

Fig. 4. Effect of genistein on phosphotyrosine content of kinase FA/GSK-3α in heat-stressed A431 cells. A431 cells were pretreated with and without 400 μM genistein at 37°C for 2 h followed by heat shock at 46°C for 45 min. The phosphotyrosine content of the immunoprecipitate of kinase FA/GSK-3α from cell extracts was determined by immunoblot analysis using anti-phosphotyrosine antibody followed by chemiluminescence detection (**A**) and quantification (**B**) as described under Materials and Methods. C, control; HS, heat-shock; Gen+HS, pretreated with genistein followed by heat shock. Data were the representative results of four independent experiments.

1990], the mitogenic signalling pathways by insulin/EGF/IGF-1 appeared to involve serine phosphorylation/inactivation of kinase FA/GSK-3α [Sutherland and Cohen, 1994; Cross et al., 1994; Saito et al., 1994; Eldar-Finkelman et al., 1995], whereas the heat stress signaling pathway appeared to involve tyrosine phosphorylation/activation of kinase FA/GSK-3α as presented here, representing a newly described control mechanism for protein kinase regulation.

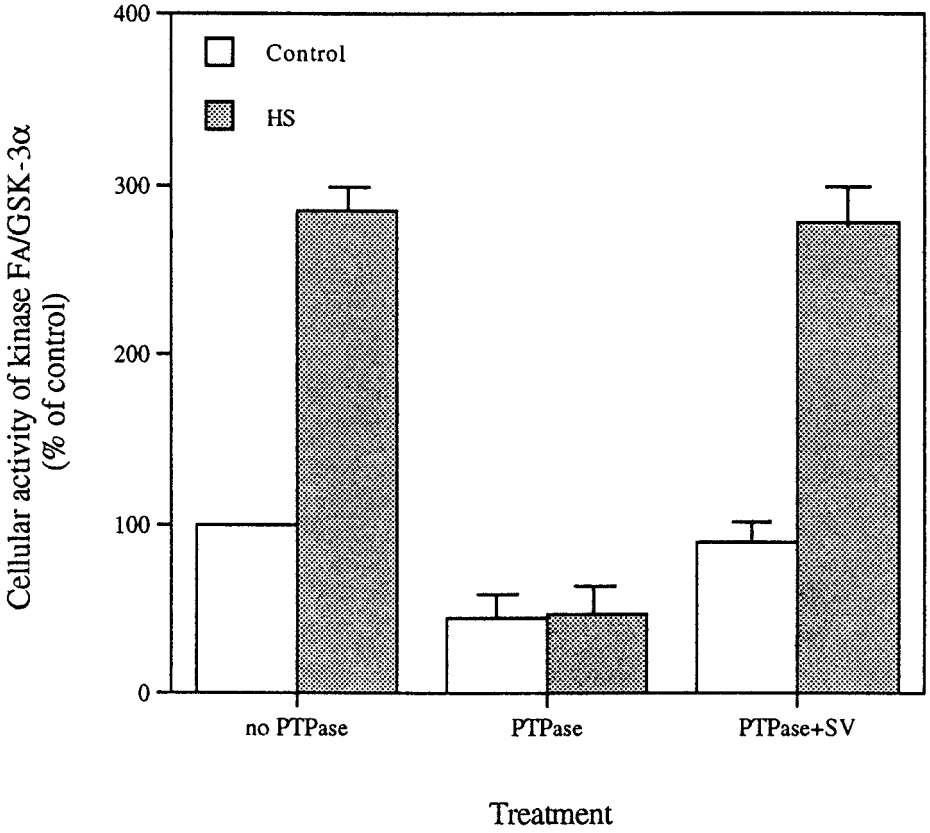

Fig. 5. Effect of tyrosine phosphatase on cellular activity of kinase FA/GSK-3α in heat-stressed A431 cells. A431 cells were heat-shocked at 46°C for 45 min. The anti-kinase FA/GSK-3α immunoprecipitates from cell extracts were next treated with and without tyrosine phosphatase in the presence and absence of 1 mM sodium orthovanadate at 37°C for 30 min followed by kinase activity assay in the immunoprecipitates as described under Materials and Methods. HS, heat shock; no PTPase, without any tyrosine phosphatase treatment; PTPase, control or heat shock followed by tyrosine phosphatase treatment; PTPase+SV, control or heat shock followed by tyrosine phosphatase treatment in the presence of sodium orthovanadate. Data were the averages of four independent experiments and expressed as means ± S.D.

Previously, kinase FA/GSK-3α was assumed to be constitutively active due to the ready detection of the kinase activity in resting cell extracts. Based on this assumption, kinase FA/GSK-3α has been proposed as a constitutively active, mitogen-inactivated protein kinase [Hughes et al., 1993; Woodgett et al., 1993]. However, as presented in this report that kinase FA/GSK-3α can be activated several-fold by heat stress, this kinase may therefore function as a cellular stress-activated protein kinase subjected to further tyrosine phosphorylation/activation and may not just exist in a constitutively active/mitogen-inactivated protein kinase as previously conceived [Hughes et al., 1993; Woodgett et al., 1993; Cross et al., 1994; Saito et al., 1994].

The tyrosine kinase inhibitor genistein [Akiyama et al., 1987; Yu and Yang, 1994a] could induce tyrosine dephosphorylation and concurrent inactivation on heat-induced cellular activation of kinase FA/GSK-3α (see Figs. 3 and 4). Moreover, protein tyrosine phosphatase could inhibit the cellular activation of kinase FA/GSK-3α in heat-stressed A431 cells and this inhibitory effect could be blocked by the tyrosine phosphatase inhibitor orthovanadate (see Fig. 5). Taken together, the results demonstrate that the heat stress induces tyrosine phosphorylation and concurrent activation of kinase FA/GSK-3α in cells. Kinase FA/GSK-3α is a proline-directed protein kinase [Hemmings et al., 1982; Hemmings and Cohen, 1983; Hunter et al., 1988; Dent et al., 1989; Vulliet et al., 1989; Fiol et al., 1990; Ramakrishna et al., 1990; Boyle et al., 1991; Plyte et al., 1992; Mandelkow et al., 1992; Yang et al., 1993, 1995a; Yu and Yang, 1994c; Singh et al., 1995], and its overexpression of cellular activity is closely correlated with human carcinoma dedifferentiation/pro-

gression [Lee et al., 1995; Yang et al., 1995b]. If heat stress indeed plays a role in tumor progression [Giraldi et al., 1994], it may act in part via activation of kinase FA/GSK-3α. This obviously presents an intriguing issue deserving further investigation. Nevertheless, the present study clearly demonstrates that kinase FA/GSK-3α is a newly described heat-inducible protein kinase subjected to tyrosine phosphorylation/activation, representing a new mode of signal transduction mechanism for the regulation of this multisubstrate/multifunctional protein kinase and a new mode of heat induction on cascade activation of a protein kinase during heat stress.

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