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Overexpression of Protein Kinase FA/GSK-3α (A Proline-Directed Protein Kinase) Correlates With Human Hepatoma Dedifferentiation/Progression

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Abstract Computer analysis of protein phosphorylation sites sequence revealed that transcriptional factors and viral oncoproteins are prime targets for regulation of proline-directed protein phosphorylation, suggesting an association of the proline-directed protein kinase (PDPK) family with neoplastic transformation and tumorigenesis. In this report, an immunoprecipitate activity assay of protein kinase FA/glycogen synthase kinase-3α (kinase FA/GSK-3α) (a member of PDPK family) has been optimized for human hepatoma and used to demonstrate for the first time significantly increased ($P < 0.01$) activity in poorly differentiated SK-Hep-1 hepatoma (24.2 ± 2.8 units/mg) and moderately differentiated Mahlavu hepatoma (14.5 ± 2.2 units/mg) when compared to well differentiated Hep 3B hepatoma (8.0 ± 2.4 units/mg). Immunoblotting analysis revealed that increased activity of kinase FA/GSK-3α is due to overexpression of the protein. Elevated kinase FA/GSK-3α expression in human hepatoma biopsies relative to normal liver tissue was found to be even more profound. This kinase appeared to be ~fivefold overexpressed in well differentiated hepatoma and ~13-fold overexpressed in poorly differentiated hepatoma when compared to normal liver tissue. Taken together, the results provide initial evidence that overexpression of kinase FA/GSK-3α is involved in human hepatoma dedifferentiation/progression. Since kinase FA/GSK-3α is a PDPK, the results further support a potential role of this kinase in human liver tumorigenesis, especially in its dedifferentiation/progression.

Key words: human hepatoma, dedifferentiation/progression, PDPK, overexpression, kinase FA/GSK-3α

Protein kinase FA was originally identified as an activating factor of Mg · ATP-depndent 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-3α (GSK-3α) [Embi et al., 1980; Hemmings et al., 1981; Woodgett, 1990]. 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, 1994; Woodgett, 1991; Plyte et al., 1992). 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., 1994]. 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]. 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,

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

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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. In consistence with this notion, clinical studies confirmed that overexpression of kinase FA/GSK-3α (a member of the 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, we extend the observations and use human hepatoma as another testing model to further demonstrate that the cellular activity and protein level of kinase FA/GSK-3α are overexpressed many-fold in well and poorly differentiated hepatoma relative to normal liver tissue. Moreover, the elevated expression of kinase FA/GSK-3α activity was found to be significantly correlated with the degree of human hepatoma dedifferentiation/progression. The possible involvement of this PDPK as a newly described differentiation-blocking and/or dedifferentiation-promoting agent in promoting the initiation and progression of human carcinoma cells is therefore proposed.

EXPERIMENTAL PROCEDURES

Materials and Methods

[γ-^{32}P]ATP was purchased from Amersham (Buckinghamshire, UK). Human hepatoma cell lines were obtained from American Type Culture Collection (Rockville, MD). Plastic wares for cell culture were from Falcon (Oxnard, CA). Antibiotics, fetal bovine sera, and complete and incomplete Freund's adjuvants were from Gibco (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin, dimethyl sulfoxide (DMSO), Tween 20, 3-[cyclohexylamino]-1-propane-sulfonic acid (CAPS), sodium pyrophosphate, sodium orthovanadate, and N-hydroxysuccinimide biotin 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) and streptoavidin conjugated with alkaline phosphatase were from Boehringer Mannheim (Mannheim, Germany). Benzamidine, NaF, aprotinin, Triton X-100, phenylmethanesulfonyl fluoride (PMSF), 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). X-ray film was from Fuji (Tokyo, Japan).

Protein Purification and Analytic Methods

Protein kinase FA/GSK-3α and myelin basic protein (MBP) 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 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 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 NH_2 terminus in order to facilitate coupling of the peptide to bovine serum albumin according to the procedure described by Reichlin [Reichlin, 1980] using glutaraldehyde as the crosslinker. 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 Sepharose 4B covalently coupled with 10 mg C-terminal peptide of kinase FA/GSK-3α as described above. After adsorption, the column was washed with 50 ml solution A (10 mM Tris at pH 7.5) and then with 50 ml solution A 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 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 the kinase FA/GSK-3α from the cell extracts without blocking the enzyme activity essentially as described in previous reports [Yu and Yang, 1994a,b].

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

Biotinylation of anti-kinase FA/GSK-3α antibody was performed according to the method described by Harlow and Lane [1988]. Briefly, 1 ml of purified anti-kinase FA/GSK-3α antibody as described above (3 mg/ml in 0.1 M sodium borate buffer at pH 8.8) was mixed with 0.1 ml of N-hydroxysuccinimide biotin (10 mg/ml in dimethyl sulfoxide) at 25°C for 4 hr and then incubated with 80 μl of 1 M NH_4Cl at 25°C for 10 min. After extensive dialysis against phosphate buffer saline (PBS) to remove uncoupled biotin, the biotinylated anti-kinase FA/GSK-3α antibody was concentrated by dialysis against PBS containing 50% glycerol and stored at −20°C.

Culture of Hepatoma Cells and Preparation of Cell Extracts

Three human hepatoma cell lines were classified into three differentiation stages based on analyses of the morphology and plasma protein secretions. Group 1 (the well differentiated Hep 3B hepatoma) [Aden et al., 1979; Knowles et al., 1980] secretes nearly all the 15 plasma proteins and its morphology is similar to the typical hepatocytes with polygonal shape, well defined boundary, and pavement-like arrangement. Group 2 (the moderately differentiated Mahlavu hepatoma) [Alexander et al., 1976] secretes only six plasma proteins and looks like elongated hepatocytes but not like fibroblasts. Group 3 (the poorly differentiated SK-Hep-1 hepatoma) [Fogh et al., 1977] does not secrete any significant amount of plasma proteins and has a spindle-shaped morphology not easily distinguished from fibroblasts. The three hepatoma cell lines were cultured at 37°C in a 95% air/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 ($\sim 2 \times 10^6$) were plated on 100 mm culture dishes. For experiments, hepatoma cells were washed twice with ice-cold PBS, lysed in 600 μl solution B (20 mM Tris-HCl at pH 7.4, 50 mM NaCl, 2 mM EDTA, 1 mM EGTA, 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, and sonicated by Sonicator (model W-380, Heat Systems-Ultrasonics, Farmingdale, NY) for 3 × 10 sec at 50% power output. The cell lysates were ultracentrifuged at 100,000 × *g* at 4°C for 30 min, and the resulting supernatants (~2 mg/ml) were used as the hepatoma cell extracts.

Tissue Preparation

Human hepatoma tissues were obtained during operations at the inpatient clinic of the Chang Gung Memorial Hospital. The tissues were partly fixed in 10% formalin and embedded in paraffin for pathologic study, and partly quick frozen in liquid nitrogen for biochemical and immunological study. The cells in human hepatoma tissues display a wide spectrum of morphologic abnormalities, depending on their degree of differentiation. Well differentiated hepatoma with micro- or macrotrabecular patterns yield in benign-appearing hepatocytes with prominent nucleoli. They are seen singly, in variable-sized clusters slender or broad, surrounded by or mixed with spindle-shaped endothelial cells. Poorly differentiated hepatoma reveals isolated and irregularly clustered pleomorphic tumor cells with large nuclei and macronucleoli. The classification of normal liver tissue, and well and poorly differentiated hepatoma tissues was defined according to Nguyen and Kline [1991].

Frozen liver tissues (~0.3 g) were homogenized in 3.5 volumes of solution B on ice by a 5 ml Teflon pestle-fitted glass homogenizer (Wheaton, Millville, NJ) with 10 up-and-down strokes, and then sonicated by Sonicator for 3 × 10 sec at 50% power output, followed by ultracentrifugation at 100,000 × *g* at 4°C for 30 min. The resulting supernatants were used as the liver tissue extracts. All three different liver tissues yield similar protein levels (~5 mg/ml) in the extracts under this condition.

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

For immunoprecipitation, 500 μl cell extracts (~2.0 mg/ml protein) or 1 ml tissue extracts (~5 mg/ml protein) were incubated with 2 μl

affinity-purified kinase FA/GSK-3α antibody (20 mg/ml pure IgG) at 4°C for 1.5 hr, and then with 100 μl protein A-Sepharose CL-4B (20% v/v) for another 1.5 hr with shaking. The immunoprecipitates were collected by centrifugation, washed three times with 0.5 M NaCl, once with 1 ml solution C (20 mM Tris-HCl at pH 7.0, 0.5 mM dithiothreitol, 1 mM PMSF, 1 mM benzamidine, and 0.5 μg/ml aprotinin), and resuspended in 100 μl solution C. For kinase FA/GSK-3α activity assay in the immunoprecipitate, 30 μl immunoprecipitate was incubated with 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 20 μl reaction mixture onto 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]. A unit of kinase FA/GSK-3α is that amount of enzyme that incorporates 1 pmol of phosphate/min into the MBP substrate.

Immunoblot

For immunoblotting analysis of kinase FA/GSK-3α from hepatoma cells or tissues, the anti-kinase FA/GSK-3α immunoprecipitates were subjected to 10% SDS-PAGE, electrotransferred to PVDF membrane, and immunoblotted with 1 μg/ml of biotinylated anti-kinase FA/GSK-3α antibody at room temperature for 4 hr, followed by incubation with streptavidin conjugated with alkaline phosphatase for another 1 hr. The 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 the 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

Figure 1 depicts the cellular activities of kinase FA/GSK-3α in the immunoprecipitates obtained from the human hepatoma cell supernatant fluids with three different differentiation stages, namely, the poorly differentiated SK-Hep-1 hepatoma cells, the moderately differentiated Mahlavu hepatoma cells, and the well differentiated Hep 3B hepatoma cells. From this figure it can be seen that the cellular activity of kinase FA/GSK-3α was dramatically increased up to ~threefold in poorly differentiated SK-Hep-1 hepatoma cells (24.2 ± 2.8 units/mg of cell protein) and ~twofold in moderately differentiated Mahlavu hepatoma cells (14.5 ± 2.2 units/mg of cell protein) when compared to well differentiated Hep 3B hepatoma cells (8.0 ± 2.4 units/mg of cell protein) (Fig. 1). More interestingly, the cellular activity of kinase FA/GSK-3α appeared to be proportionally increased following the hepatoma cell dedifferentiation (Fig. 1). This is the first indication for an association of kinase FA/GSK-3α with human hepatoma cell dedifferentiation. Immunoblotting analysis of the cell extracts (Fig. 2) from these three hepatoma cells further revealed that the increased cellular activity of kinase FA/GSK-3α in poorly differentiated SK-Hep-1 and moderately differentiated Mahlavu hepatoma cells is due to overexpression of the protein. More importantly, the overexpressed protein levels of kinase FA/GSK-3α, which are in agreement with the overexpressed cellular activities of kinase FA/GSK-3α as shown in Figure 1, also appeared to be proportionally correlated with the degree of dedifferentiation of the hepatoma cells (Fig. 2). Most importantly, the elevated kinase FA/GSK-3α expression in human hepatoma tissues relative to normal human liver tissues was found to be even more profound. The cellular activities (Fig. 3) and protein levels (Fig. 4) of this kinase appeared to be ~fivefold overexpressed in well differentiated human hepatoma tissues and ~13-fold overexpressed in poorly differentiated hepatoma tissues when compared to normal liver tissues (Figs. 3, 4), demonstrating a pathophysiologically relevant involvement of kinase FA/GSK-3α in human liver tumorigenesis in vivo. It is important to note that although the microscopic appearances of the well differentiated human hepatoma tissues are almost indistinguishable from those of the normal liver tissues, the protein levels and cellular activities of kinase FA/GSK-3α in well differentiated hepatoma tissues are already more than fivefold overexpressed when compared to normal liver tissues (Figs. 3, 4, N and C1). The results further point out a potential involvement of this kinase in the initiation of the human liver tumor promotion. All the results taken together provide initial evidence to demonstrate that overexpression of protein kinase FA/GSK-3α is indeed associated with hu-

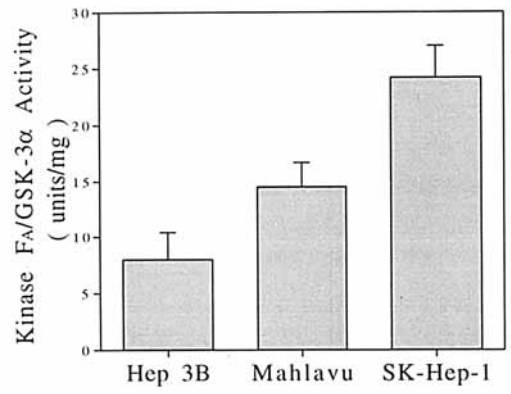

Fig. 1. Comparison of the cellular activities of kinase FA/GSK-3α from three different differentiation stages of human hepatoma cells. The cellular activities of kinase FA/GSK-3α from cell extracts (~1 mg of cell protein) of three different differentiation stages of human hepatoma cells as indicated were immunoprecipitated using 40 μg anti-kinase FA/GSK-3α antibody. The kinase FA/GSK-3α activities in the immunoprecipitates were measured as described in Experimental Procedures. Data were taken from the averages of four independent experiments and expressed as means ± SD. *Hep 3B,* well differentiated Hep 3B hepatoma cells; *Mahlavu,* moderately differentiated Mahlavu hepatoma cells; and *SK-Hep-1,* poorly differentiated SK-Hep-1 hepatoma cells.

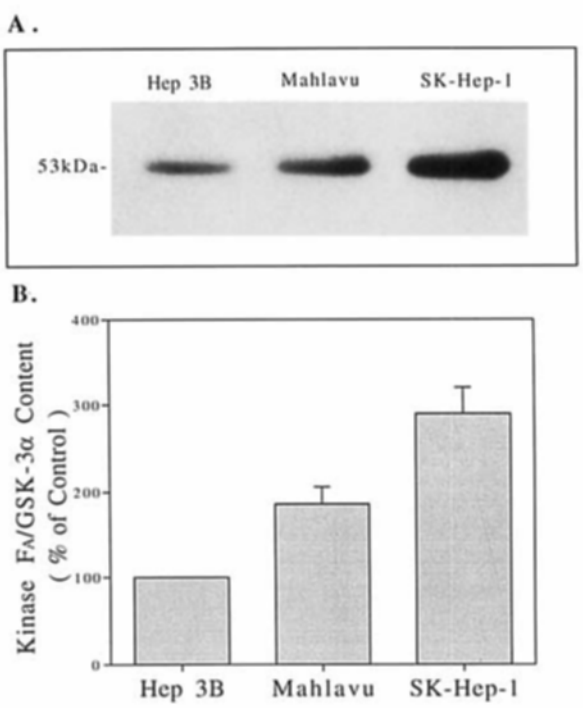

Fig. 2. Immunoblotting analysis of kinase FA/GSK-3α from three different differentiation stages of human hepatoma cells. Crude extracts (~1 mg of cell protein) of three different differentiation stages of human hepatoma cells as indicated were immunoprecipitated using 40 μg anti-kinase FA/GSK-3α antibody. The immunoprecipitated proteins were subjected to 10% SDS-PAGE and immunoblotted with 1 μg/ml of biotinylated anti-kinase FA/GSK-3α antibody (**A**) followed by densitometric quantification of the relative amount of kinase FA/GSK-3α (% of control) on the immunoblot (**B**) as described in Experimental Procedures. A is the representative results of four independent experiments, expressed as means ± SD in B. Symbols are as in Figure 1.

man hepatocellular carcinoma dedifferentiation and progression.

DISCUSSION

In this report, we demonstrate that protein kinase FA/GSK-3α is overexpressed and dysregulated in human hepatocellular carcinoma as compared with normal controls. 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]. In comparisons with cyclin-dependent cell division cycle control kinases [Vulliet et al., 1989; Hall and Vulliet, 1991], mitogen-activated protein kinases [Gonzalez et al., 1991; Mukhopadhyay et al., 1992], and stress-activated protein kinases [Kyriakis et al., 1994], protein kinase FA/GSK-3α appeared to represent a particular member of the 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]. It has been proposed that overexpression, dysregulation, or viral subversion of some certain PDPKs could be associated with neoplastic transformation and tumorigenesis. The results presented here, that protein kinase FA/GSK-3α, a member of the PDPK family, is indeed greatly overexpressed and dysregulated in human hepatoma, strongly support this notion. Since the protein levels and cellular activities of kinase FA/GSK-3α appeared to be closely correlated with the degree of dedifferentiation/progression of human hepatoma, the results further support an association of kinase FA/GSK-3α with human hepatocellular carcinoma dedifferentiation/progression. Since kinase FA/GSK-3α

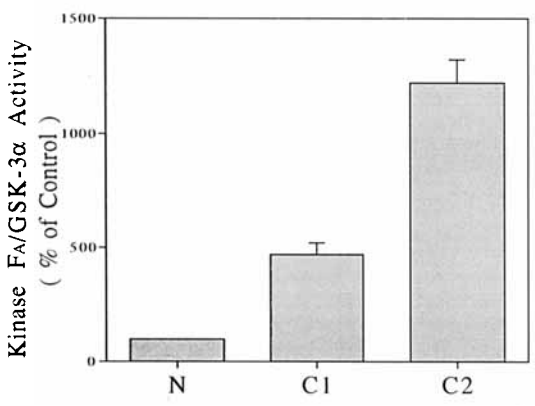

Fig. 3. Comparison of the elevated cellular activities of kinase FA/GSK-3α in well and poorly differentiated human liver carcinoma tissues relative to normal liver tissues. The cellular activities of kinase FA/GSK-3α from crude extracts (~5 mg of tissue protein) of normal liver tissues, and well and poorly differentiated liver carcinoma tissues were immunoprecipitated using 40 μg anti-kinase FA/GSK-3α antibody. The kinase activities in the immunoprecipitates were measured as described in Experimental Procedures. Data were taken from the averages of four independent experiments and expressed as means ± SD. N, from normal liver tissues; C1, from well differentiated liver carcinoma tissues; and C2, from poorly differentiated liver carcinoma tissues.

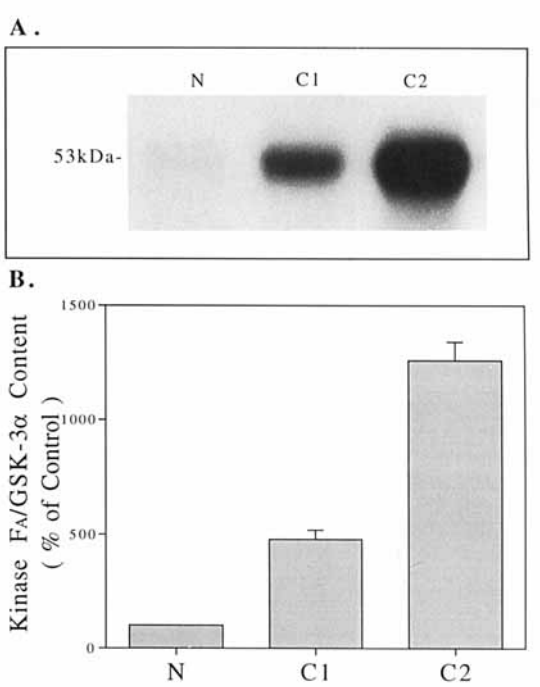

Fig. 4. Immunoblotting analysis of kinase FA/GSK-3α in well and poorly differentiated human liver carcinoma tissues relative to normal liver tissues. Crude extracts (~5 mg of tissue protein) of normal liver tissues, and well and poorly differentiated human liver carcinoma tissues were immunoprecipitated using 40 μg anti-kinase FA/GSK-3α antibody. The immunoprecipitated proteins were subjected to 10% SDS-PAGE and immunoblotted with 1 μg/ml of biotinylated anti-kinase FA/GSK-3α antibody (**A**) followed by densitometric quantification of the relative amount of kinase FA/GSK-3α (% of control) on the immunoblot (**B**) as described in Experimental Procedures. A is the representative results from four independent experiments, expressed as means ± SD in B. Symbols are as in Figure 3.

activity is inversely proportional to the degree of hepatoma differentiation, this kinase may function as a negatively acting protein kinase influencing hepatoma differentiation. Together with the previous results, that kinase FA/GSK-3α is a PDPK and its elevated expression levels are closely correlated with the states of dedifferentiation/progression of human thyroid carcinoma [Lee et al., 1995] and human cervical carcinoma [Yang et al., 1995b], we finally came to the conclusion that kinase FA/GSK-3α represents a newly described human carcinoma differentiation-blocking and/or dedifferentiation-promoting agent involved in promoting the initiation and progression of human cancers.

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, this kinase has been proposed as a constitutively active, mitogen-inactivated protein kinase [Hughes et al., 1993; Woodgett et al., 1993; Woodgett, 1991]. However, as presented in this report, that kinase FA/GSK-3α could be activated up to ~1,300% of normal controls during human hepatoma dedifferentiation/progression, kinase FA/GSK-3α therefere may function as a mitogen-activated protein kinase and may not function only as a constitutively active/mitogen-inactivated protein kinase, as previously conceived [Hughes et al., 1993; Woodgett et al., 1993; Woodgett, 1991].

From the clinical viewpoint, since the protein levels and cellular activities of kinase FA/GSK-3α are significantly and quantitatively correlated with the degree of human hepatoma dedifferentiation/progression, this kinase may possibly be used as a marker protein for clinical diagnosis of the status of human hepatoma during pre- and postdiagnosis of the disease. Moreover, the well differentiated liver carcinoma tissue is almost indistinguishable from normal liver tissue from the cytological appearances. However, the levels of kinase FA/GSK-3α are already more than fivefold overexpressed in the well differentiated hepatoma relative to normal liver tissue (Figs. 3, 4). The results, taken together with the fact that kinase FA/GSK-3α is a PDPK, further indicate that this kinase may play an important role in human liver tumorigenesis and can be a very

sensitive marker for early diagnosis of human hepatoma. This obviously presents an important issue deserving further investigation. Nevertheless, the present study clearly demonstrates that protein kinase FA/GSK-3α (a member of the PDPK family) is activated manyfold in human hepatoma, and the activation state of this kinase is correlated with the state of dedifferentiation and progression of the human hepatocellular carcinoma.

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REFERENCES

Aden DP, Fogel A, Plotkin S, Damjanov I, Knowles BB (1979): Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature 282:615–616.

Alexander AA, Bey EM, Geddes EW, Lecatsa G (1976): Establishment of a continuously growing cell line from primary carcinoma of the liver. S Afr Med J 50:2124–2128.

Boyle WJ, Smeal T, Defize LHK, Angel P, Woodgett JR, Karin M, Hunter T (1991): Activation of protein kinase C decreased phosphorylation of c-jun at sites that negatively regulate its DNA-binding activity. Cell 64:573–584.

Dent P, Campbell DG, Hubbard MJ, Cohen P (1989): Multisite phosphorylation of the glycogen-binding subunit of protein phosphatase-1_G by cyclic AMP–dependent protein kinase and glycogen synthase kinase-3. FEBS Lett 248: 67–72.

Embi N, Rylatt DB, Cohen P (1980): Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. Eur J Biochem 107:519–527.

Fiol CJ, Wang A, Roeske RW, Roach PJ (1990): Ordered multisite protein phosphorylation analysis of glycogen synthase kinase-3 action using model peptide substrate. J Biol Chem 265:6061–6065.

Fogh J, Fogh JM, Thomas O (1977): One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. J Natl Cancer Inst 59:221–225.

Gillespie PG, Hudspeth AJ (1991): Chemiluminescence detection of proteins from single cells. Proc Natl Acad Sci USA 88:2563–2567.

Gonzalez F, Raden D, Davis R (1991): Identification of substrate recognition determinants for human ERK1 and ERK2 protein kinases. J Biol Chem 266:22159–22163.

Hall F, Vulliet PR (1991): Proline-directed protein phosphorylation and cell cycle regulation. Curr Opin Cell Biol 3:176–184.

Harlow E, Lane D (1988): "Antibodies: A Laboratory Manual." Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, pp 340–341.

Hemmings BA, Cohen P (1983): Glycogen synthase kinase 3 from rabbit skeletal muscle. Methods Enzymol 99:337–345.

Hemmings BA, Yellowlees D, Kernohan JC, Cohen P (1981): Purification of glycogen synthase kinase 3 from rabbit skeletal muscle. Copurification with the activating factor (FA) of the (Mg-ATP) dependent protein phosphatase. Eur J Biochem 119:443–451.

Hemmings BA, Aitken A, Cohen P, Rymond M, Hofmann F (1982): Phosphorylation of the type-II regulatory subunit of cyclic AMP–dependent protein kinase by glycogen synthase kinase 3 and glycogen synthase kinase 5. Eur J Biochem 127:473–481.

Hughes K, Nikolakaki E, Plyte SE, Totty NF, Woodgett JR (1993): Modulation of the glycogen synthase kinase-3 family by tyrosine phosphorylation. EMBO J 12:803–808.

Hunter T, Angel P, Boyle WJ, Chiu R, Freed E, Gould KL, Isacke CM, Karin M, Lindberg RA, van der Geer P (1988): Targets for signal-transducing protein kinases. Cold Spring Harbor Symp Quant Biol 53:131–142.

Knowles BB, Howe CC, Aden DP (1980): Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science 209:497–499.

Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Ahmad MF, Avruch J, Woodgett JR (1994): The stress-activated protein kinase subfamily of c-Jun kinase. Nature 369:156–160.

Laemmli UK (1970): Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227:680–685.

Lee T-T, Ho Y-S, Yu J-S, Yang S-D (1995): Overexpression of cellular activity and protein level of protein kinase FA/GSK-3α correlates with human thyroid tumor cell dedifferentiation. J Cell Biochem 58:1–7.

Lin BTY, Gruenwald S, Morla AO, Lee WH, Wang JYJ (1991): Retinoblastoma cancer suppressor gene product is a substrate of the cell cycle regulator cdc2 kinase. EMBO J 10:857–864.

Mandelkow E-M, Drewes G, Biernat J, Gustke N, Van Lint J, Vandenheede JR, Mandelkow E (1992): Glycogen synthase kinase-3 and the Alzheimer-like state of microtubule-associated protein tau. FEBS Lett 314:315–321.

Moreno S, Nurse P (1990): Substrates for $p34^{cdc2}$: In vivo veritas? Cell 61:549–551.

Mukhopadhyay NK, Price DJ, Kyriakis JM, Pelech S, Sanghera J, Avruch J (1992): An array of insulin-activated, proline-directed serine/threonine protein kinase phosphorylate the S6 kinase. J Biol Chem 267:3325–3335.

Nguyen G-K, Kline TS (1991): "Essentials of Aspiration Biopsy Cytology." New York: IGAKU-SHOIN Medical Publishers, Inc., pp 127–138.

Pines T, Hunter T (1990): $p34^{cdc2}$: The S and M kinase. New Biol 2:389–401.

Plyte SE, Hughes K, Nikolakaki E, Pulverer BJ, Woodgett JR (1992): Glycogen synthase kinase-3: Functions in oncogenesis and development. Biochem Biophys Acta 1114: 147–162.

Ramakrishna S, D'Angelo G, Benjamin WB (1990): Sequence of sites on ATP-citrate lyase and phosphatase inhibitor 2 phosphorylated by multifunctional protein kinase (a glycogen synthase kinase 3 like kinase). Biochemistry 29:7617–7624.

Reichlin M (1980): Use of glutaraldehyde as a coupling agent for proteins and peptides. Methods Enzymol 70:159–165.

Roach PJ (1991): Multisite and hierarchical protein-phosphorylation. J Biol Chem 266:14139–14142.

Suzuki M (1989): SPXX, a frequent sequence motif in gene regulatory proteins. J Mol Biol 207:61–84.

Vandenheede JR, Yang S-D, Goris J, Merlevede W (1980): ATP · Mg-dependent protein phosphatase from rabbit muscle. Purification of the activating factor and its regulation as a bifunctional protein also displaying synthase kinase activity. J Biol Chem 255:11768–11774.

Vulliet PR, Hall FL, Mitchell JP, Hardie DG (1989): Identification of a novel proline-directed serine/threonine protein kinase in rat phenochromocytoma. J Biol Chem 264: 16292–16298.

Wang QM, Park I-K, Fiol CJ, Roach PJ, DePaoli-Roach AA (1994): Isoform differences in substrate recognition by glycogen synthase kinase-3α and kinase-3β in the phosphorylation of phosphatase inhibitor-2. Biochemistry 33: 143–147.

Warburton D, Berndt N, Hall F (1993): "Signal Transduction in Lung Cells." In Brody et al. (eds): Marcel Dekker, Inc., pp 245–261.

Williams RT, Carbonaro-Hall DA, Hall FL (1992): Copurification of $p34^{cdc2}/p58^{cyclinA}$ proline-directed protein kinase and the retinoblastoma tumor susceptibility gene product: Interaction of an oncogenic serine/threonine protein kinase with a tumor-suppressor protein. Oncogene 7:423–432.

Woodgett JR (1990): Molecular cloning and expression of glycogen synthase kinase-3/Factor A. EMBO J 9:2431–2438.

Woodgett JR (1991): A common denominator linking glycogen metabolism, nuclear oncogenes and development. Trends Biochem Sci 16:177–181.

Woodgett JR, Plyte SE, Pulverer BJ, Mitchell JA, Hughes K (1993): Roles of glycogen synthase kinase-3 in signal transduction. Biochem Soc Trans 21:905–907.

Yang S-D (1986): Identification of the ATP · Mg-dependent protein phosphatase activator FA as a myelin basic protein kinase in brain. J Biol Chem 261:11786–11791.

Yang S-D (1991): Characteristics and regulation of the ATP · Mg-dependent protein phosphatase activating factor (protein kinase FA). Adv Protein Phosphatases 6:133–157.

Yang S-D (1994): Signal transduction and biological functions of protein phosphatase activating factor (protein kinase FA/GSK-3α). J Chin Biochem Soc. 23:123–134.

Yang S-D, Vandenheede JR, Goris J, Merlevede W (1980): ATP · Mg-dependent phosphorylase phosphatase from rabbit muscle. Purification of the enzyme and its regulation by the interaction with an activating protein factor. J Biol Chem 255:11759–11767.

Yang S-D, Song J-S, Yu J-S, Shiah S-G (1993): Protein kinase FA/GSK-3α phosphorylates tau on Ser^{235}-Pro and Ser^{404}-Pro that are abnormally phosphorylated in Alzheimer's disease brain. J Neurochem 61:1742–1747.

Yang S-D, Huang J-J, Huang T-J (1995a): Protein kinase FA/glycogen synthase kinase-3α predominantly phosphorylates the in vivo sites of Ser^{502}, Ser^{506}, Ser^{603}, and Ser^{666} in neurofilament. J Neurochem 64:1848–1854.

Yang S-D, Yu J-S, Lee T-T, Ni M-H, Yang C-C, Ho Y-S, Tsen T-Z (1995b): Association of protein kinase FA/GSK-3α (a proline-directed kinase and a regulator of protooncogenes) with human cervical carcinoma dedifferentiation/progression. J Cell Biochem 59: 143–150.

Yu J-S, Yang S-D (1993): Immunological and biochemical study on tissue and subcellular distributions of protein kinase FA (an activating factor of ATP · Mg-dependent protein phosphatase). A simplified and efficient procedure for high quantity purification from brain. J Protein Chem 12:665–674.

Yu J-S, Yang S-D (1994a): Tyrosine dephosphorylation and inactivation of protein kinase FA/GSK-3α by genistein in A431 cells. J Cell Biochem 56:131–141.

Yu J-S, Yang S-D (1994b): Okadaic acid, a serine/threonine phosphatases inhibitor, induces tyrosine dephosphorylation/inactivation of protein kinase FA/GSK-3α in A431 cells. J Biol Chem 269:14341–14344.

Yu J-S, Yang S-D (1994c): Protein kinase FA/GSK-3α predominantly phosphorylates the in vivo site Thr^{97}-Pro in brain myelin basic protein. Evidence for Thr-Pro and Ser-Arg-X-X-Ser as consensus sequences motif. J Neurochem 62:1596–1603.