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# Endothelin-1 and Insulin Induce Cellular Inactivation of Protein Kinase $F_A$/Glycogen Synthase Kinase-3α in a Common Signaling Pathway

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**Key Words**
Endothelin-1
Insulin
Protein Kinase $F_A$/GSK-3α
MAP kinase signaling pathway
Rat adipocytes

**Abstract**
In this study, we investigate the effects of endothelin-1 (ET-1) and insulin on the cellular activity of protein kinase $F_A$/glycogen synthase kinase-3α (kinase $F_A$/GSK-3α) in rat adipocytes. The cellular activity of kinase $F_A$/GSK-3α is inhibited to ~ 50% of control within 30 min when cells are treated with 1 n*M* ET-1 at 37 °C; in addition, significant inhibition to ~60% of control is observed at as low as 1 p*M* ET-1. Conversely, ET-1 at concentrations up to 1 n*M* has no direct effect on purified kinase $F_A$/GSK-3α in vitro. Immunoblotting analysis further reveals that the protein level of this kinase is not significantly changed when treated with 1 n*M* ET-1 for 30 min. Similar to ET-1, insulin as low as 10 n*M* can also induce inactivation of kinase $F_A$/GSK-3α to ~ 50% of control in adipocytes when processed under identical conditions. Most importantly, when treated with both insulin and ET-1, the activity of kinase $F_A$/GSK-3α can be decreased only to ~ 50% of control. Taken together, the results provide initial evidence that ET-1 and insulin may regulate this important multisubstrate/multifunctional protein kinase in a common signaling pathway in cells.

## Introduction

Protein kinase $F_A$ was originally identified as an activating factor of Mg.ATP-dependent protein phosphatase [18, 23], but has subsequently been demonstrated to function as a protein kinase identical to glycogen synthase kinase-3α (GSK-3α) [9, 18, 21]. 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 [10], myelin basic protein [24, 30], the G subunit of phosphatase-1 [5, 6], ATP-citrate lyase [15], acetyl-CoA carboxylase [11], microtubule-associated protein-2 and tau protein [26, 27], brain clathrin-coated vesicles [28] and the transcription factors/protooncogenes such as *c-jun* [2, 7], *c-myb* and *c-myc* [13], and CREM [8]. Due to its unique feature as a multisubstrate protein kinase and as an activating factor of a multisubstrate protein phosphatase, $F_A$/GSK-3α may simulta-

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neously modulate phosphorylation and dephosphorylation states of many key regulatory proteins involved in regulating diverse cell functions [22, 25]. In this study, we investigate the regulation of kinase $F_A$/GSK-3α in isolated rat adipocytes treated with ET-1 and insulin by immunodetection in an anti-kinase $F_A$/GSK-3α immunoprecipitate. Here, we show that ET-1 and insulin can regulate this important multisubstrate/multifunctional protein kinase in a common signaling pathway.

## Materials and Methods

*Materials*

[γ-$^{32}$P]ATP was purchased from Amersham (UK). ET-1 was from Peptide Institute (Japan). Collagenase was from Worthington Biochem (USA). Polyvinylidene fluoride (PVDF) membrane (Immobilon-P) was from Millipore (USA). Insulin, sodium orthovanadate, Tween 20 and goat anti-rabbit IgG antibody conjugated with alkaline phosphatase were from Sigma (USA). Monoclonal anti-phosphotyrosine antibody (RC20B) conjugated with biotin was from Transduction Laboratories (USA). Strepavidin conjugated with alkaline phosphatase and molecular weight marker proteins were from Boehringer Mannheim (Germany). BCA protein assay reagent was from Pierce (USA). Alkaline phosphatase conjugate substrate kit was from Bio-Rad (USA). Protein A-Sepharose CL-4B was from Pharmacia (Sweden).

*Protein purification*

Protein kinase $F_A$/GSK-3α [24, 29] and myelin basic protein (MBP) [30] were purified from porcine brain as described in previous reports [24, 29, 30].

*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 [21] as the antigen. Production, affinity purification, identification and characterization of anti-kinase $F_A$/GSK-3α antibody were detailed in previous reports [29, 31, 32]. In this study, the antibody can potently and specifically immunoblot kinase $F_A$/GSK-3α from the rat adipocyte extracts on SDS-PAGE. The antibody can also efficiently immunoprecipitate all the kinase $F_A$/GSK-3α from the rat adipocyte extracts and without blocking the kinase activity, as described in previous reports [29, 31, 32] (data not further illustrated).

*Preparation of Isolated Rat Adipocytes*

Male Sprague-Dawley rats weighing 200–250 g were sacrificed by decapitation, and the epididymal fat pads were collected. Isolated adipocytes were obtained using a method modified from Rodbell [16] by shaking finely minced tissue at 37 °C for 1 h in Krebs-Ringer bicarbonate (KRB) buffer containing 1 m*M* pyruvate, 1% bovine serum albumin, and 0.1% collagenase. Cells were then filtered through nylon mesh (400 μm), centrifuged at 100 rpm for 1 min, washed twice in KRB buffer containing 1 m*M* pyruvate and 1% bovine serum albumin (solution A), and finally resuspended in solution A.

*ET-1 and Insulin Treatment and Cell Extract Preparation of Isolated Rat Adipocytes*

One milliliter of the isolated rat adipocytes (~ $10^6$ cells/ml) was treated with various concentrations of ET-1 and insulin at 37 °C in a $CO_2$ incubator for various time intervals as indicated. The adipocytes were then centrifuged at 100 rpm for 1 min, washed twice in ice-cold phosphate-buffered saline (PBS), and homogenized in 1 ml of solution B (20 m*M* Tris-HCl at pH 7.0, 1 m*M* EDTA, 1 m*M* EGTA, 1% Triton X-100, 1 m*M* benzamidine, 1 m*M* phenylmethylsulfonyl fluoride, 1 m*M* TLCK, 50 m*M* NaF, 20 m*M* sodium pyrophosphate and 0.2 m*M* sodium orthovanadate) on ice by a sonicator (model W-380, Heat Systems-Ultrasonics) for 3 × 10 s at 40% power output. The homogenates were then centrifuged at 160,000 *g* for 25 min at 4 °C. The resulting supernatants were used as the cell extracts.

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

For immunoprecipitation, 800 μl of cell extracts was incubated with 2 μl of affinity-purified kinase $F_A$/GSK3α (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 B) for another 1 h with shaking. The immunoprecipitates were collected by centrifugation, washed three times with 1 ml of solution B containing 0.5 *M* NaCl, once with 1 ml solution C (20 m*M* Tris-HCl at pH 7.0, 0.5 m*M* dithiothreitol, 1 m*M* phenylmethylsulfonyl fluoride, 1 m*M* benzamidine, 0.5 mg/ml aprotinin), and resuspended in 50 μl of solution C. 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 m*M* Tris-HCl at pH 7.0, 0.5 m*M* dithiothreitol, 0.2 m*M* [γ-$^{32}$P]ATP, 20 m*M* $MgCl_2$, and 4 mg/ml MBP at 30 °C for 10 min. Next, $^{32}$P incorporation into MBP was measured by spotting 30 μl of reaction mixture on phosphocellulose paper (1 × 2 cm) (Whatman), washing three times with 75 m*M* $H_3PO_4$, and counting in liquid scintillation analyzer (Model 1600CA, Packard) as described in previous reports [24, 31, 32].

*Immunoblots*

Proteins were transferred from unstained SDS-gels to Immobilon-P membrane and the membrane was subjected to immunoblotting with 1 μg/ml anti-kinase $F_A$/GSK-3α antibody as described in previous reports [31, 33]. The kinase $F_A$/GSK-3α protein was detected by the color development reagent kit. For immunoblotting by anti-phosphotyrosine antibody, the samples obtained from immunoprecipitation were analyzed by 10% SDS-PAGE, electrotransferred to Immobilon-P membrane and then subjected to immunoblotting with anti-phosphotyrosine antibody conjugated with biotin (RC20B) at 1:1,000 dilution. The phosphotyrosine-containing proteins were detected by the strepavidin-conjugated alkaline phosphatase and the color development reagent kit.

*Analytic Methods*

Protein concentrations were determined by using the BCA protein assay reagent from Pierce. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [12] using 10% gels.

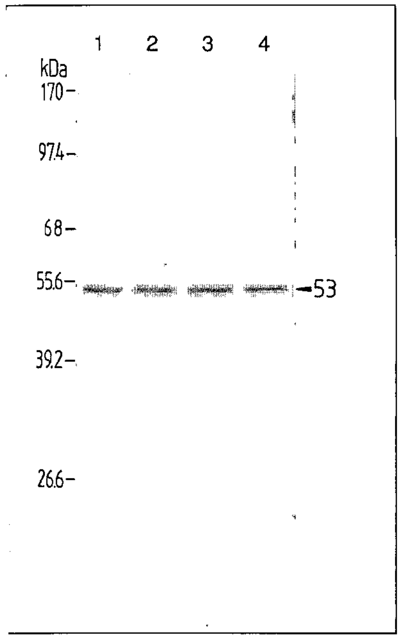


**Fig. 1.** Immunoblot of kinase $F_A$/GSK-3α from cell extracts of isolated rat adipocytes treated with ET-1. Isolated rat adipocytes were treated with 1 n*M* ET-1 at 37 °C for various time intervals as indicated, and 100 μg of the cell extracts were immunoblotted by anti-kinase $F_A$/GSK-3α antibody on 10% SDS-PAGE as described in 'Materials and Methods'. Lanes 1–4, immunoblot of extracts of isolated rat adipocytes treated with 1 n*M* ET-1 for 0, 5, 15, and 30 min, respectively.

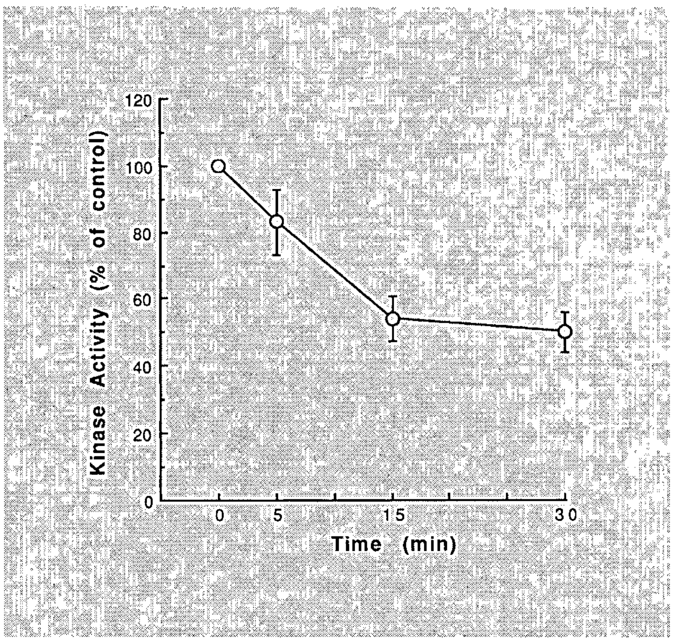


**Fig. 2.** Time course effect of ET-1 on the cellular activity of kinase $F_A$/GSK-3α in isolated rat adipocytes. Isolated rat adipocytes were treated with 1 n*M* ET-1 at 37 °C for various time intervals as indicated. The cell extracts were immunoprecipitated by 20 μg anti-kinase $F_A$/GSK-3α antibody, followed by kinase activity assay in the immunoprecipitates as described under 'Materials and Methods'. Data are the averages of three independent experiments and expressed as means ± SD. $p < 0.01$.

## Results and Discussion

The anti-kinase $F_A$/GSK-3α antibody produced and affinity-purified as described in Materials and Methods was found to be very specific and potent toward immunoblotting kinase $F_A$/GSK-3α at a molecular weight of 53 kD from isoladet rat adipocytes (fig. 1, lane 1), demonstrating the immunospecificity of anti-kinase $F_A$/GSK-3α antibody produced here. When isolated rat adipocytes were treated with 1 n*M* ET-1 at 37 °C for various time intervals as indicated, followed by immunoblotting of kinase $F_A$/GSK-3α from cell extracts using the same anti-kinase $F_A$/GSK-3α antibody, there was also only one single protein band at a molecular weight of 53 kD detectable in the immunoblot (fig. 1, lanes 2–4). Furthermore, no significant change occurred in the immunoblotted protein level of this kinase in cells treated with ET-1 (fig. 1). Moreover, ET-1 at concentrations up to 1 n*M* was found to have no direct effect on the activity of purified $F_A$/GSK-3α in vitro (not illustrated). However, when adipocytes were treated with 1 n*M* ET-1 at 37 °C for various time points as indicated, followed by immunoprecipitation of this kinase from cell extracts using the antibody as described above, the cellular activity of kinase $F_A$/GSK-3α, which is detectable in the immunoprecipitates from the cell extracts, was found to be inactivated to ~ 50% of control value within 30 min (fig. 2). Taken together, the results point out that a post-translational modification of pre-existing protein could possibly be involved in the ET-1-mediated inactivation of protein kinase $F_A$/GSK-3α in adipocytes (fig. 1, 2). Interestingly, the ET-1-induced inactivation of kinase $F_A$/GSK-3α in rat adipocytes was found to be dose dependent and significant inactivation to ~ 60% of control could be observed at as low as 1 p*M* (fig. 3). This observation indicates that protein kinase $F_A$/GSK-3α is a highly sensitive target subjected to regulation by ET-1. Similar to ET-1, insulin as low as 10 n*M* could also induce inactivation of kinase $F_A$/GSK-3α to ~ 50% of control in adipocytes when processed under identical conditions (fig. 4, lane 3), which is in agreement with the previous reports [11, 14].

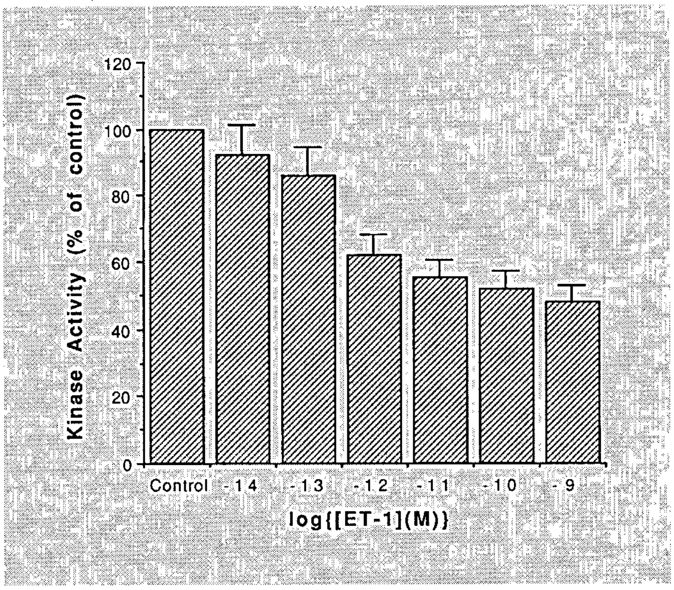


**Fig. 3.** Dose effect of ET-1 on the cellular activity of kinase $F_A$/GSK-3α in rat adipocytes. Isolated rat adipocytes were treated with various concentrations of ET-1 as indicated at 37 °C for 30 min. The cell extracts were immunoprecipitated by 20 μg anti-kinase $F_A$/GSK-3α antibody, followed by kinase activity assay in the immunoprecipitates as described in 'Materials and Methods'. Data are the averages of three independent experiments and expressed as means ± SD. $p < 0.01$.

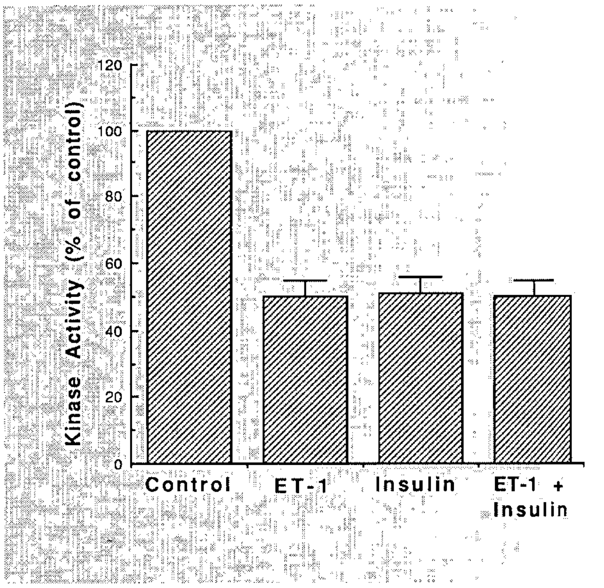


**Fig. 4.** Effect of insulin on ET-1-mediated inactivation of kinase $F_A$/GSK-3α in rat adipocytes. Isolated rat adipocytes were first treated with and without 1 n*M* ET-1 at 37 °C for 30 min and then with and without 10 n*M* insulin for another 30 min. The cell extracts were immunoprecipitated by 20 μg anti-kinase $F_A$/GSK-3α antibody, followed by kinase activity assay in the immunoprecipitates as described in 'Materials and Methods'. Data are the averages of three independent experiments and expressed as means ± SD. $p < 0.01$.

Most importantly, when adipocytes were treated with both insulin and ET-1, the cellular activity of kinase $F_A$/GSK-3α could only be decreased to ~50% of control (fig. 4, lane 4). This finding would imply that ET-1 and insulin may regulate this important protein kinase in a common signaling pathway. Phosphotyrosine analysis further revealed that no tyrosine phosphorylation is involved in the ET-1-mediated inactivation of kinase $F_A$/GSK-3α in a similar pattern as described in figure 1 (data not further illustrated). The result again is in agreement with the insulin-mediated inactivation of kinase $F_A$/GSK-3α without involving tyrosine phosphorylation as reported in the literature [4, 17, 20].

Mitogen-activated protein kinase-activated protein kinase-1β (MAPKAP-1β) has been reported to be involved in the insulin-mediated inactivation of kinase $F_A$/GSK-3α [4, 17, 20] and mitogen-activated protein kinase (MAP kinase) can be activated by ET-1 [1, 19]. This taken together with the present study that ET-1 and insulin induce inactivation of kinase $F_A$/GSK-3α in a common signaling pathway suggest that ET-1 and insulin may induce inactivation of this important multisubstrate/multifunctional protein kinase in a common MAP kinase signaling pathway in cells. On the other hand, the insulin-stimulated glucose transport can be inhibited by ET-1 in rat adipocytes [3]. Although the physiological significance of ET-1 inhibition on kinase $F_A$/GSK-3α and on insulin-stimulated glucose transport remain to be established. results in this study and in a previous report [3] further suggest that insulin and ET-1 may act in a common or antagonistic signaling pathway. This obviously presents an intriguing issue deserving further investigation.

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## References


1 Bogoyevitch MA, Glennon PE, Andersson MB, Clerk A, Lazou A, Marchalls CJ, Parker PJ, Sugden PH. Endothelin-1 and fibroblast growth factors stimulate the mitogen-activated protein kinase signaling cascade in cardiac myocytes. J Biol Chem 269:1110–1119;1994.

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

3 Chou YC, Perng JC, Juan CC, Jang SY, Kowk CF, Chen WL, Fong JC, Ho LT. Endothelin-1 inhibits insulin-stimulated glucose uptake in isolated rat adipocytes. Biochem Biophys Res Commun 202:688–693;1994.

4 Cross DAE, Alessi DR, Vandenheede JR, McDowell HE, Hundal HS, Cohen P. The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor 1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: Evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf. Biochem J 303:21–26;1994.

5 Dent P, Campbell DG, Hubbard MJ, Cohen P. 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; 1989.

6 Fiol CJ, Haesman JH, Wang Y, Roach PJ, Roeske RW, Kowulczuk M, DePaoli-Roach AA. Phosphoserine as a recognition determinant for glycogen synthase kinase-3: Phosphorylation of a synthetic peptide based on the G-component of protein phosphatase-1. Arch Biochem Biophys 267:797–802;1988.

7 de Groot RP, Auwerx J, Bourous M, Sassone-Corsi P. Negative regulation of Jun/AP-1: Conserved function of glycogen synthase kinase 3 and the *Drosophila* kinase *shaggy*. Oncogene 7: 841–847;1992.

8 De Groot RP, den Hertog J, Vandenheede JR, Goris J, Sassone-Corsi P. Multiple and cooperative phosphorylation events regulate the CREM activator function. EMBO J 12:3903–3911;1993.

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

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

11 Hughes K, Ramakrishna S, Bejamin WB, Woodgett JR. Identification of multifunctional ATP-citrate lyase kinase as the α-isoform of glycogen synthase kinase-3. Biochem J 288: 309–314;1992.

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

13 Plyte SE, Hughes K, Nikolakaki E, Pulverer BJ, Woodgett JR. Glycogen synthase kinase-3: functions in oncogenesis and development. Biochim Biophys Acta 1114:147–152;1992.

14 Ramakrishna S, Bejamin WB. Insulin action rapidly decreases multifunctional protein kinase activity in rat adipocyte tissue. J Biol Chem 263:12677–12681;1988.

15 Ramakrishna S, D'Angelo G, Benjamin W. 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;1990.

16 Rodbell M. Metabolism of isolated fat cell. J Biol Chem 239:375–380;1964.

17 Stambolic V, Woodgett JR. Mitogen inactivation of glycogen synthase kinase-3β in intact cells via serine 9 phosphorylation. Biochem J 303:701–704;1994.

18 Vandenheede JR, Yang S-D, Goris J, Merlevede W. 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;1980.

19 Wang Y, Simonson MS, Pouyssegur J, Dunn MJ. Endothelin rapidly stimulates mitogen-activated protein kinase activity in rat mesangial cells. Biochem J 287:589–594;1992.

20 Welsh GI, Foulstone EJ, Young SW, Tavare JM, Proud CG. Wortmannin inhibits the effects of insulin and serum on the activities of glycogen synthase kinase-3 and mitogen-activated protein kinase. Biochem J 303:15–20; 1994.

21 Woodgett JR. Molecular cloning and expression of glycogen synthase kinase-3/factor A. EMBO J 9:2431–2438;1990.

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

23 Yang SD, Vandenheede JR, Goris J, Merlevede W. ATP.Mg-dependent phosphorylase phosphatase from rabbit muscle. J Biol Chem 255:11759–11767;1980.

24 Yang S-D. Identification of the ATP.Mg-dependent protein phosphatase activator $F_A$ as a myelin basic protein kinase in the brain. J Biol Chem 261:11786–11791;1986.

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

26 Yang S-D, Yu J-S, Lai Y-G. Identification and characterization of the ATP-Mg-dependent protein phosphatase activator ($F_A$) as a microtubule protein kinase in the brain. J Protein Chem 10:171–181;1991.

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

28 Yu J-S, Yang S-D. Identification and characterization of protein kinase $F_A$/glycogen synthase kinase 3 in clathrin-coated brain vesicles. J Neurochem 60:1714–1721;1993.

29 Yu J-S, Yang S-D. Immunological and biochemical study on tissue and subcellular distributions of protein kinase $F_A$ (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;1993.

30 Yu J-S, Yang S-D. Protein kinase $F_A$/glycogen synthase kinase-3 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;1994.

31 Yu J-S, Yang S-D. Okadaic acid, a serine/threonine phosphatase inhibitor, induces tyrosine dephosphorylation/inactivation of kinase $F_A$/GSK-3α in A431 cells. J Biol Chem 269: 14341–14344;1994.

32 Yu J-S, Yang S-D. Tyrosine dephosphorylation and concurrent inactivation of protein kinase $F_A$/GSK-3α by genistein in A431 cells. J Cell Biochem 56:131–141;1994.