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Overexpression of Cellular Activity and Protein Level of Protein Kinase F_A/GSK-3α Correlates With Human Thyroid Tumor Cell Dedifferentiation

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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 F_A/glycogen synthase kinase-3α (kinase F_A/GSK-3α) (a member of the PDPK family) has been optimized for human thyroid tissue and used to demonstrate for the first time significantly increased ($P < 0.001$) activity in thyroid carcinoma (24.2 ± 2.8 units/mg of protein) (n = 7), thyroid adenoma (14.5 ± 2.2 units/mg of protein) (n = 6), and thyroid hyperplasia (8.0 ± 2.4 units/mg of protein) (n = 5) when compared to five normal controls (4.1 ± 1.8 units/mg of protein). Immunoblotting analysis further revealed that increased activity of kinase F_A/GSK-3α in thyroid tumor cells is due to overexpression of the protein synthesis of the enzyme. Taken together, the results provide initial evidence that overexpression of protein level and cellular activity of kinase F_A/GSK-3α is involved in human thyroid tumor cell dedifferentiation, supporting an association of PDPK with neoplastic transformation and tumorigenesis. Since kinase F_A/GSK-3α may function as a possible regulator of transcription factors/protooncogenes, kinase F_A/GSK-3α may therefore play an important role in thyroid cell carcinogenesis, especially in its differentiation.

Key words: kinase F_A/GSK-3α, overexpression, thyroid, tumor cell dedifferentiation, carcinogenesis

Protein kinase F_A was originally identified as an activating factor of Mg.ATP-dependent protein phosphatase [Yang et al., 1980; Vandenheede et al., 1980] but has subsequently been identified as a protein kinase identical to glycogen synthase kinase-3α (GSK-3α) [Hemmings et al., 1981; Woodgett, 1990]. In addition to Mg.ATP-dependent protein phosphatase and glycogen synthase as its substrates, protein kinase F_A/GSK-3α was further identified as a multisubstrate protein kinase that could act on many substrates, including the R_{II} subunit of cAMP-dependent protein kinase [Hemmings et al., 1982], phosphatase inhibitor-2 [Depaoli-Roach, 1984; Jurgensen et al., 1984], myelin basic protein [Yang, 1986], the nerve growth factor receptor [Taniuchi et al., 1986], the G subunit of phosphatase-1 [Fiol et al., 1988; Dent et al., 1989], neural cell adhesion molecules [Mackie et al., 1989], ATP-citrate lyase [Ramakrishna et al., 1990], neurofilament proteins [Guan et al., 1991], acetyl-CoA carboxylase [Hughes et al., 1992], microtubule associated protein-2 and tau protein [Yang et al., 1991, 1993; Hanger et al., 1992; Mandelkow et al., 1992], and brain clathrin-coated vesicles [Yu and Yang, 1993a]. Due to its unique feature as a multisubstrate protein kinase and as an activating factor of a multisubstrate protein phophatase, F_A/GSK-3α may simultaneously modulate phosphorylation and dephosphorylation states of many key regulatory proteins involved in the regulation of diverse cell functions [Yang, 1991; Woogett, 1991]. Recently, kinase F_A/GSK-3α has further been iden-

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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tified as a possible regulator of the transcription factors/protooncogenes, such as c-jun [Boyle et al., 1991; de Groot et al., 1992], c-myb and c-myc [Plyte et al., 1992], CREM [de Groot et al., 1993], and CREB [Wang et al., 1994]. This raised another possibility that kinase F_A/GSK-3α could be involved in wider aspects of cellular regulation, such as control of nuclear transcription and tumor promotion. On the other hand, F_A/GSK-3α has been demonstrated as a particular member of the so-called proline-directed protein kinase family [Hemmings and Cohen, 1983; Vulliet et al., 1989; Ramakrishna et al., 1990; Yang et al., 1993; Yu and Yang, 1994a]. Based on computer-assisted sequence analysis of transcriptional factors and viral oncoproteins, proline-directed protein phosphorylation sites appeared to be a major regulatory mechanism [Suzuki, 1989], and proline-directed protein kinases such as kinase F_A/GSK-3α may therefore be associated with neoplastic transformation and tumorigenesis. However, the clinical correlation of kinase F_A/GSK-3α with human cancer diseases has not yet been studied. An association of kinase F_A/GSK-3α with human cancer therefore remains to be established.

In this report, we tried to use human thyroid tumors as testing models and demonstrated that the cellular activity and protein level of kinase F_A/GSK-3α in human thyroid tumors appeared to be manyfold higher than those of normal controls and that the expression of kinase F_A/GSK-3α activity was significantly correlated with the degree of dedifferentiation of thyroid tumor cells, providing initial evidence that dysregulation and overexpression of kinase F_A/GSK-3α is associated with malignant transformation and tumor promotion.

EXPERIMENTAL PROCEDURES

Materials

[γ-^{32}P]ATP was purchased from Amersham (Buckinghamshire, UK). Polyvinylidene fluoride (PVDF) membrane (Immobilon-P) was from Millipore (Bedford, MA). Sodium orthovanadate, Tween 20, and goat antirabbit IgG antibody conjugated with alkaline phosphatase were from Sigma (St. Louis, MO). Molecular weight marker proteins were from Boehringer Mannheim (Mannheim, Germany). BCA protein assay reagent was from Pierce (Rockford, IL). The alkaline phosphatase conjugate substrate kit was from Bio-Rad. (California) Protein A-Sepharose CL-4B was from Pharmacia (Uppsala, Sweden).

Protein Purification

Myelin basic protein (MBP) was purified from porcine brain following the procedure described in previous reports [Yang et al., 1987; Yu and Yang, 1994a].

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

The anti-kinase F_A/GSK-3α antibody was produced by using 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] as the antigen. Production, identification, and characterization of anti-kinase F_A/GSK-3α were detailed in previous reports [Yu and Yang, 1993b, 1994b,c]. In this report, the antibody can potently and specifically immunoblot kinase F_A/GSK-3α from the human thyroid tissue extracts on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The antibody can also efficiently immunoprecipitate all the kinase F_A/GSK-3α from the thyroid tissue extracts and without blocking the kinase activity essentially as described in previous reports [Yu and Yang, 1994b,c] (data not further illustrated).

Tissue Preparation

Human thyroid tissue specimens were obtained from dissected tissues during operations at the in-patient 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. Five normal control (from three female and two male carcinoma patients during operations), five hyperplasia, six adenoma, and seven carcinoma (four follicular, two papillary, and one papillary-follicular) were collected and categorized according to the World Health Organization Histologic Classification of Thyroid Tumors. None of the subjects had taken medication within 1 month of the test. In agreement with the previous report [Yang et al., 1992], the biological dysfunction of kinase F_A/GSK-3α is unrelated to the age and sex of the patients (see Table I, normal control values). The serum TSH concentrations were not significantly different among all of the subjects tested. Frozen tissues were homogenized in 3.5 volumes of solution A (20 mM Tris-HCl at pH 7.0, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM benzamidine, 1

TABLE I. Protein Kinase F_A/GSK-3α Activity in Normal and Different Malignant Lesions of the Thyroid Tissue*

Thyroid tissues		Kinase F_A/GSK-3α activity in immunoprecipitates (units/mg)
Normal control	(n = 5)	4.1 ± 1.8
Hyperplasia (Grade I)	(n = 5)	8.0 ± 2.4
Adenoma (Grade II)	(n = 6)	14.5 ± 2.2
Carcinoma (Grade III)	(n = 7)	24.2 ± 2.8

*The thyroid tissue extracts were first adjusted to identical protein concentrations (0.5 mg of tissue protein in 500 μl of solution A) and then immunoprecipitated by 20 μg of anti-kinase F_A/GSK-3α antibody, followed by kinase F_A/GSK-3α activity assay in the immunoprecipitates as described in Experimental Procedures. Data were the average of 5–7 independent experiments and expressed as means ± SD (n indicates the number of cases).

mM phenylmethylsulfonyl fluoride, 1 mM TLCK, 50 mM NaF, 0.2 mM sodium orthovanadate) on ice by a 5 ml Teflon pestle-fitted glass homogenizer (Wheaton, Millville, NJ) with ten up-and-down strokes. After centrifugation at 400*g* for 1 min at 4°C to remove cell debris, the resulting cloudy supernatants were further sonicated on ice with a sonicator (model W-380; Heat Systems-Ultrasonics, Farmingdale, NY) for 3 × 10 s at 50% power output and then centrifuged at 20,000*g* for 20 min at 4°C. The resulting supernatants were used as the thyroid tissue extracts.

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

Before immunoprecipitation, protein concentrations of the tissue extracts were first diluted to equal amounts with solution A. For immunoprecipitation, 500 μl of tissue extracts (1 mg/ml protein) was incubated with 2 μl of affinity-purified kinase F_A/GSK-3α (10 mg/ml pure IgG) at 4°C) for 1 h and then with 100 μl of protein A-Sepharose CL-4B (20% v/v, in solution A) for another 1 h with shaking. The immunoprecipitates were collected by centrifugation, washed three times with 1 ml of 0.5 M NaCl and once with 1 ml solution B (20 mM Tris-HCl at pH 7.0, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.5 μg/ml aprotinin), and resuspended in 100 μl of solution B. For kinase F_A/GSK-3α activity assay in the immunoprecipitate, 15 μl of immunoprecipitate prepared as described above was incubated with 30 μl of a mixture containing 20 mM Tris-HCl at pH 7.0, 0.5 mM dithiothreitol, 0.2 mM [γ-^{32}P]ATP, 20 mM $MgCl_2$, and 4 mg/ml MBP at 30°C for 10 min. ^{32}P incorporation into MBP was measured by spotting 30 μl of reaction mixture on phosphocellulose paper (1 × 2 cm) (Whatman, Maidstone, UK), washing three times with 75 mM H_3PO_4, and counting in a liquid scintillation analyzer (Model 1600CA; Packard, Meviden, CT) essentially as described in previous reports [Yang, 1986; Yu and Yang, 1994b,c]. A unit of kinase F_A/GSK-3α is that amount of enzyme that incorporates 1 pmol of phosphate/min into the MBP substrate.

Immunoblots

Proteins were transferred from unstained SDS gels to Immobilon-P membrane in a Transphor (Hoefer) at 350 mA in transfer buffer (10 mM 3-[cyclohexylamino]-1-propane-sulfonic acid (CAPS) at pH 10 and 20% methanol) at 4°C for 2 h. The membrane 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 membrane was incubated with 1 μg/ml anti-kinase F_A/GSK-3α antibody in TTBS buffer containing 3% nonfat dried milk at room temperature for 4 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. Finally, the kinase F_A/GSK-3α protein was detected by the color development reagent kit.

Analytic Methods

Protein concentrations were determined by using the BCA protein assay reagent from Pierce. SDS-PAGE was performed by the method of Laemmli [1970] using 10% gels. Molecular weight markers used are as follows: α_2-macroglobulin (170,000), β-galatosidase (116,400), fructose-6-phosphate kinase (85,200), glutamate dehydrogenase (55,600), aldolase (39,200), and triosephosphate isomerase (26,600). Quantification of the relative amounts of kinase F_A/GSK-3α on immunoblot was performed by densitometric scanning using a Video Densitometer (Molecular Dynamics, Sunnyvale, CA).

Statistical Analysis

Results are means ± SD for n observations. Student's *t*-test was used to calculate the statistical significance of the differences.

RESULTS

Figure 1 depicts the biological activities of protein kinase F_A/GSK-3α in the immunoprecipitates obtained from the thyroid tissue supernatant fluids with different differentiation stages of thyroid tumors, namely thyroid hyperplasia, adenoma, and carcinoma, respectively, and normal control using a specific and potent anti-kinase F_A/GSK-3α produced and affinity-purified as described in Experimental Procedures. From this figure it can be seen that the cellular activity of kinase F_A/GSK-3α was dramatically increased up to approximately sixfold in thyroid tumors when compared to normal controls. More interestingly, the cellular activity of kinase F_A/GSK-3α appeared to be proportionally increased following dedifferentiation of thyroid tumors (Fig. 1). This is the first indication for an association of kinase F_A/GSK-3α with human thyroid tumor cell dedifferentiation. To confirm this point, we further analyzed 18 thyroid tumors with three different differentiation stages and five normal controls. As shown in Table I, the cellular activity of kinase F_A/GSK-3α appeared to be statistically and significantly increased in thyroid carcinoma (24.2 ± 2.8 units/mg of protein), thyroid adenoma (14.5 ± 2.2 units/mg of protein), and thyroid hyperplasia (8.0 ± 2.4 units/mg of protein) when compared to normal controls (4.1 ± 1.8 units/mg of protein) (Table I). Most importantly, the cellular activity of kinase F_A/GSK-3α appeared to be statistically and proportionally correlated with the degree of differentiation of the tumor cells (Table I).

Immunoblotting analysis of the tissue extracts (Fig. 2) from three typical thyroid tumors at different stages, namely hyperplasia, adenoma, and carcinoma, and one normal control further revealed that the increased cellular activity of kinase F_A/GSK-3α in thyroid tumor cells is due to overexpression of protein synthesis of the kinase. Most importantly, the overexpressed protein levels of kinase F_A/GSK-3α, which are in agreement with the overexpressed cellular activities of kinase F_A/GSK-3α as shown in Figure 1 and Table I, also appeared to be proportionally correlated with the degree of dedifferentiation of the thyroid tumor cells (Fig. 2A). Quantification

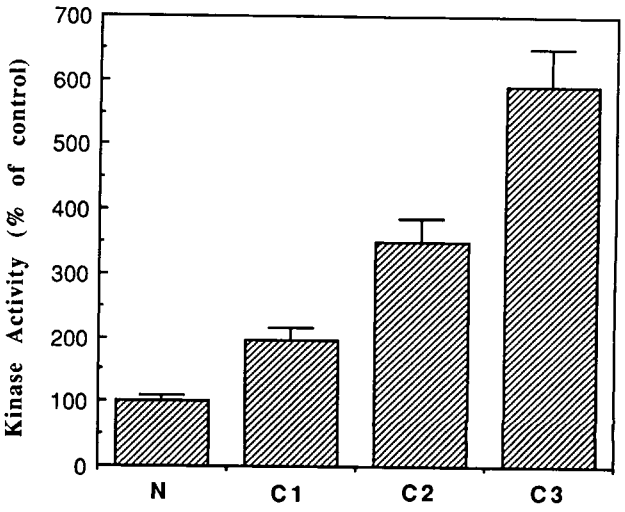

Fig. 1. Cellular activity of kinase F_A/GSK-3α immunoprecipitated from crude extracts of normal control and different malignant lesions of human thyroid tissue. Crude extracts (0.5 mg of tissue protein in 500 μl of solution A) of normal control and different malignant lesions of human thyroid tissue were immunoprecipitated by 20 μg anti-kinase F_A/GSK-3α antibody, followed by kinase activity assay in the immunoprecipitates as described in Experimental Procedures. *Lane N:* Normal control. *Lane C1:* Hyperplasia. *Lane C2:* Adenoma. *Lane C3:* Carcinoma. Data were taken from the averages of three independent experiments using one single tissue and expressed as means ± SD.

of kinase F_A/GSK-3α on immunoblot by densitometric analysis (Fig. 2B) further revealed that the increased protein level for kinase F_A/GSK-3α is correlated with an increase in biological activity of kinase F_A/GSK-3α following the thyroid tumor cell dedifferentiation when both are expressed as a percentage of controls (see Figs. 1, 2B). The same observations could also be extended to the 23 samples as described above (data not further illustrated). All the results taken together provide initial evidence to demonstrate that overexpression and dysregulation of protein kinase F_A/GSK-3α is indeed associated with human thyroid tumor cell dedifferentiation.

DISCUSSION

In this report, we demonstrate that protein kinase F_A/GSK-3α is consistently and statistically overexpressed and dysregulated in human thyroid tumor cells as compared with normal controls. Based on computer-assisted sequence analysis of transcriptional factors and viral oncoprotein proteins [Suzuki, 1989] as well as analysis of site-specific protein phosphorylation both in vitro and in vivo [Moreno and Nurse, 1990;

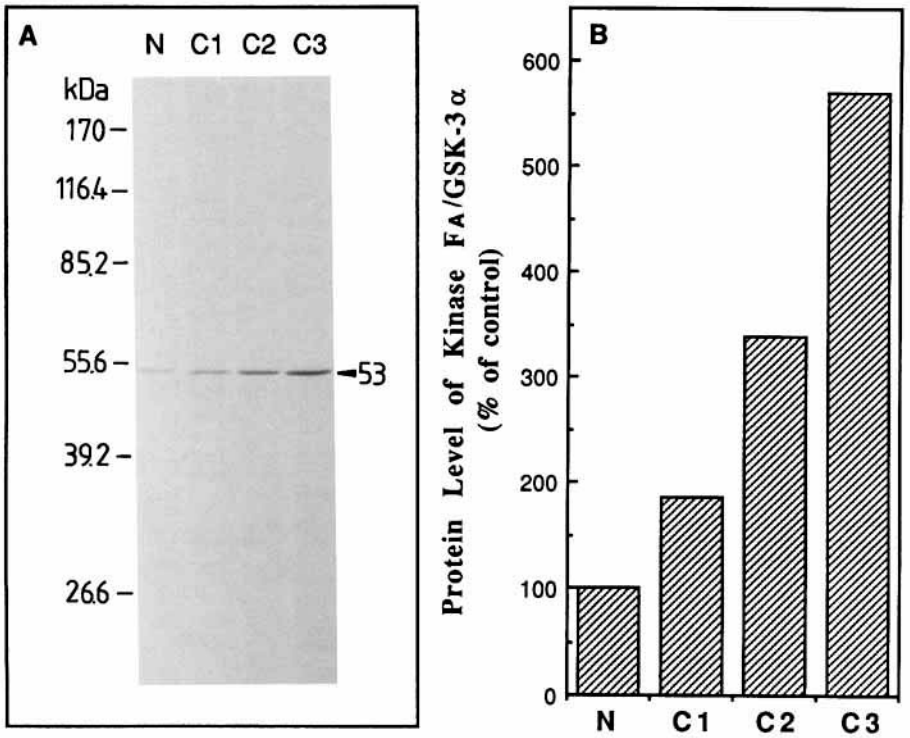

Fig. 2. Immunoblotting analysis of kinase F_A/GSK-3α from crude extracts of normal control and different malignant lesions of human thyroid tissue. Crude extracts (200 μg protein each) of normal control and different malignant lesions of human thyroid tissue were subjected to 10% SDS-PAGE followed by (**A**) immunoblotting with anti-kinase F_A/GSK-3α antibody and (**B**) quantification of the relative amount of kinase F_A/GSK-3α on the immunoblot as described in Experimental Procedures. *Lane N:* Normal control. *Lane C1:* Hyperplasia. *Lane C2:* Adenoma. *Lane C3:* Carcinoma.

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 Valliet, 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 F_A/GSK-3α appeared to represent a particular member of the proline-directed protein kinase family [Hemmings and Cohen, 1983; Vulliet et al., 1989; Ramakrishna et al., 1990; Yang et al., 1993; Yu and Yang, 1994a]. It has been proposed that overexpression, dysregulation, or viral subversion of some certain proline-directed protein kinases could be associated with neoplastic transformation and tumorigenesis. The results presented here that protein kinase F_A/GSK-3α, a member of the proline-directed protein kinase family, is indeed greatly overexpressed and dysregulated in human thyroid tumor cells strongly support this notion. Since the cellular levels and biological activities of kinase F_A/GSK-3α appeared to be closely correlated with the degree of dedifferentiation of the tumor cells, the results presented here further support an association of kinase F_A/GSK-3α with human thyroid tumor cell dedifferentiation. Since kinase F_A/GSK-3α activity is inversely proportional to the degree of tumor cell differentiation, this kinase may function as a negatively acting protein kinase influencing cellular differentiation and may, therefore, represent a newly described differentiation-blocking and/or dedifferentiation-promoting agent involved in promoting the progression of tumor cells. Until recently, protein kinase F_A/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 F_A/GSK-3α has been concluded to be a mitogen-inactivated protein kinase [Woodgett, 1991; Hughes et al., 1993; Woodgett et al., 1993]. However, as presented in this report that kinase F_A/GSK-3α could be activated up to ~600% of normal controls during human thyroid tumor cell dedifferentiation, the results

point out that kinase F_A/GSK-3α may also function as a mitogen-activated protein kinase. It is possible that human papillomaviruses which play an important role in thyroid tumor cell carcinogenesis may function as a mitogen to induce activation of kinase F_A/GSK-3α in thyroid tumor cells. This obviously presents an intriguing issue deserving further investigation.

From the clinical viewpoint, since the cellular levels and biological activities of protein kinase F_A/GSK-3α are significantly and quantitatively correlated with the degree of human thyroid tumor cell dedifferentiation, this kinase may possibly be used as a specific marker protein for clinical diagnosis of the status of thyroid cancer during pre- and postdiagnosis of the disease. This obviously presents another important issue deserving further investigation. Nevertheless, the present study clearly demonstrates that protein kinase F_A/GSK-3α (a possible regulator of transcription factors/protooncogenes and a particular member of the proline-directed protein kinase family) is consistently and statistically activated manyfold in human thyroid tumor cells and that the activation state of the kinase is proportionally correlated with the state of dedifferentiation of human thyroid tumor.

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