

Interaction between ERK and GSK3 β Mediates Basic Fibroblast Growth Factor-induced Apoptosis in SK-N-MC Neuroblastoma Cells*

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The Ewing's sarcoma family of tumors (ESFT) includes Ewing's sarcoma (ES), Askin's tumor of the chest wall, and peripheral primitive neuroectodermal tumor. Basic fibroblast growth factor (FGF2) suppresses the growth of ESFT cells and causes their apoptosis. The underlying mechanism is unclear. Using a human peripheral primitive neuroectodermal tumor cell line, SK-N-MC, we demonstrated FGF2 stimulated phosphorylation of ERK1 and ERK2 (pERK1/2) and GSK3 β (pGSK3 β (Tyr-216)), all of which were primarily retained in the cytoplasm. FGF2 promoted the association between ERK and pGSK3 β (Tyr-216). Inhibitors for GSK3 β (TDZD and LiCl) and ERK (PD98059) protected cells from FGF2-induced apoptosis. On the other hand, inhibitors of GSK3 β , but not PD98059 decreased ERK/pGSK3 β (Tyr-216) association and caused a nuclear translocation of pERK1/2. Similarly, expression of a kinase-deficient (K85R) GSK3 β or GSK3 β -small interfering RNA inhibited FGF2-regulated ERK/pGSK3 β (Tyr-216) association and translocated pERK to the nucleus. Both K85R GSK3 β and small interfering RNA offered protection against FGF2-induced cell death. In contrast, overexpression of wild-type GSK3 β sensitized cells to FGF2 cytotoxicity. Hydrogen peroxide and ethanol enhanced FGF2-stimulated pGSK3 β (Tyr-216), ERK/pGSK3 β (Tyr-216) association, and cytoplasmic retention of pERK1/2. As a result, they potentiated FGF2-induced cell death. Taken together, our results suggested that FGF2-induced accumulation of pERK1/2 in the cytoplasm is toxic for SK-N-MC cells. The formation of an ERK-GSK3 β complex retained pERK1/2 in the cytoplasm. In contrast, disruption of the ERK-GSK3 β complex resulted in nuclear translocation of pERK1/2 and offered protection.

The Ewing's sarcoma family of tumors (ESFT)³ including Ewing's sarcoma, Askin's tumor of the chest wall, and peripheral primitive neuroectodermal tumor are common bone and soft tissue tumors among children and young adults. It is generally believed that ESFT are derived from pluripotent neural crest cells (1). They are malignant tumors of childhood and adolescence (1). The outcomes of treatment of these tumors are poor; less than 20% of patients with metastatic disease are long-term survival patients (2). Therefore, development of new treatment strategies for these tumors is important.

Basic fibroblast growth factor (bFGF or FGF2) belongs to the FGF family, which consists of up to 23 members (3, 4). FGFs and their cell surface receptors (FGFR) make up a large and complex family of signaling molecules that play an important role in a variety of processes of embryonic development and tissue homeostasis, as well as pathogenesis of some morphogenetic disorders and cancers. FGF2 is ubiquitously expressed, but is most abundant in the nervous system (5). In embryonic tissues, FGF2 plays a critical role in morphogenesis by regulating cell proliferation, differentiation, and cell migration. In adult organisms, FGF2 plays an important role in the function of the nervous system, tissue repair, wound healing, and tumor angiogenesis (3, 4).

FGF2 is generally viewed as a mitogen or pro-survival factor. Dysregulation of FGF signaling has been implicated in tumorigenesis and malignant progression (6). However, the response to FGF2 depends on cell type and developmental status (7, 8). For example, FGF2 causes apoptosis in chondrocytes and breast cancer cells (9, 10). FGF2 suppresses the growth of ESFT cells by inducing apoptosis of tumor cells *in vivo* (2). FGF2-induced death of ESFT cells is confirmed *in vitro* using various ESFT cell lines (2, 11–13). However, the cellular and molecular mechanisms underlying FGF2-mediated death of ESFT cells remain unclear.

Key components of FGF2 signaling are mitogen-activated protein kinases (MAPKs) (3). In mammals, three major groups

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³ The abbreviations used are: ESFT, Ewing's sarcoma family of tumors; ERK1/2, extracellular signal-regulated kinases 1 and 2; bFGF2, basic fibroblast growth factor; GSK3 β , glycogen synthase kinase 3 β ; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK1/2, MAPK kinase 1 and 2; p38 MAPK, 38-kDa mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FITC, fluorescein isothiocyanate; CA-MEK1, constitutively active MEK1; siRNA, small interfering RNA.

of MAPKs have been identified: extracellular signal-regulated kinases (ERKs), p38 MAPK, and c-Jun N-terminal kinase (JNK). The ERKs are stimulated by receptor tyrosine kinases and G protein-coupled receptors, and their activation generally leads to mitogenic or growth response. JNK and p38 MAPK are stimulated by cellular stresses, such as free radicals and inflammatory agents, leading to apoptotic cell death. Although ERKs have been generally known for their mitogenic and survival promoting functions, many studies indicate that ERK activation may lead to cell death (14). It appears that the subcellular localization of ERK plays an important role in determining the function of ERKs (15).

In the present study, we used human SK-N-MC cells, which were derived from soft tissue peripheral primitive neuroectodermal tumors, to investigate the mechanism of FGF2-induced apoptosis. SK-N-MC cells endogenously express FGF2 and FGF receptor (FGFR-1) (2). We demonstrate here that FGF2 induces a sustained phosphorylation of ERK1 and ERK2 (pERK1/2), whereas it has a modest effect on JNK and p38 MAPK. The FGF2-induced pERK1/2 is predominantly retained in the cytoplasm and forms a complex with GSK3 β . The cytoplasmic accumulation of pERK is accountable for FGF2-induced death of SK-N-MC cells. Our findings underscore a novel mechanism by which FGF2 induces cell death.

EXPERIMENTAL PROCEDURES

Materials—Antibodies against GSK3 β (catalog number 9332), Histone 3 (catalog number 9715), pERK1/2 (catalog number 9101), pJNK (catalog number 9251), poly(ADP-ribose) polymerase (catalog number 9542), and cyclin D1 (catalog number 2926) were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-tubulin antibody (catalog number T6199) was purchased from Sigma. Anti-pGSK3 β (Ser-9) (catalog number 44-600G) and pGSK3 β (Tyr-216) antibodies (catalog number 44-604G) were obtained from Invitrogen. Anti-p-p38 antibody (catalog number 7973) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). GSK3 β inhibitors (TDZD-8 and LiCl) and MEK1 inhibitor (PD98059) were purchased from Calbiochem (La Jolla, CA). Annexin-V FITC Apoptosis Detection Kit was obtained from Biovision Inc. (Mountain View, CA). Other chemicals were obtained from Sigma.

Cell Culture—Human SK-N-MC cells obtained from ATCC were grown in Eagle's minimal essential medium containing 10% fetal bovine serum, 2 mM L-glutamine and 25 μ g/ml gentamicin, 100 units/ml penicillin, and 100 μ g/ml streptomycin at 37 °C with 5% CO₂.

Determination of Cell Viability and Apoptosis—Cell viability was determined by MTT assay as previously described (16). Apoptosis was quantified by the expression of Annexin-V using an Annexin-V FITC Apoptosis Detection Kit as previously described (17). Briefly, cells (1×10^6) were suspended in 500 μ l of binding buffer (10 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 1.8 mM CaCl₂) and treated with 10 μ l of FITC-conjugated anti-Annexin-V antibody and 10 μ l of propidium iodide for 10 min in the dark at room temperature. The samples were analyzed by flow cytometry (excitation = 488 nm; emission = 530 nm).

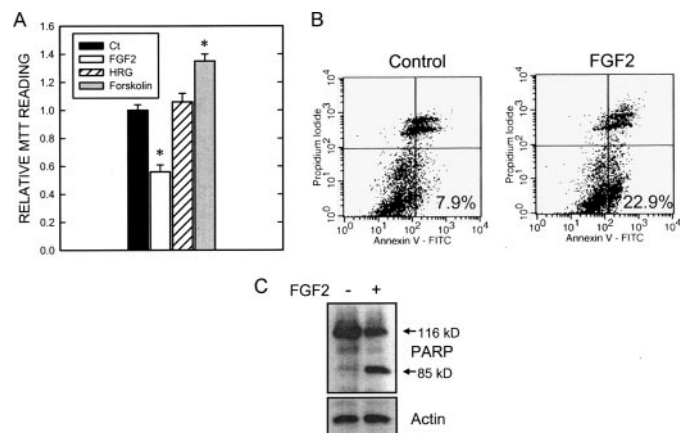


FIGURE 1. FGF2-induced death of SK-N-MC cells. *A*, SK-N-MC cells were treated with FGF2 (10 ng/ml), heregulin β 1 (30 ng/ml), or forskolin (10 μ M) for 48 h. Cell viability was determined by MTT as described under "Experimental Procedures." Each data point (\pm S.E.; bar) is the mean of three experiments. Asterisk denotes significant difference from controls. *B*, SK-N-MC cells were treated with FGF2 (10 ng/ml) for 12 h. Annexin-V-positive cells were analyzed via flow cytometry as described under "Experimental Procedures." *C*, SK-N-MC cells were treated with FGF2 (10 ng/ml) for 12 h. The expression of poly(ADP-ribose) polymerase (PARP) was determined with immunoblotting.

Preparation of Cell Lysates of Whole Cell and Cytoplasmic and Nuclear Fractions—For the lysates of whole cells, cells were washed with phosphate-buffered saline (pH 7.4) and lysed with RIPA buffer (150 mM NaCl, 50 mM Tris (pH 8.0), 1% Nonidet P-40 (Nonidet P-40), 0.1% SDS, 0.5% deoxycholic acid sodium, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 3% aprotinin) on ice for 10 min, solubilized cells were centrifuged, the supernatant was collected and designated as the whole cell lysates. For extraction of cytoplasmic and nuclear fractions, cells were lysed in phosphate-buffered saline that contained 5 mM EDTA, 1% Nonidet P-40, 1 mM dithiothreitol (Sigma), 10 μ g/ml leupeptin, and 1 mM Pefabloc SC. After 5 min incubation on ice, the lysate was centrifuged at 12,000 \times g for 5 min. The supernatant was designated the cytoplasmic fraction. The pelleted nuclei were sonicated in nuclear extraction buffer (20 mM Tris-HCl, pH 7.5, 1% SDS, 5 mM EGTA, 0.5% Triton X-100, 150 mM NaCl, 1 mM dithiothreitol, 10 mg/ml leupeptin, and 1 mM Pefabloc SC) and centrifuged at 12,000 \times g for 5 min. The supernatant was collected and designated the nuclear fraction.

Immunoblotting—The immunoblotting procedure has been previously described (16). Briefly, after the protein concentrations were determined, aliquots of the protein samples (30 μ g) were loaded into the lanes of a SDS-polyacrylamide gel. The protein samples were separated by electrophoresis, and the separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with either 5% bovine serum albumin or 5% nonfat milk in 0.01 M phosphate-buffered saline (pH 7.4) and 0.05% Tween 20 (TPBS) at room temperature for 1 h. Subsequently, the membranes were probed with primary antibodies directed against target proteins for 2 h at room temperature or overnight at 4 °C. After three quick washes in TPBS, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences) diluted at 1:2000

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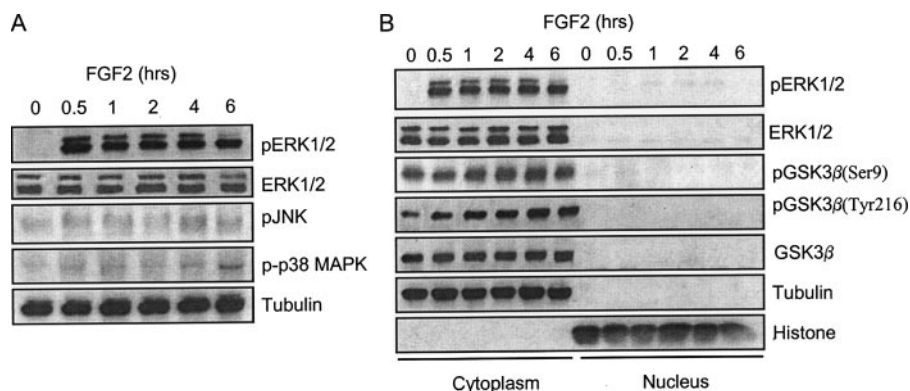


FIGURE 2. Effect of FGF2 on the phosphorylation of MAPKs and GSK3 β . A, SK-N-MC cells were maintained in serum-free medium overnight and then exposed to FGF2 (10 ng/ml) for the indicated times. Total cell lysates were collected and examined for the expression of phosphorylated ERK1/2, JNK, and p38 MAPK. B, SK-N-MC cells were maintained in serum-free medium overnight and then exposed to FGF2 (10 ng/ml) for the indicated times. The cytoplasmic and nuclear proteins were collected. The expression of ERK1/2 and GSK3 β as well as phosphorylated ERK1/2 (pERK1/2) and pGSK3 β (Ser-9 and Tyr-216) was examined with immunoblotting. The purity of cytoplasmic and nuclear protein was verified by the expression of tubulin and histone H3, respectively. The experiment was replicated three times.

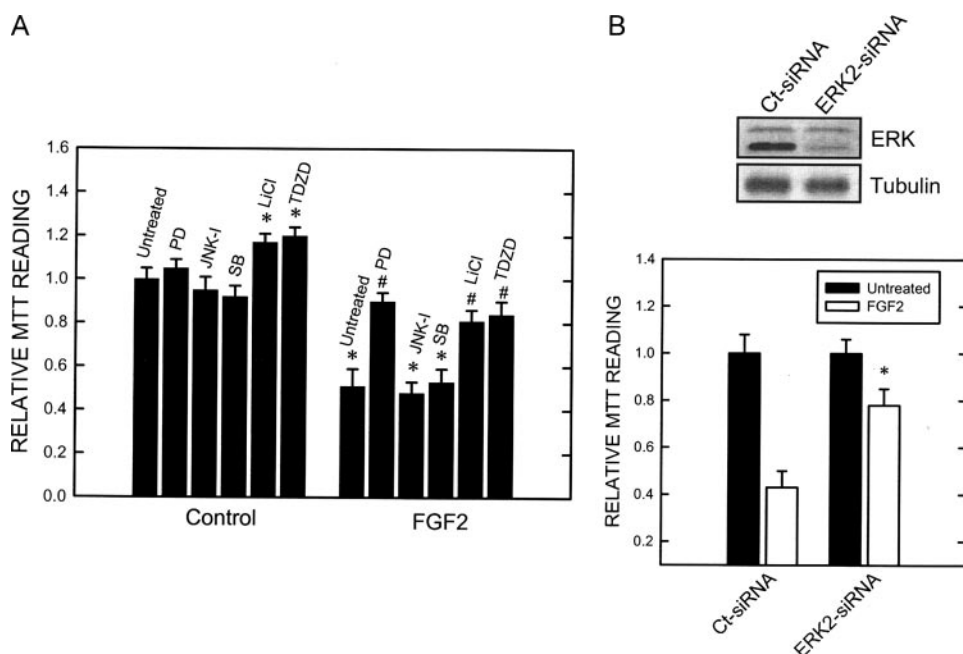


FIGURE 3. Effect of inhibitors of MAPKs and GSK3 β as well as ERK2 siRNA on FGF2-induced death of SK-N-MC cells. A, SK-N-MC cells were pretreated with inhibitors of MEK1 (PD98059, 50 μ M), JNK (JNK-I, 1 μ M), p38 MAPK (SB203580, 10 μ M), and GSK3 β (LiCl, 20 mM; TDZD, 10 μ M) for 30 min. After that, cells were exposed to FGF2 (10 ng/ml) for 48 h. Cell viability was determined by MTT as described under "Experimental Procedures." The experiment was replicated three times. Asterisk denotes significant difference from untreated controls. Number sign denotes significant difference from the group treated with FGF2 but without inhibitors. B, SK-N-MC cells were treated with an ERK2 siRNA for 48 h. The expression of ERK1/2 was determined with immunoblotting (top panel). After transfection with ERK2 siRNA or control siRNA for 48 h, cells were treated with FGF2 (0 or 10 ng/ml) for 48 h. Cell viability was determined by MTT (bottom panel). Each data point (\pm S.E.; bar) is the mean of three replications. Asterisk, ERK2 siRNA-mediated protection; denotes significant difference from the control siRNA-treated group (open bar in ERK2 siRNA-treated group versus open bar in control siRNA-treated group).

in TPBS for 1 h. The immune complexes were detected by the enhanced chemiluminescence method (Amersham Biosciences). In some cases, the blots were stripped and re-probed with either an anti-tubulin or an anti-Histone H3 antibody. The density of immunoblotting was quantified with the software of Quantity One (Bio-Rad). The expression of target proteins was normalized to the levels of either tubulin or Histone H3.

Cell Transfection and Establishing Stable Transfectants—Stable transfectants expressing various GSK3 β constructs were established as previously described (18). V5-tagged GSK3 β constructs (wild-type and K85R) carried by vector pcDNA3 were generous gifts from Dr. Thilo Hagen (University Hospital Nottingham, Nottingham, UK). Constitutively active MEK1 plasmid (CA-MEK1) was obtained from Stratagene (pFC-MEK1, catalog number 219065). Cell transfection was carried out with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Stable cell clones expressing exogenous GSK3 β were selected by treatment of G418 (600 μ g/ml) for 3–4 weeks. Positive clones were verified by the expression of the V5 tag protein, as well as the evidence of GSK3 β overexpression. The clones expressing the highest levels of exogenous GSK3 β were selected for subsequent experiments.

GSK3 β Small Interfering RNA (siRNA)—GSK3 β siRNA (catalog number 6301; Cell Signaling Technology, Inc.) or Silencer Negative Control siRNA (catalog number 4611; Ambion, Inc., Austin, TX) were transfected into SK-N-MC cells with either oligofectamine reagent (Invitrogen) or Nucleofector™ (Amaya Inc., Gaithersburg, MD) according to the manufacturer's protocol. At 48 h after transfection, the cells were subjected to immunoblotting analysis for GSK3 β expression.

Statistical Analysis—Differences among treatment groups were tested using analysis of variance. Differences in which *p* was less than 0.05 were considered statistically significant. In cases where significant differences were detected, specific post hoc comparisons between

treatment groups were examined with Student-Newman-Keuls tests.

RESULTS

ERK1/2 Mediate FGF2-induced Death of SK-N-MC Cells—We examined the effect of FGF2, forskolin, and heregulin β 1 on the viability of SK-N-MC cells. Forskolin and heregulin β 1 were selected for comparison because forskolin-induced pERK1/2

was mainly localized in the nucleus (15), whereas heregulin β 1 did not affect pERK1/2 (data not shown). As determined by MTT assay, FGF2 decreased the viability of SK-N-MC cells, whereas forskolin enhanced the survival of SK-N-MC cells; heregulin β 1 had little effect on cell viability (Fig. 1A). FGF2-induced apoptosis of SK-N-MC cells was confirmed by Annexin-V staining and the cleavage of poly(ADP-ribose) polymerase (Fig. 1, B and C).

FGF2 caused a prolonged phosphorylation of ERK1 and ERK2, whereas it had a modest effect on the phosphorylation of JNK and p38 MAPK (Fig. 2A). FGF2-induced pERK1/2 persisted for at least 6 h. The majority of FGF2-induced pERK1/2 was retained in the cytoplasm (Fig. 2B). GSK3 β is a substrate of ERK, and ERK phosphorylates GSK3 β at serine 9 (19). Full activity of GSK3 β generally requires phosphorylation at tyrosine 216, and conversely, phosphorylation at serine 9 inhibits GSK3 β activity (19). We thus sought to determine whether FGF2 affected GSK3 β phosphorylation. As shown in Fig. 2B, FGF2 had little effect on the phosphorylation of GSK3 β at serine 9 (pGSK3 β (Ser-9)), but enhanced its phosphorylation at tyrosine 216 (pGSK3 β (Tyr-216)); both phosphorylated and non-phosphorylated forms of GSK3 β were localized to the cytoplasm (Fig. 2B). ERK1/2 are substrates of MEK1 (14). PD98059, a selective inhibitor of MEK1, effectively blocked FGF2-induced ERK phosphorylation (Fig. 5A). PD98059, but not inhibitors for p38 MAPK and JNK, protected SK-N-MC cells against FGF2-induced cell death (Fig. 3A), suggesting pERK1/2 were required for FGF2 cytotoxicity. To confirm that ERK-mediated FGF2 induced cell death, we knocked down the expression of ERK2 by an ERK2 siRNA. As shown in Fig. 3B, ERK2 siRNA significantly protected cells against FGF2 cytotoxicity. Inhibitors of GSK3 β (LiCl and TDZD) also offered significant protection against FGF2-induced cell death (Fig. 3A), suggesting that GSK3 β is also involved in FGF2-induced cell death. It was noted that GSK3 β inhibitors by themselves promoted cell survival (Fig. 3A).

FGF2 Modulates the Interaction between ERK1/2 and GSK3 β

It has been shown that pERK was retained in the cytoplasm by forming a heterotrimeric complex with other cytosolic proteins, and cytoplasmic retention of pERK1/2 is cytotoxic to SK-N-MC cells (15). We demonstrated here that FGF2 induced an accumulation of pERK1/2 in the cytoplasm. We therefore sought to determine the mechanisms underlying the cytoplasmic retention of pERK1/2. Our results indicated that FGF2 promoted the association between ERK and GSK3 β ; the association mostly occurred between ERK and pGSK3 β (Tyr-216) (Fig. 4A), suggesting that ERK1/2 preferably associated with active GSK3 β . To test this hypothesis, we examined the effect of GSK3 β inhibitors on ERK/pGSK3 β (Tyr-216) association. As shown in Fig. 4B, LiCl and TDZD disrupted FGF2-induced ERK/GSK3 β association. A similar result was obtained using another specific GSK3 β inhibitor (L803) (data not shown). On the other hand, the phosphorylation status of ERK did not affect the ERK/GSK3 β association because both ERK and pERK associated with pGSK3 β (Tyr-216) to a similar extent; PD98059 had little effect on pGSK3 β (Tyr-216) as well as ERK/pGSK3 β (Tyr-216) association (Fig. 4B). To further verify the active form of GSK3 β is required for the interaction, we transfected

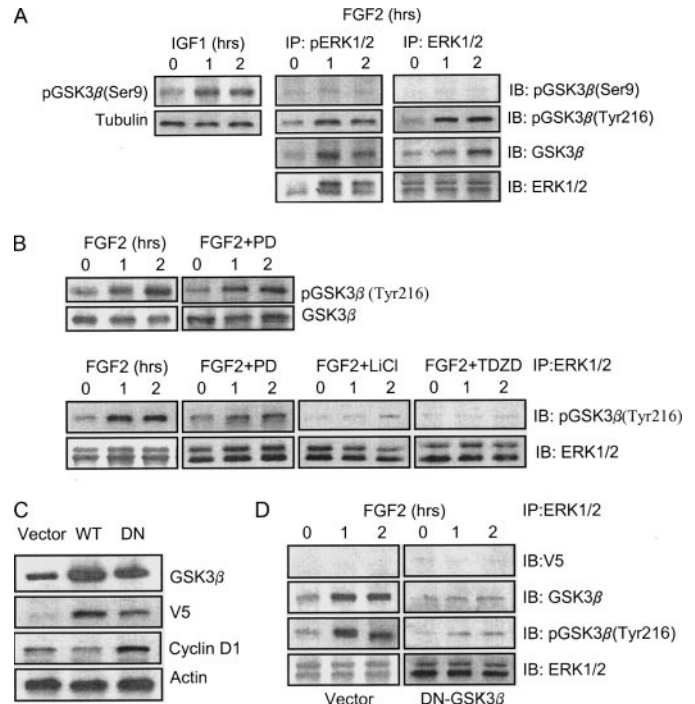


FIGURE 4. FGF2-mediated association between ERK1/2 and GSK3 β . A, SK-N-MC cells were treated with FGF2 (10 ng/ml) for the indicated times. Total protein lysates were collected and immunoprecipitated (IP) with either anti-ERK1/2 or anti-pERK1/2 antibody. The immunoprecipitates were then immunoblotted (IB) for the expression of GSK3 β , pGSK3 β (Ser-9 and Tyr-216), and ERK. IGF1-induced pGSK3 β (Ser-9) served as a positive control. The experiment was replicated three times. B, SK-N-MC cells were pretreated with inhibitors of MEK1 (PD98059, 50 μ M), JNK (JNK-1, 1 μ M), p38 MAPK (SB20358, 10 μ M), and GSK3 β (LiCl, 20 mM; TDZD, 10 μ M) for 30 min. After that, cells were exposed to FGF2 (10 ng/ml) for the indicated times. Cell lysates were immunoprecipitated with an anti-ERK1/2 antibody and immunoblotted using an anti-pGSK3 β (Tyr-216) antibody. The experiment was replicated three times. C, SK-N-MC cells were transfected with either an empty vector or GSK3 β constructs (wild type, WT; dominant negative, DN) for 48 h. The expression of GSK3 β , V5 tag protein, cyclin D1, and actin was examined with immunoblotting. D, SK-N-MC cells were transfected with either an empty vector or a dominant negative GSK3 β construct for 48 h. After that, cells were treated with FGF2 (10 ng/ml) for the indicated times. Cell lysates were immunoprecipitated with an anti-ERK1/2 antibody and immunoblotted for the expression of V5 tag protein, GSK3 β , pGSK3 β (Tyr-216), and ERK1/2. The experiment was replicated three times.

SK-N-MC cells with a kinase-deficient GSK3 β (K85R) mutant. The mutant functions as a dominant negative protein and inhibits the activation of endogenous GSK3 β (20). Overexpression of the K85R mutant in SK-N-MC cells was verified by the expression of the K85R tag protein and high levels of GSK3 β , as well as up-regulation of cyclin D1 (Fig. 4C) because GSK3 β phosphorylates cyclin D1 and induces its degradation (21). In contrast, transfection of wild-type GSK3 β decreased cyclin D1 expression. As shown in Fig. 4D, overexpression of the K85R GSK3 β mutant inhibited FGF2-mediated ERK/GSK3 β association.

Because inhibitors of GSK3 β disrupted ERK/pGSK3 β (Tyr-216) association, we sought to determine their effect on the distribution of pERK1/2. As shown in Fig. 5, A and B, inhibitors of GSK3 β significantly enhanced the nuclear localization of pERK1/2. The K85R GSK3 β mutant failed to associate with ERK. Like GSK3 β inhibitors, overexpression of this mutant greatly enhanced pERK1/2 translocation to the nucleus (Fig. 5, C and D). The role of GSK3 β in the regulation of pERK1/2 distribution was further confirmed by knocking down GSK3 β

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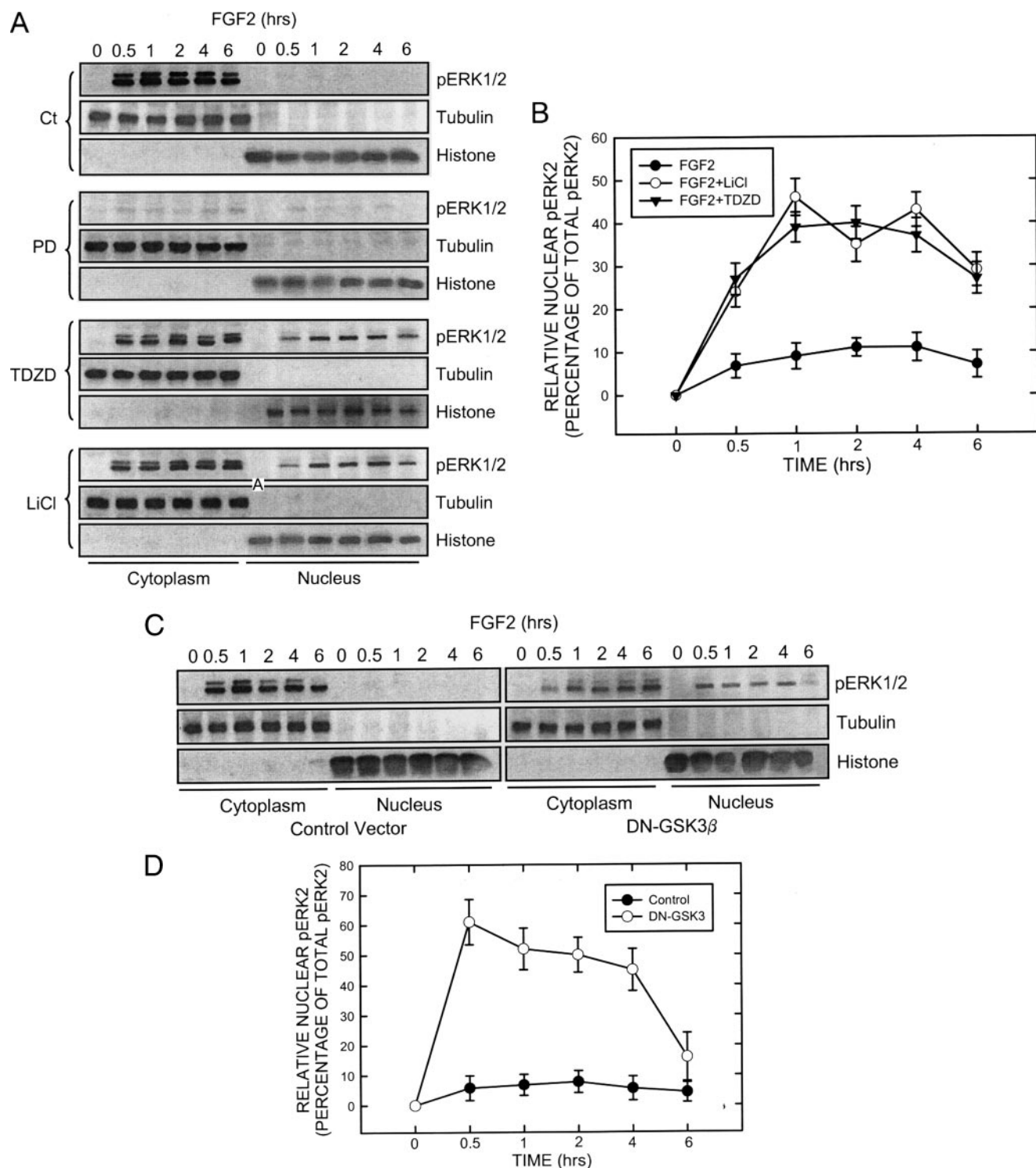


FIGURE 5. Effect of GSK3 β inhibitors and dominant negative (DN) GSK3 β on the distribution of pERK1/2. A, SK-N-MC cells were maintained in serum-free medium overnight and pretreated with inhibitors of MEK1 (PD98059, 50 μ M) and GSK3 β (LiCl, 20 mM; TDZD, 10 μ M) for 30 min. After that, cells were exposed to FGF2 (10 ng/ml) for the indicated times. The expression of phosphorylated ERK (pERK1/2) in both the cytoplasm and nucleus were examined with immunoblotting. B, FGF2-induced up-regulation of pERK2 (42 kDa) was much larger than pERK1 (44 kDa). The relative amounts of pERK2 in the nucleus were measured microdensitometrically, normalized to the level of total cellular pERK2, and expressed as the percentage of total pERK. Each data point (\pm S.E.; bar) is the mean of three replications. C, SK-N-MC cells were transfected with either an empty vector or a dominant negative (DN) GSK3 β construct (kinase-deficient mutant) for 48 h. After that, cells were treated with FGF2 (10 ng/ml) for the indicated times. Cytoplasmic and nuclear protein were collected. The expression of pERK1/2, tubulin, and histone H3 was determined by immunoblotting. D, the relative amounts of pERK2 in the nucleus were measured microdensitometrically as described above. Each data point (\pm S.E.; bar) is the mean of three replications.

using a specific GSK3 β siRNA. As shown in Fig. 6A, GSK3 β siRNA effectively knocked down the expression of GSK3 β . Like GSK3 β inhibitors and the K85R GSK3 β mutant, GSK3 β siRNA

enhanced pERK1/2 translocation to the nucleus (Fig. 6C). Consistent with the protective effect of GSK3 β inhibitors (Fig. 3), overexpression of the K85R GSK3 β mutant (Fig. 7A) and

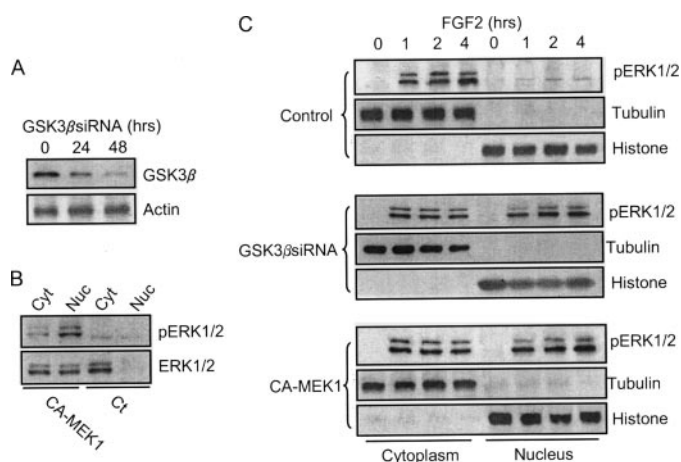


FIGURE 6. Effect of GSK3 β siRNA on the distribution of pERK. *A*, SK-N-MC cells were treated with a GSK3 β siRNA for 24 or 48 h as described under "Experimental Procedures." The expression of GSK3 β was determined with immunoblotting. *B*, SK-N-MC cells were transfected with CA-MEK1 for 48 h. The expression of cytoplasmic (Cyt) and nuclear (Nuc) pERK1/2 and ERK1/2 was determined with immunoblotting. *C*, after being treated with GSK3 β siRNA, control siRNA, or CA-MEK1 for 48 h, SK-N-MC cells were exposed to FGF2 (10 ng/ml) for the indicated times. The expression of cytoplasmic and nuclear pERK1/2 was determined as described above. The experiment was replicated three times.

GSK3 β siRNA (Fig. 7*B*) also significantly ameliorated FGF2-mediated cell death. Contrarily, overexpression of wild-type GSK3 β sensitized SK-N-MC cells to FGF2 cytotoxicity (Fig. 7*A*). To further demonstrate that cytoplasmic pERK1/2 were cytotoxic for SK-N-MC cells, we transfected cells with a CA-MEK1. Because MEK1 had little effect on pGSK3 β (Tyr-216) and bypassed FGF2-initiated signaling, CA-MEK1 expression resulted in pERK localization in the nucleus (Fig. 6, *B* and *C*). It was noted that CA-MEK1 only induced weak pERK expression (Fig. 6*B*). On the other hand, CA-MEK1 did increase pGSK3 β (Ser-9), which resulted in reduced association between GSK and ERK (data not shown); this likely caused pERK nuclear translocation. As shown in Fig. 7*C*, FGF2 cytotoxicity was significantly diminished in cells expressing CA-MEK1. Taken together, FGF2-induced formation of the ERK·GSK3 β complex caused a cytoplasmic retention of pERK, which was cytotoxic to SK-N-MC cells.

Hydrogen Peroxide and Ethanol Potentiate FGF2-induced Cell Death by Modulating ERK/GSK3 β Interaction—To further establish the role of cytoplasmic pERK1/2 in the regulation of cell death, we investigated the effect of hydrogen peroxide on ERK/GSK3 β association and subcellular distribution of pERK because oxidative stress-induced cell death may be mediated by ERK activation (14). We sought to determine whether oxidative stress may promote the death of SK-N-MC cells by retaining pERK in the cytoplasm. Both hydrogen peroxide and ethanol cause cellular oxidative stress. We treated SK-N-MC cells with FGF2 with/without hydrogen peroxide or ethanol and examined the expression of pGSK3 β (Tyr-216), formation of ERK/pGSK3 β (Tyr-216) complex, and subcellular distribution of pERK1/2. As shown in Fig. 8*A*, although hydrogen peroxide or ethanol alone had a modest or little effect on pGSK3 β (Tyr-216), they greatly enhanced FGF2-stimulated pGSK3 β (Tyr-216). Hydrogen peroxide and ethanol also drastically promoted FGF2-induced ERK/pGSK3 β (Tyr-216) association (Fig. 8*B*).

Hydrogen peroxide and ethanol alone did not cause ERK1/2 phosphorylation and alteration in their localization (data not shown). Consistent with our prediction, hydrogen peroxide and ethanol increased FGF2-mediated pERK1/2 in the cytoplasm while decreasing their amount in the nucleus, indicating a promotion of cytosolic retention of pERK1/2 (Fig. 8*C*). As a result, hydrogen peroxide and ethanol potentiated FGF2 cytotoxicity (Fig. 9). Taken together, these results suggested that promotion of the ERK/pGSK3 β (Tyr-216) complex by hydrogen peroxide and ethanol enhanced cytoplasmic retention of pERK1/2 and subsequently potentiated FGF2-induced cell death.

DISCUSSION

This is the first report to show that ERK mediates FGF2-induced death of SK-N-MC cells. We demonstrate that FGF2 causes phosphorylation of ERK1/2 and the phosphorylated ERK1/2 are primarily retained in the cytoplasm. Accumulation of cytosolic pERK1/2 is cytotoxic for SK-N-MC cells.

ERK1/2 belong to a family of MAPKs that is comprised of ERKs, c-Jun N-terminal kinase/stress-activated protein kinases, and p38 kinases. ERK1/2 are the most extensively studied ERKs and are regulated by the dual-specificity kinase MEK1 and MEK2. ERK1/2 are generally stimulated by receptor tyrosine kinases and G protein-coupled receptors; they are also activated in response to various stress stimuli through divergent mechanisms involving the Ras-Raf-MEK pathway (14). Depending upon the cell type, the stimulus and the duration of activation, a variety of biological responses, *i.e.* cell proliferation, survival, migration, differentiation or cell death, are associated with ERK1/2 activation (14).

In resting cells, ERK1/2 are mainly kept in the cytoplasm by the microtubule cytoskeleton, which serves as a major docking matrix (22). Other putative cytosol-anchoring proteins of ERKs include MAP kinase phosphatases (23, 24). ERKs are also retained in the cytosol by their association with MEK1/2 (25). After phosphorylation by MEK1/2, pERK1/2 are detached from the cytosolic anchors and rapidly translocated into the nucleus (26). Once in the nucleus, pERK1/2 phosphorylate various substrates, such as transcription factors, thereby transmitting signals received by cell surface receptors to the nucleus. The translocation of pERK1/2 from the cytoplasm to the nucleus is believed to be mandatory for efficient regulation of cell survival and proliferation (23, 27). However, pERK1/2 may be abnormally retained in the cytoplasm, resulting in low transcription activity, lack of a mitogenic response, or cytotoxicity (15, 28–31). The cytoplasmic retention of pERK1/2 may be caused by forming a complex with other cytosolic proteins. For example, it is demonstrated that pERK1/2 form stable heterotrimeric complexes with the G protein-coupled receptors and β -arrestin2, which are retained in the cytoplasm (15, 28–31).

GSK3 β is a multifunctional serine/threonine kinase. The activation of GSK3 β is regulated by site-specific phosphorylation. Full activity of GSK3 β generally requires phosphorylation at Tyr-216, and conversely, phosphorylation at Ser-9 inhibits GSK3 β activity (19). GSK3 β directly interacts with ERK1/2 (32). In the hepatoma cell line, ERK/GSK3 β association "primes" GSK3 β for its subsequent phosphorylation at Ser-9 by

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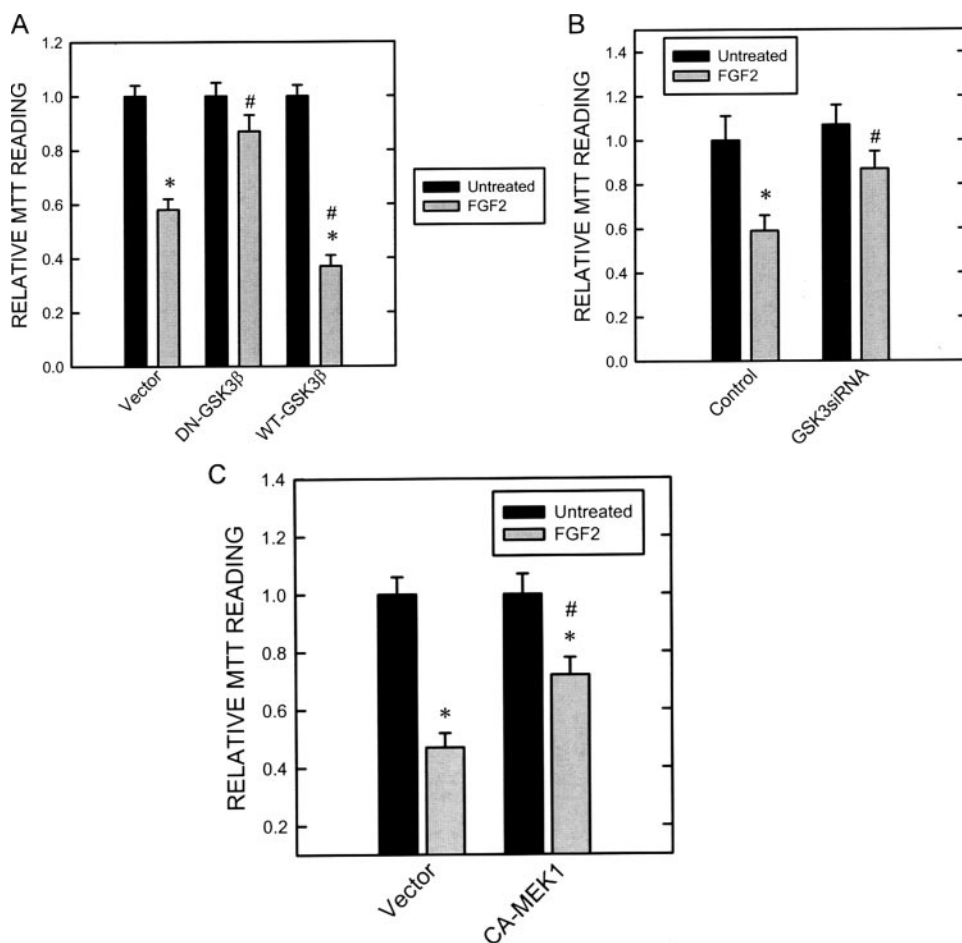


FIGURE 7. Effect of dominant negative (DN), wild-type (WT) GSK3 β , GSK3 β siRNA, and CA-MEK1 on FGF2-induced cell death. A, SK-N-MC cells stably expressing dominant negative or wild-type GSK3 β constructs were established as described under "Experimental Procedures." These cells were treated with FGF2 (0 or 10 ng/ml) for 48 h. Cell viability was determined by MTT as described under "Experimental Procedures." Each data point (\pm S.E.; bar) is the mean of three experiments. Asterisk, FGF2-induced death; denotes significant difference from untreated controls (black bar versus gray bar within group). #, DN-GSK3 β -mediated protection; denotes significant difference from FGF2-exposed cultures in the empty vector-treated group (gray bar in DN-GSK3 β -treated group versus gray bar in empty vector-treated group). B, after transfection with GSK3 β siRNA or control siRNA for 48 h, cells were treated with FGF2 (0 or 10 ng/ml) for 48 h. Cell viability was determined by MTT. C, after transfection with CA-MEK1 or empty vector for 48 h, cells were treated with FGF2 (0 or 10 ng/ml) for 48 h. Cell viability was determined by MTT. Each data point (\pm S.E.; bar) is the mean of three experiments. Asterisk, FGF2-induced cell death (black bar versus gray bar within group). #, GSK3 β siRNA or CA-MEK1 mediate protection (gray bars in GSK3 β siRNA- or CA-MEK1-treated group versus gray bars in empty vector-treated group).

p90RSK (33). In human colon cancer cell lines, ERK/GSK3 β association inhibits ERK activity in a protein kinase C-dependent manner (34). We demonstrate here that ERK1/2 preferably associate with pGSK3 β (Tyr-216) in SK-N-MC cells and inhibitors of GSK3 β disrupt the association, suggesting that an active GSK3 β is required for the association. This argument is further supported by the result showing that a kinase-deficient GSK3 β fails to associate with ERK1/2. FGF2-stimulated ERK/GSK3 β association in SK-N-MC cells is likely mediated by its effect on pGSK3 β (Tyr-216) because FGF2 does not stimulate GSK3 β phosphorylation at Ser-9, but causes its phosphorylation at Tyr-216 (Fig. 2B). Compared with pGSK3 β (Ser-9), regulation of GSK3 β activity by pGSK3 β (Tyr-216) is less common. Stimulation of pGSK3 β (Tyr-216) can be mediated by alterations in intracellular calcium levels or by Fyn, a member of the Src tyrosine family (35, 36). Recent evidence indicates that

pGSK3 β (Tyr-216) is regulated by MEK1/2 (37). FGF2 is shown to regulate intracellular calcium levels and activate Src family kinases including Fyn (38). FGF2 likely activates MEK1/2 in SK-N-MC cells because FGF2-induced pERK1/2 is blocked by PD98059, a MEK1 inhibitor. However, MEK1/2 seem minimally involved in FGF2 regulation of pGSK3 β (Tyr-216) in SK-N-MC cells because PD98059 has little effect on FGF2-stimulated pGSK3 β (Tyr-216) expression.

It appears that ERK1/2-GSK3 β association is important for cytoplasmic retention of pERK1/2 because disruption of the ERK1/2-GSK3 β complex by specific GSK3 β inhibitors, dominant negative protein, or down-regulation of GSK3 β by siRNA promotes nuclear translocation of pERK1/2. In contrast, agents that promote ERK/GSK3 β association, such as hydrogen peroxide and ethanol, retain pERK1/2 in the cytoplasm. Cytoplasmic accumulation of pERK1/2 is toxic for SK-N-MC cells. This is supported by the results showing that forced nuclear localization of pERK1/2 by inhibition of GSK3 β or expression of a CA-MEK1 offer protection against FGF2 cytotoxicity. ERK1/2 are believed to mediate oxidative stress-induced apoptosis (14). Oxidative stress can cause Fyn activation, phosphorylation of GSK3 β at Tyr-216, and disruption of intracellular calcium levels (39–41). Hydrogen peroxide and ethanol are oxidative stress inducers, and they enhance both FGF2-stimulated pGSK3 β (Tyr-216) and ERK/GSK3 β association. As predicted, they potentiate FGF2-induced cytoplasmic retention of pERK1/2 and subsequent cell death. The result further supports the theory that association of ERK1/2 with active GSK3 β results in cytosolic retention of ERK1/2 and promotes cell death. A recent study shows that hydrogen peroxide alone can induce pGSK3 β (Tyr-216) in human Hepa-1 cells (40). Our results indicate that hydrogen peroxide only produces a modest increase in the pGSK3 β (Tyr-216) level, but drastically potentiated FGF2-induced pGSK3 β (Tyr-216) in SK-N-MC cells (Fig. 8). The discrepancy may be due to the use of different model systems.

FGF2 and oxidative stress initiate different signal pathways. pGSK3 β (Tyr-216) appears to be a converging point for FGF2 and oxidative stress-mediated signaling, and therefore mediates the interaction between FGF2 and oxidative stress. Oxida-

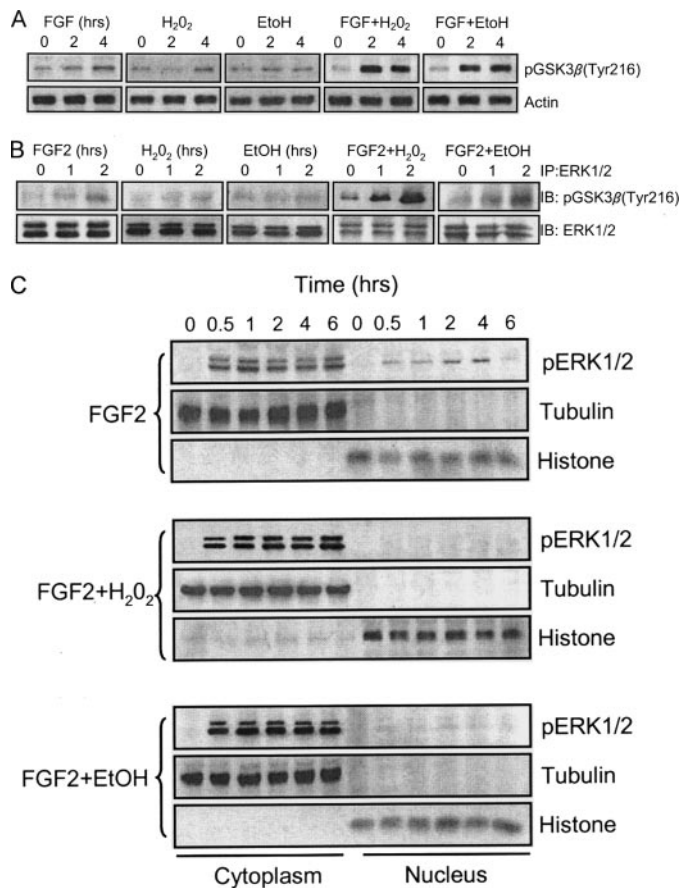


FIGURE 8. Effect of hydrogen peroxide and ethanol on FGF2-mediated ERK/GSK3 β interaction and pERK1/2 distribution. *A*, SK-N-MC cells were treated with FGF2 (10 ng/ml) with/without hydrogen peroxide (50 μ M) or ethanol (400 mg/dl) for the indicated times. The expression of pGSK3 β (Tyr-216) was examined with immunoblotting as described above. *B*, SK-N-MC cells were treated with FGF2 (10 ng/ml) with/without hydrogen peroxide (50 μ M) or ethanol (400 mg/dl) for the indicated times. Total protein lysates were immunoprecipitated (IP) with anti-ERK1/2 antibody and immunoblotted (IB) for the expression of pGSK3 β (Tyr-216) and ERK1/2. The experiment was replicated three times. *C*, SK-N-MC cells were treated with FGF2 (10 ng/ml) with/without hydrogen peroxide (50 μ M) or ethanol (400 mg/dl) for the indicated times. The distribution of pERK1/2 was determined as described above. The experiment was replicated three times.

tive stress potentiates the effect of FGF2 by enhancing pGSK3 β (Tyr-216) expression. It is currently unclear why active GSK3 β is required for ERK/GSK3 β interaction. It cannot be excluded that other cytoplasmic proteins may be part of the complex; an active GSK3 β may be necessary for recruiting these proteins for the complex formation. The investigation of this mechanism is currently underway. Our finding that ERK/GSK3 β association causes cytosolic retention of pERK1/2 provides additional insight into the regulation of the function of ERK. Similar to FGF2, dopamine or agonists for dopamine receptor cause ERK1/2-dependent apoptosis of SK-N-MC cells (15). The majority of pERK1/2 stimulated by dopamine or dopamine receptor agonist is retained in the cytoplasm and forms a stable heterotrimeric complex with D1 dopamine receptor and β -arrestin2. In contrast, forskolin, which induces pERK1/2 localization in the nucleus, enhances survival of SK-N-MC cells (15) (Fig. 1).

The mechanisms of cytosolic pERK1/2-induced cell death are unclear. If abnormally retained in the cytoplasm, pERKs

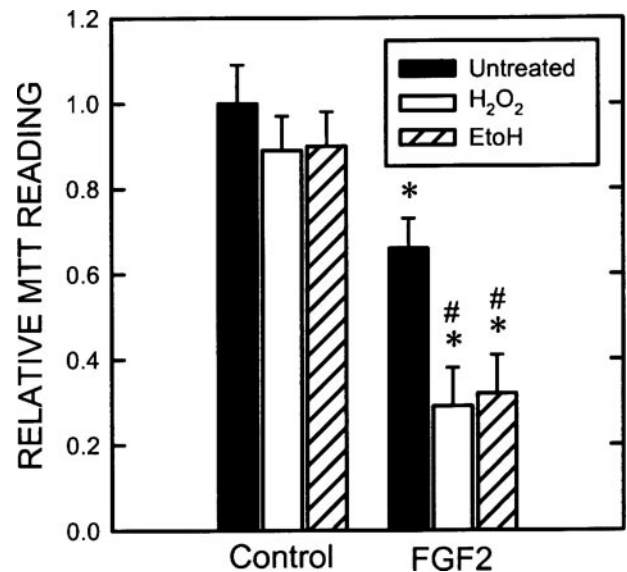


FIGURE 9. Effect of hydrogen peroxide and ethanol on FGF2-mediated death. SK-N-MC cells were treated with FGF2 (10 ng/ml) with/without hydrogen peroxide (50 μ M) or ethanol (400 mg/dl) for 48 h. Cell viability was determined by MTT as described under "Experimental Procedures." The experiment was replicated three times. Asterisk, FGF2-induced cell death; denotes significant difference from untreated control (black bar versus black bar). #, the potentiation of hydrogen peroxide or ethanol on cell death; denotes significant difference from the group treated with FGF2 but without hydrogen peroxide and ethanol.

may phosphorylate multiple proteins in plasma membranes as well as cytoplasmic and cytoskeletal substrates (42), resulting in inappropriate cellular events. For example, cytosolic ERK may directly target mitochondria and interfere with mitochondrial functions that cause the release of cytochrome *c* and subsequent caspase activation and apoptosis (43–45). Cytosolic pERK1/2 may suppress survival signaling, such as the phosphatidylinositol 3-kinase/AKT pathway, and promote cell death (46). The significance of cytosolic retention of pERKs is underscored by findings in postmortem tissues of neurodegenerative diseases where neuronal inclusion bodies were found to contain substantially high levels of aggregated pERK1/2. pERK1/2 were found to be localized in the cytoplasm and not in the nucleus of degenerating neurons of Parkinson disease, Alzheimer disease, and Pick disease (47–49). GSK3 β is believed to play an important role during neurodegeneration (32). Our study, which shows that ERK/GSK3 β association causes cytosolic retention of pERK, provides new insight into mechanisms of GSK3 β -mediated neurotoxicity.

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