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# Grape seed extract inhibits EGF-induced and constitutively active mitogenic signaling but activates JNK in human prostate carcinoma DU145 cells: possible role in antiproliferation and apoptosis

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**A loss of functional androgen receptor and an enhanced expression of growth factor receptors and associated ligands are causal genetic events in prostate cancer (PCA) progression. These genetic alterations lead to an epigenetic mechanism where a feedback autocrine loop between membrane receptor and ligand (e.g. EGFR–TGFα) results in a constitutive activation of MAPK-Elk1–AP1-mediated mitogenic signaling in human PCA at an advanced and androgen-independent stage. We rationalized that inhibiting these epigenetic events could be useful in controlling advanced PCA growth. Recently, we found that grape seed extract (GSE), a dietary supplement rich in flavonoid procyanidins, inhibits advanced and androgen-independent human PCA DU145 cell growth in culture and nude mice. Here, we performed detailed mechanistic studies to define the effect of GSE on EGFR–Shc–MAPK–Elk1–AP1-mediated mitogenic signaling in DU145 cells. Pretreatment of serum-starved cells with GSE resulted in 70% to almost complete inhibition of EGF-induced EGFR activation and 50% to complete inhibition of Shc activation, which corroborated with a comparable decrease in EGF-induced Shc binding to EGFR. Conversely, EGF-induced ERK1/2 phosphorylation was inhibited only by lower doses of GSE; in fact, higher doses showed an increase. Additional studies showed that GSE alone causes a dose- and time-dependent increase in ERK1/2 phosphorylation in starved DU145 cells that is inhibited by an MEK1 inhibitor PD98059. Independent of this increase in ERK1/2 phosphorylation, GSE showed a strong inhibition of ERK1/2 kinase activity to Elk1 in both cellular and cell-free systems. GSE treatment of cells also inhibited both EGF-induced and constitutively active Elk1 phosphorylation and AP1 activation. GSE treatment also showed DNA synthesis inhibition in starved and EGF-stimulated cells as well as loss of cell viability and apoptotic death that was further increased by adding MEK1 inhibitor. Since GSE strongly induced apoptosis independent of its affect on an increase in phospho-ERK1/2, we hypothesized that apoptotic effect of GSE could be by other mechanism(s) including its effect on stress-associated MAPK, the JNK. Indeed, GSE-treated cells showed a strong and sustained increase in phospho-JNK1/JNK2 levels, JNK activity and phospho-cJun levels. An inhibition of GSE-induced JNK activation by a novel JNK inhibitor SP600125 resulted in a significant reversal of GSE-induced apoptotic death suggesting the involvement of JNK activation by GSE in its apoptosis response. Together, these results suggest that anticancer effects of GSE in PCA be mediated via impairment of EGFR–ERK1/2–Elk1–AP1-mediated mitogenic signaling and activation of JNK causing growth inhibition and apoptosis, respectively.**
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**Keywords:** prostate cancer; MAPK; AP1; apoptosis; grape seed extract

## Introduction

Prostate cancer (PCA) is one of the most common malignancies and the second leading cause of cancer-related deaths in men (Greenlee *et al*., 2001). The early stages of PCA growth are androgen dependent and are generally responsive to androgen ablation therapy. However, following such therapy, the disease almost invariably progresses to an androgen-independent state, rendering androgen ablation therapy ineffective (Harris and Reese, 2001). Several different molecular alterations causally contribute to androgen independence in PCA. The one that has received extensive attention is an aberrant expression and interaction, via an autocrine loop, of receptor tyrosine kinases and associated ligands, such as epidermal growth factor receptor (EGFR) and its ligands epidermal growth factor (EGF) and transforming growth factor α (TGFα) (Agarwal, 2000). EGF is one of the most important mitogens for many epithelial cells, and typically promotes cell proliferation through a well-characterized EGFR–Shc–Grb2/SOS–Ras–Raf–ERK pathway (Levitzki and Gazit, 1995; Zi *et al*., 1998). EGFR is a ligand-activated receptor tyrosine kinase that ultimately transduces mitogenic signals to the nucleus via mitogen-activated protein kinases (MAPK), which are well

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recognized to play an important role in orchestrating intracellular events essential for cell functioning and growth, and apoptosis (Ullrich and Schlessinger, 1990). At least three MAP kinases subgroups have been identified in MAPK super family that include ERK (extracellular signal-regulated kinase), JNK (c-*jun* N-terminal protein kinase, also known as stress-activated protein kinase, SAPK), and p38 (Jiang *et al*., 1996). Since control of MAP kinase activities is essential for normal cell growth and differentiation, disruption in MAPK signaling pathway leads to pathophysiological changes including oncogenesis and apoptosis (Clarke, 1994). With regard to PCA, several recent studies have shown constitutively active mitogenic and cell survival signaling in human PCA cells as well as tumor tissue (Gioeli *et al*., 1999; Palayoor *et al*., 1999; Agarwal, 2000; Zi *et al*., 2000).

Since there is no cure for advanced and androgen-independent PCA and its metastasis, research directed towards development of new treatments for this malignancy is imperative. The need for new agents with novel mechanism of action to prevent cancer is possibly one of the most important areas of chemoprevention research. In this regard, use of microchemicals present in diet and in several herbs and plants are gaining widespread attention for the prevention and or/intervention of various cancers (Sporn and Suh, 2000). Evidences from experimental and epidemiological studies have already established a link between consumption of certain diets or diet-related constituents and prevention of cancer (Wattenberg, 1997). Among these, flavonoids and isoflavones are receiving increased attention in recent years (Dragsted, 1998; Agarwal *et al*., 2000). Grape seed extract (GSE) is one such naturally occurring class of chemicals that is rich in procyanidins; a diverse group of flavonoid compounds also present in several fruits and vegetables (Escribano-Bailon *et al*., 1992; Bartolome *et al*., 1996). Wine may also be a rich source of procyanidins (Halpern *et al*., 1998), and consumption of, particularly, red wine is shown to impart greater benefits in the prevention of coronary heart disease than other alcoholic beverages (Sato *et al*., 2001).

In several of our recent studies, we found that GSE exerts preventive effects against skin cancer (Zhao *et al*., 1999), and inhibits the growth and induces apoptotic death of DU145 cells in culture (Agarwal *et al*., 2000) and nude mice tumor xenografts (unpublished data). Accordingly, here we performed detailed mechanistic studies to define antiproliferative and apoptotic effects of GSE on DU145 cells. Our results clearly show that anticancer effects of GSE in PCA DU145 cells are mediated at least in part via inhibition of EGFR–ERK1/2–Elk1–AP1-mediated mitogenic signaling and activation of JNK causing growth inhibition and apoptosis, respectively. These findings suggest a new intervention strategy for PCA by GSE exploring its dual mechanisms of action.

## Results

### *Effect of GSE on EGF-induced EGFR and Shc activation as well as EGFR–Shc binding*

Using DU145 cells, we first assessed the effect of GSE on EGF-caused EGFR activation. Serum starvation of cells for 24 h almost completely diminished EGFR activation as confirmed by a very low reactivity of immunoprecipitated EGFR with antiphosphotyrosine antibody (Figure 1a). Treatment of serum-starved cells with EGF caused a strong activation of EGFR; however, pretreatment of 24 h starved DU145 cells with GSE (10, 25, 50 and 75 $\mu$g/ml) for 2 h followed by EGF (50 ng/ml) stimulation for 30 min resulted in a strong inhibition (70% to almost complete) of EGF-induced

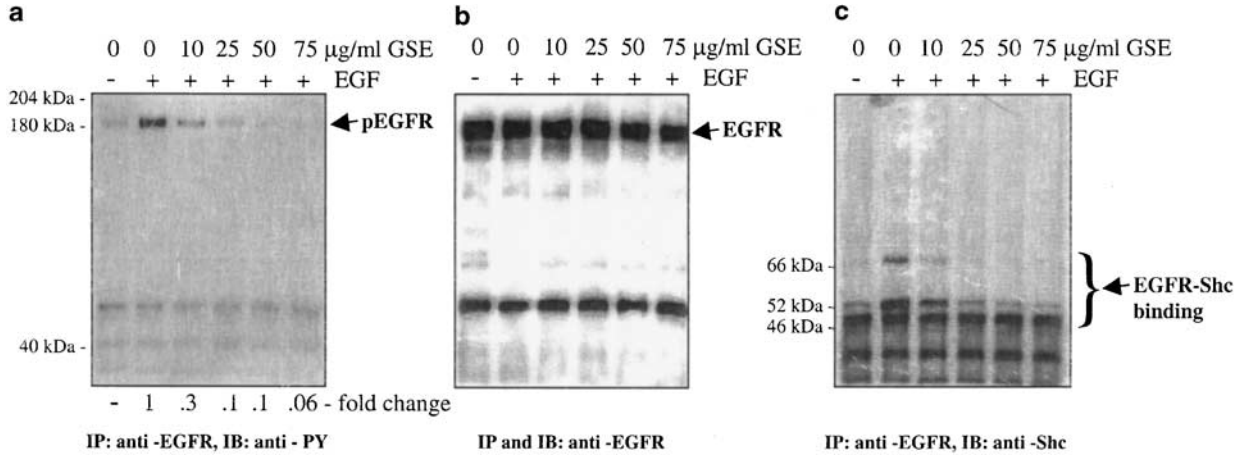


**Figure 1** Effect of GSE on EGF-induced EGFR activation and EGFR–Shc binding in DU145 cells. Cells were grown to 70% confluency and serum starved for 24 h, followed by either no treatment, EGF alone (50 ng/ml) for 30 min, or indicated doses of GSE for 2 h followed by EGF stimulation. Cell lysates were prepared, EGFR was immunoprecipitated using anti-EGFR antibody, and following SDS–PAGE and Western blotting, membranes were probed with anti-phosphotyrosine (**a**), anti-EGFR (**b**) or anti-Shc (**c**) antibody followed by peroxidase-conjugated appropriate secondary antibody and visualization by ECL detection system

EGFR activation (Figure 1a). The observed inhibitory effect of GSE on EGF-induced EGFR activation was not due to a decrease in total EGFR protein level (Figure 1b).

One of the immediate downstream substrates to EGFR activation is Shc, which acts as an adaptor protein between EGFR and other SH2 containing proteins in EGFR-mediated mitogenic signaling involving Grb2/SOS/Ras/Raf/ERK pathway (Levitzki and Gazit, 1995; Zi *et al*., 1998). Based on observed inhibitory effect of GSE on EGF-induced EGFR activation, next we assessed its effect on EGF-induced Shc activation. Serum starvation of DU145 cells almost completely diminished phosphorylated levels of 66 and 52 kDa, but not 46 kDa, Shc isoforms, and treatment of starved cells with EGF showed a strong activation (phosphorylation) of both 66 and 52 kDa Shc isoforms and a modest activation of 46 kDa isoform of Shc (Figure 2a). Similar to EGFR results, pretreatment of starved cells with GSE for 2 h showed a strong and dose-dependent decrease in EGF-stimulated tyrosine phosphorylation of both 66 and 52 kDa Shc isoforms; there was also a modest decrease in the tyrosine phosphorylation of 46 kDa Shc isoform following GSE treatment (Figure 2a). Compared to EGF-treated control, GSE treatment resulted in 50% to complete and 40–90% inhibition in EGF-caused tyrosine phosphorylation of 66 and 52 kDa Shc isoforms, respectively (Figure 2a), which was not due to a change in the overall Shc protein levels following GSE treatments (Figure 2b). Since EGFR activation followed by its binding with Shc is important for the activation of downstream signaling pathway (Rozakis-Adcock *et al*., 1992), we also assessed the effect of GSE on EGF-induced EGFR–Shc binding. As shown in Figures 1c and 2c, GSE pretreatment also resulted in comparable inhibition in EGF-induced binding of EGFR to both 66 and 52 kDa isoforms of Shc (Figure 1c) or an overall binding of Shc to EGFR (Figure 2c). Together, these results suggest that GSE inhibits EGFR activation that leads to a decrease in Shc binding to EGFR followed by an inhibition of Shc activation.

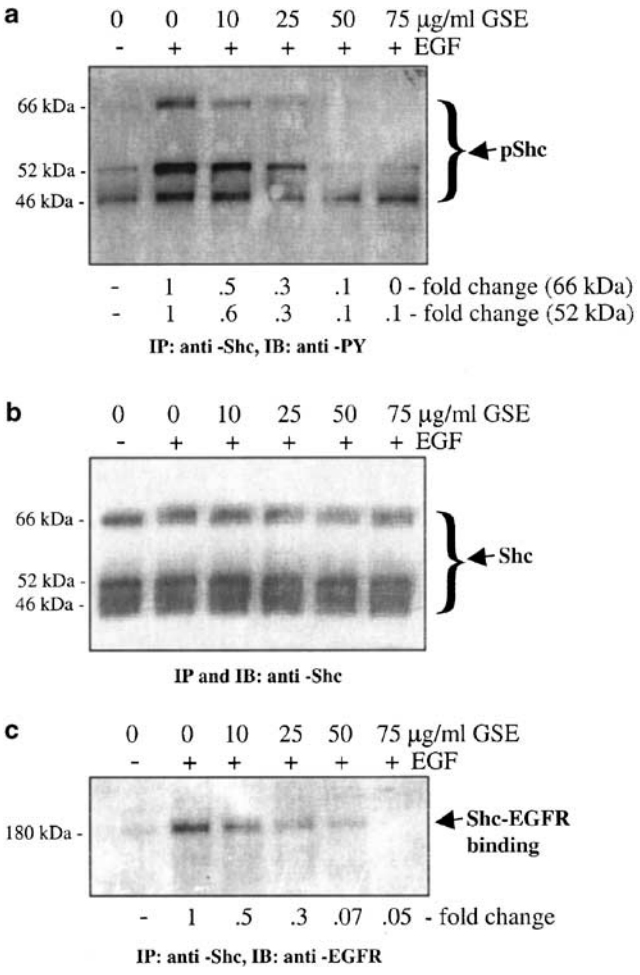


**Figure 2** Effect of GSE on EGF-induced Shc activation and Shc–EGFR binding in DU145 cells. Cells were grown to 70% confluency and serum-starved for 24 h, followed by either no treatment, EGF alone (50 ng/ml) for 30 min, or indicated doses of GSE for 2 h followed by EGF stimulation. Cell lysates were prepared, Shc was immunoprecipitated using anti-Shc antibody, and following SDS–PAGE and Western blotting, membranes were probed with antiphosphotyrosine (**a**), anti-Shc (**b**) or anti-EGFR (**C**) antibody followed by peroxidase-conjugated appropriate secondary antibody and visualization by ECL detection system

*Effect of GSE on EGF-induced ERK1/2 activation and kinase activity, and Elk1-AP1 activation*

EGF-induced activation of EGFR ultimately activates MAPK/ERK1/2, which translocate to the nucleus and activate transcription factors for cell growth and proliferation (Goldman *et al*., 1990; Cohen, 1997; Grasso *et al*., 1997). Based on inhibitory effect of GSE on EGF-induced EGFR and Shc activation, we next investigated whether GSE impairs ERK1/2-mediated mitogenic signal transduction pathway. Identical treatments of serum-starved DU145 cells with EGF without or with GSE were done, and cell lysates were examined by Western immunoblotting with antibodies that recognize either phosphorylated or total forms of ERK1/2. As shown in Figure 3a (upper panel), 24 h of serum starvation completely diminished phospho-ERK1/2 levels, and EGF treatment of starved cells caused a strong activation of ERK1/2. However, pretreatment of starved cells with different doses of GSE caused a strong inhibition in EGF-induced ERK1/2 phosphorylation only at low doses; an increase in GSE doses started showing a reversal of the observed inhibition at low doses, and the highest dose examined (75 $\mu$g/ml) showed an increase in phospho-ERK1/2 levels compared to EGF alone positive control (Figure 3a, upper panel). The observed changes in phospho-ERK1/2 levels in this experiment were not because of an overall change in total ERK1/2 protein level (Figure 3a, lower panel).

Based on the data showing GSE does not inhibit EGF-induced ERK1/2 phosphorylation to the same

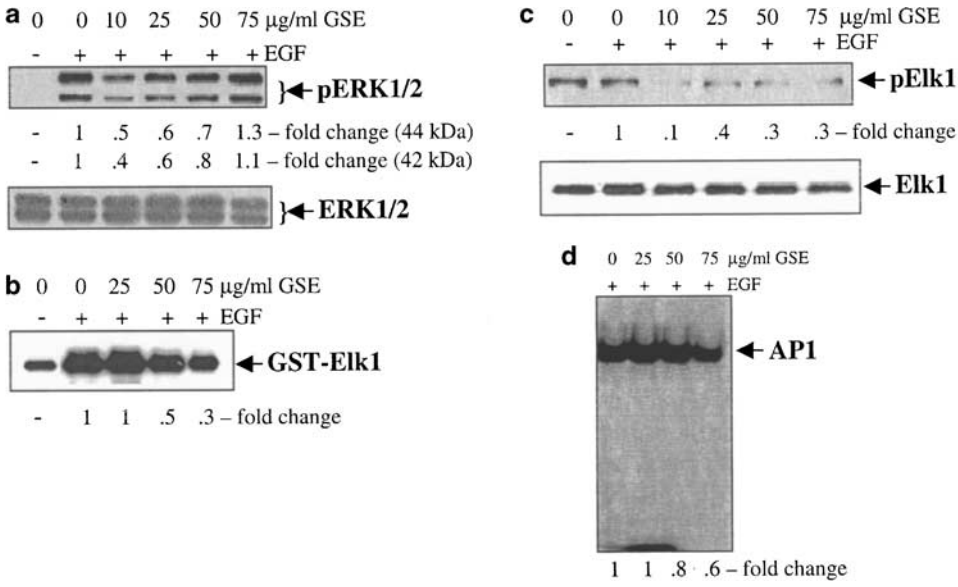


**Figure 3** Effect of GSE on EGF-induced ERK1/2–Elk1–AP1 activation in DU145 cells. Cells were grown to 70% confluency and serum starved for 24 h, followed by either no treatment, EGF alone (50 ng/ml) for 30 min, or indicated doses of GSE for 2 h followed by EGF stimulation, and cell lysates and nuclear fractions were prepared. Equal amounts of protein from cell lysates were resolved on 12% SDS–PAGE followed by Western blotting, and membranes were probed with antiphospho-ERK1/2 or total ERK1/2 antibody (**a**) followed by peroxidase-conjugated appropriate secondary antibody and visualization by ECL detection system. ERK1/2 kinase activity to Elk1 (**b**) was determined by immunoprecipitating active ERK1/2 with monoclonal phospho-ERK1/2 antibody, followed by incubation with GST-Elk1 protein in the presence of ATP and kinase buffer, and phosphorylation of Elk1 was measured by Western blotting using antiphospho-Elk1 antibody. To analyse phospho- and total Elk1 levels (**c**), equal amounts of protein from nuclear extracts were resolved on 12% SDS–PAGE followed by Western blotting, and membranes were probed with antiphospho-Elk1 or anti-Elk1 antibody followed by peroxidase-conjugated appropriate secondary antibody and visualization by ECL detection system. In the studies assessing effect of GSE on EGF-induced AP1 activation (**d**), EMSA was performed using nuclear extract as described in Materials and methods. Gel was then dried and subjected to autoradiography

intensity as EGFR–Shc activation at most of the doses examined, we next assessed whether it has similar lack of effect on ERK1/2 kinase activity to Elk1. As shown in Figure 3b, sufficient ERK1/2 kinase activity was evidenced even in 24 h serum-starved DU145 cells that is consistent with a recent study showing a constitutively active ERK1/2 in DU145 cells (Putz *et al*., 1999; Segawa *et al*., 2001). However, treatment of starved cells with EGF resulted in a strong increase in ERK1/2 kinase activity to Elk1 (Figure 3b). Interestingly, in contrast to ERK1/2 phosphorylation data, pretreatment of starved cells for 2 h with different doses of GSE followed by EGF stimulation resulted in a significant inhibition of ERK1/2 kinase activity to Elk1 in a dose-dependent manner, which accounted for almost 70% decrease at 75 $\mu$g/ml GSE dose (Figure 3b). It is important to re-emphasize here that this dose of GSE, in fact, showed an increase in EGF-induced ERK1/2 phosphorylation (Figure 3a, upper panel).

After phosphorylation, ERK1/2 translocate from the cytoplasm to the nucleus, where they phosphorylate various transcription factors including Elk1 (Liu *et al*., 2001). The transcription factor Elk1 is a substrate for ERK1/2, and phosphorylation at several COOH terminal sites, including serine 383, is critical for its transcriptional potential (Marais *et al*., 1993; Whitmarsh *et al*., 1995). Accordingly, we next assessed the effect of GSE on EGF-induced Elk1 phosphorylation. Similar to a constitutive kinase activity of ERK1/2 to Elk1, 24 h serum-starved DU145 cells showed high levels of phospho-Elk1, and EGF stimulation of starved cultures did not show any further increase in phospho-Elk1 protein levels (Figure 3c, upper panel). However, pretreatment of starved cells with different doses of GSE resulted in a strong decrease in phospho-Elk1 protein levels (Figure 3c, upper panel); higher doses of GSE (50 and 75 $\mu$g/ml) showed $\sim$70% decrease. Since EGF stimulation did not show an increase in constitutive phospho-Elk1 protein levels, it was not possible to dissect whether the observed decrease by GSE was that of constitutive or EGF-mediated phospho-Elk1 protein levels; the densitometric analysis data shown, however, are relative to the EGF-treated sample (Figure 3c, upper panel). The observed changes in phospho-Elk1 protein levels were not because of an overall alteration in total Elk1 protein level (Figure 3c, lower panel).

Transcription factor, AP1, is an immediate downstream target of Elk1 activation, and in fact, is the most important component of EGFR-ERK1/2-Elk1-mediated mitogenic signaling in activating the transcription of genes involved in cell growth and proliferation (Karin *et al*., 1997; Mukhopadhyay *et al*., 2001). Accordingly, based on the results discussed above, we also examined the effect of GSE on EGF-induced AP1-DNA binding activity in DU145 cells. As shown in Figure 3d, EGF treatment of 24 h serum-starved cells

resulted in a strong activation of AP1; however, pretreatment of cells with GSE caused a 20–40% inhibition of EGF-induced AP1 activation. In the studies analysing the specificity of AP1 band, addition of unlabeled AP1 probe resulted in a decrease in AP1 specific band (data not shown). In other assay systems to determine that AP1 band in gel-shift assay is indeed the case, nuclear extracts were first incubated with anti-cJun or anti-cFos antibody followed by EMSA, which showed a strong super-shift in both cases to higher molecular weight bands suggesting that the observed AP1 band consists of these two subunits (data not shown). The contradictory effects of GSE in terms of an increase in phospho-ERK1/2, but an inhibition of ERK1/2 kinase activity to Elk1 as well as Elk1 and AP1 activation, were further assessed in detail employing starved cells without EGF stimulation.

### *Effect of GSE on EGFR–Shc–ERK1/2–Elk1–AP1 activation in serum-starved DU145 cells*

Based on the data discussed above showing that GSE strongly inhibits EGF-induced EGFR–Shc–Elk1–AP1 activation, but not ERK1/2 phosphorylation to the similar magnitude, next we assessed the effect of GSE alone on this mitogenic signaling cascade in serum-starved DU145 cells. There were two rationales for this experiment. First, in another study by us, we showed that treatment of serum-starved DU145 cells with epigallocatechin-3-gallate, an active compound in green tea, causes a strong increase in ERK1/2 phosphorylation via an oxidative response involving production of hydrogen peroxide (Bhatia and Agarwal, 2001). Production of hydrogen peroxide by epigallocatechin-3-gallate is also reported in lung epithelial cells as a chemical mechanism of its apoptotic activity (Yang *et al*., 1998). Since GSE is rich in procyanidins that are monomer to polymers of catechins and epicatechins, the basic structure of epigallocatechin-3-gallate (Zhao *et al*., 1999), it was reasoned that a similar mechanism could be operational in this case. Secondly, we realized that since this mitogenic signaling pathway is constitutively active in DU145 cells (Putz *et al*., 1999; Segawa *et al*., 2001), it is equally important to define the inhibitory effect of GSE on constitutive activation of different signaling molecules in this cascade.

First we assessed the effect of GSE alone on EGFR and Shc activation in serum-starved DU145 cells. Similar GSE doses and treatment periods did not result in EGFR and Shc activation (data not shown). However, as shown in Figure 4a (upper panel), GSE treatment of starved DU145 cells for only 2.5 h (the treatment time of GSE + EGF study) resulted in a robust increase in phospho-ERK1/2 levels in a dose-dependent manner without any noticeable change in total ERK1/2 levels (Figure 4a, lower panel). In the studies assessing whether the observed increase in phospho-ERK1/2 by GSE is transient or sustained, starved cells were either treated with vehicle or 50 $\mu$g/ml dose of GSE for different time periods, and total cell lysates were analysed for phospho-ERK1/2 levels. As shown in Figure 4b, GSE treatment resulted in an

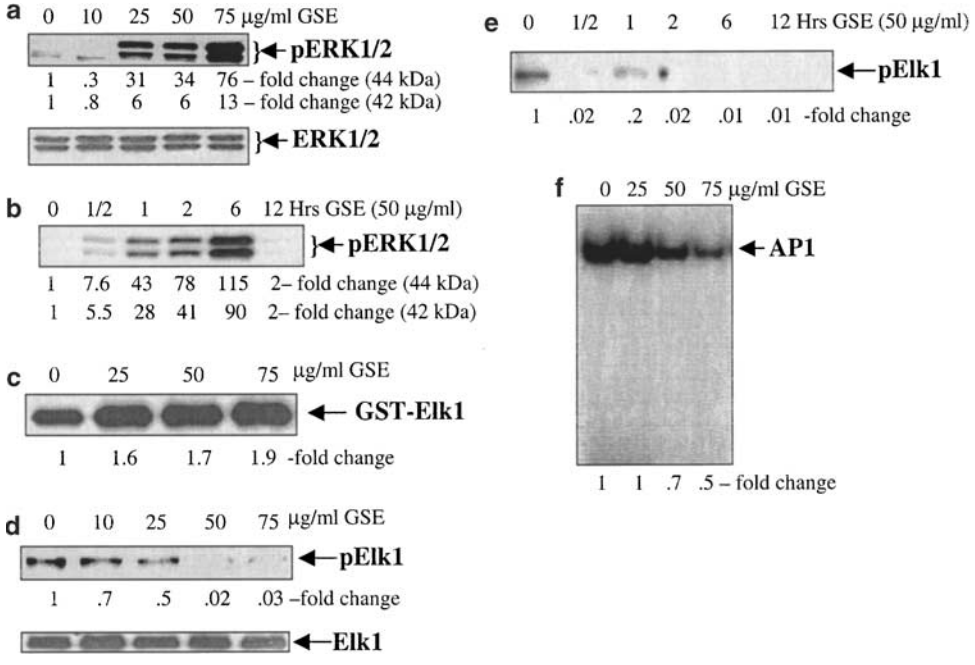


**Figure 4** Effect of GSE on ERK1/2–Elk1–AP1 activation in serum-starved DU145 cells. Cells were grown to 70% confluency and serum starved for 24 h followed by treatment with indicated doses of GSE for 2.5 h. Total cell lysates and nuclear fractions were then prepared and analysed for phospho- and total ERK1/2 (**a**), ERK1/2 kinase activity to Elk1 (**c**), phospho- and total Elk1 levels (**d**) and AP1 activation (**f**) as detailed in Materials and methods. To analyse the time kinetics of GSE-induced ERK1/2 phosphorylation and a decrease in phospho-Elk1, starved cells were treated with 50 $\mu$g/ml GSE, and total cell lysates and nuclear extracts were analysed for phospho-ERK1/2 (**b**) and phospho-Elk1 (**e**) levels, respectively, at the indicated times following GSE treatments by immunoblotting as detailed in Materials and methods

increase in ERK1/2 phosphorylation as early as 30 min of treatment. The maximum increase, however, was observed following 6 h of GSE treatment that returned to the control levels by 12 h after GSE treatment (Figure 4b). There was no change in phospho-ERK1/2 levels in vehicle treated cells during all the time points of 0.5–12 h (data not shown for all these time points; only 0.5 h control shown in Figure 4b). The observed increase in phospho-ERK1/2 was not due to an overall change in total ERK1/2 levels (data not shown). In terms of ERK1/2 kinase activity to Elk1, interestingly only 1.6–1.9-fold increase was evidenced in GSE-treated starved cells as compared to DMSO-treated control (Figure 4c). However, treatment of starved cells with different doses of GSE resulted in almost complete decrease in constitutive phospho-Elk1 level in a dose-dependent manner without any noticeable change in total Elk1 protein (Figure 4d). GSE treatment of starved cells also showed a time-dependent decrease in phospho-Elk1 protein levels, where all time points studied showed almost complete decrease (Figure 4e). The effect of GSE on phospho-Elk1 in serum-starved DU145 cells was also consistent with a strong inhibition of constitutive AP1 activation by GSE at 50 and 75 $\mu$g/ml doses (Figure 4f).

### *GSE induces ERK1/2 phosphorylation via MEK1, but directly inhibits kinase activity of ERK1/2 to Elk1 in DU145 cells*

The above results raised two important questions: (1) whether GSE directly induces ERK1/2 phosphorylation or it is because of an upstream effect, and (2) why GSE inhibits activation of Elk1-AP1 though induces phospho-ERK1/2? To answer the first question, we focused our attention on MEK1, an immediate upstream effector for ERK1/2 activation. Serum-starved cells were first treated with MEK1 inhibitor PD98059 followed by GSE. As shown in Figure 5a (upper panel), compared to starved cells, PD98059 did not show any change in phospho-ERK1/2 levels, but GSE treatment caused a strong increase that is completely blocked by PD98059 providing convincing evidence that the observed increase in phospho-ERK1/2 by GSE is because of an activation of MEK1. In other studies, PD98059 also showed inhibition of both EGF and GSE + EGF-induced phospho-ERK1/2 levels (Figure 5a). Together, the above data suggest that independent of EGFR–Shc involvement, GSE activates MEK1 in starved DU145 cells, which though strongly induces phospho-ERK1/2 levels causes only a mild increase in ERK1/2 kinase activity. Whereas an exact nature of this effect of GSE on phospho-ERK1/2 induction remains to be established, it does not activate downstream effectors in ERK1/2 signaling pathway. In fact, GSE showed a strong inhibition of constitutively active phospho-Elk1 and AP1. These observations raised two possibilities, first whether the assay conditions in ERK1/2 kinase activity were optimum, and secondly whether GSE directly impairs ERK1/2 kinase activity resulting in inhibition of Elk1 and AP1 activation. In order to

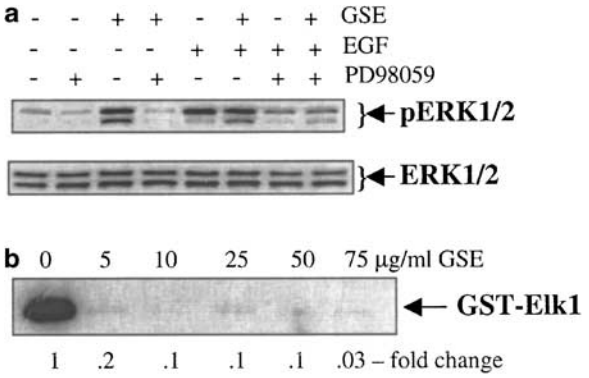


**Figure 5** GSE-caused increase in ERK1/2 phosphorylation occurs via MEK1 in serum-starved DU145 cells, and GSE directly inhibits ERK1/2 kinase activity to Elk1 in a cell-free system. To analyse whether GSE-induced phosphorylation of ERK1/2 is via an activation of MEK1 (**a**), serum-starved cells were treated with PD98059 (50 $\mu$M), GSE (50 $\mu$g/ml) and/or EGF (50 ng/ml) as described in Materials and method. Total cell lysates were then subjected to SDS–PAGE and Western blotting, and phospho- and total ERK1/2 levels determined using appropriate primary antibody followed by peroxidase-conjugated secondary antibody and ECL detection. To assess whether GSE directly inhibits ERK1/2 kinase activity to Elk1 (**b**), a cell-free system was used where active ERK1/2 was immunoprecipitated from serum-starved untreated DU145 cells, and indicated doses of GSE were added in kinase assay incubation containing GST-Elk1 protein, ATP and kinase buffer. Phosphorylation of Elk1 was measured by Western blotting using antiphospho-Elk1 antibody

address optimum assay conditions, we did several additional kinase assays where varying total protein lysates, amount of primary antibody, amount of GST-Elk1 substrate, amount of ATP in kinase assay, or the exposure times of the blot to film were varied. These assays with several permutation combinations employed clearly showed that a lack of increase in kinase activity to Elk1 by GSE (corroborative to a robust increase in phospho-ERK1/2) is not because of either of the above-assessed factors in the kinase assay (data not shown). We next conducted the study to assess the direct effect of GSE on impairing ERK1/2 kinase activity. The active ERK1/2 was immunoprecipitated from the total cell lysate prepared from control cells and GSE was added *in vitro* into the kinase assay. As shown in Figure 5b, GSE showed a direct effect on inhibiting ERK1/2 kinase activity towards Elk1. As low as 5 $\mu$g/ml GSE dose caused 80% inhibition of kinase activity; higher doses resulted in 90% and complete inhibition of ERK1/2 kinase activity (Figure 5b). These data provide convincing evidence that GSE has multiple effects on mitogenic signaling, for example, it inhibits EGF-caused EGFR–Shc–ERK1/2 activation, induces ERK1/2 phosphorylation via MEK1, and directly inhibits ERK1/2 kinase activity to Elk1 that is possibly involved in GSE-caused inhibition of Elk1 and AP1 activation.

### *Effect of GSE on DNA synthesis, cell viability and apoptosis induction*

Based on the data showing that GSE inhibits both EGF-stimulated and constitutively active mitogenic signaling

in DU145 cells, we next assessed the biological relevance of these findings. First, we focused our attention on DNA synthesis and cell viability. As shown in Figure 6a, compared to DMSO-treated control starved cells, GSE treatment resulted in a strong inhibition (59%) of DNA synthesis. A higher inhibition (66%) in DNA synthesis by GSE was observed in the starved cells stimulated with EGF; as expected, compared to starved cells, EGF addition showed an increase in DNA synthesis in the starved cells (Figure 6a). Similar to GSE, treatment of starved cells with MEK1 inhibitor PD98059 also resulted in a strong inhibition of DNA synthesis, though it was lesser than that observed with GSE (Figure 6a). Interestingly, when starved cells were treated with both PD98059 and GSE, much stronger inhibition (82%) in DNA synthesis was evidenced (Figure 6a). When serum-starved cells were treated with PD98059 + EGF, comparable DNA synthesis was observed to that in serum-starved cells (Figure 6a). Although it was less than that observed following EGF stimulation, it was higher than that in PD98059 alone treated starved cells (Figure 6a). However, when cells were also treated with GSE in PD98059 + EGF study, they showed comparable DNA synthesis inhibition (82%) as observed in PD98059 + GSE treatment (Figure 6a). In the additional studies assessing whether the observed inhibition in DNA synthesis under different treatment conditions is because of a loss in cell viability, as shown in Figure 6b, an inhibition in DNA synthesis by GSE in starved cells and those stimulated with EGF seems to be due to a loss in cell viability. However, in the other two treatment conditions also involving PD98059, an inhibition in DNA synthesis by GSE was in part due to a loss of cell viability (Figure 6b).

In view of the cell viability data, next we assessed whether GSE induces apoptotic death under various treatment conditions employed in the present study. Representative dot plots of Annexin V/PI staining clearly show that in each case GSE causes strong apoptotic death (Figure 7a). A quantitative estimation of these data clearly show that compared to DMSO-treated control starved cells showing negligible percent apoptotic cells, GSE treatment at 50 $\mu$g/ml dose for 12 h resulted in 18% apoptotic cells (Figure 7b). However, when starved cells were stimulated with EGF, the apoptosis observed even in starved cells was totally abolished (Figure 7b). Interestingly, treatment with GSE + EGF showed a very strong apoptotic cell population (39%, Figure 7b). This observation suggests that whereas EGF protects apoptotic death of starved cells, a combination of EGF + GSE exerts a strong synergistic/additive effect on apoptosis induction by a pathway yet to be characterized. Next, we assessed whether selective inhibition of MEK1–ERK1/2-mediated mitogenic signaling produces additional effect on GSE caused apoptotic death. As shown in Figure 7b, treatment of starved cells with MEK1 inhibitor PD98059 caused a significant increase in apoptotic cell population (8.4%) compared to starved cells showing 3.1% apoptotic cells. However, treatment of starved cells with PD98059 + GSE showed a very strong

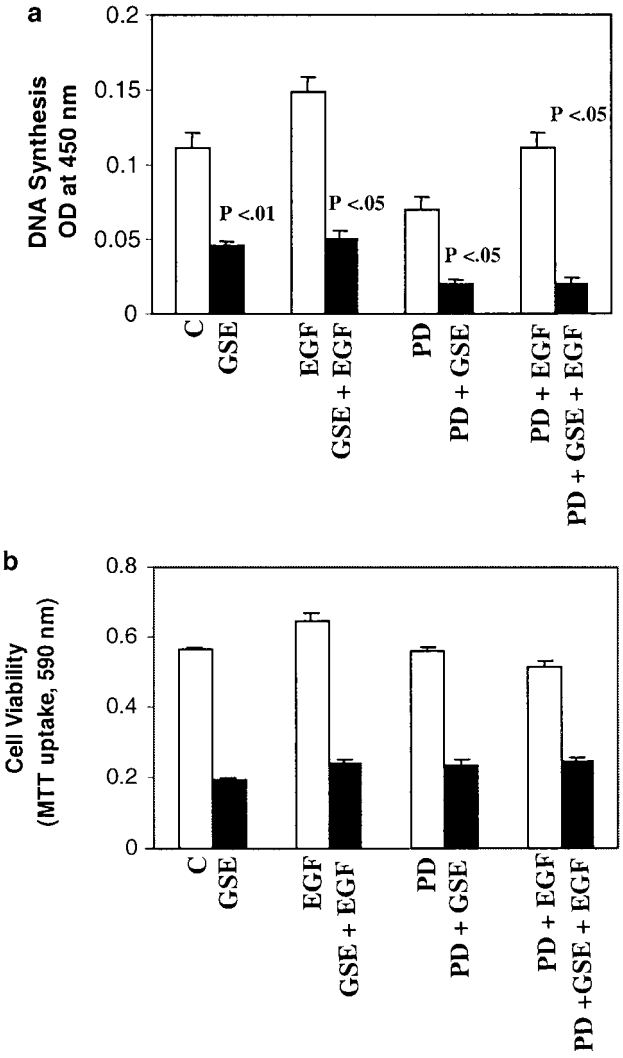


**Figure 6** Effect of GSE on DNA synthesis and cell viability in DU145 cells. Cells were grown in 96-well plates, serum starved for 24 h followed by treatment with PD98059 (50 $\mu$M), GSE (50 $\mu$g/ml) and/or EGF (50 ng/ml) as described in Materials and methods. They were then incubated with BrdU for 2–3 h followed by anti-BrdU antibody and substrate, and BrdU incorporation (**a**) was measured using ELISA reader. For cell viability (**b**), MTT assay was done under identical treatments as detailed in Materials and methods. The data are representative of three independent experiments with similar results

apoptosis, accounting for 40% apoptotic cells. In other studies, treatment of starved cells with PD98059 + EGF showed similar response as untreated starved cells but higher than EGF stimulated samples; cotreatment with GSE in this study (PD98059 + GSE + EGF), however, showed 23.7% apoptotic cell population (Figure 7b).

### *Effect of GSE on JNK activation*

JNK and p38 stress kinase pathways are strongly implicated in the induction of growth arrest/ apoptosis by a wide variety of signals (Kyriakis and Avruch, 1996). Since we observed an increase in phospho-ERK1/2 levels by GSE in serum-starved DU145 cells that could

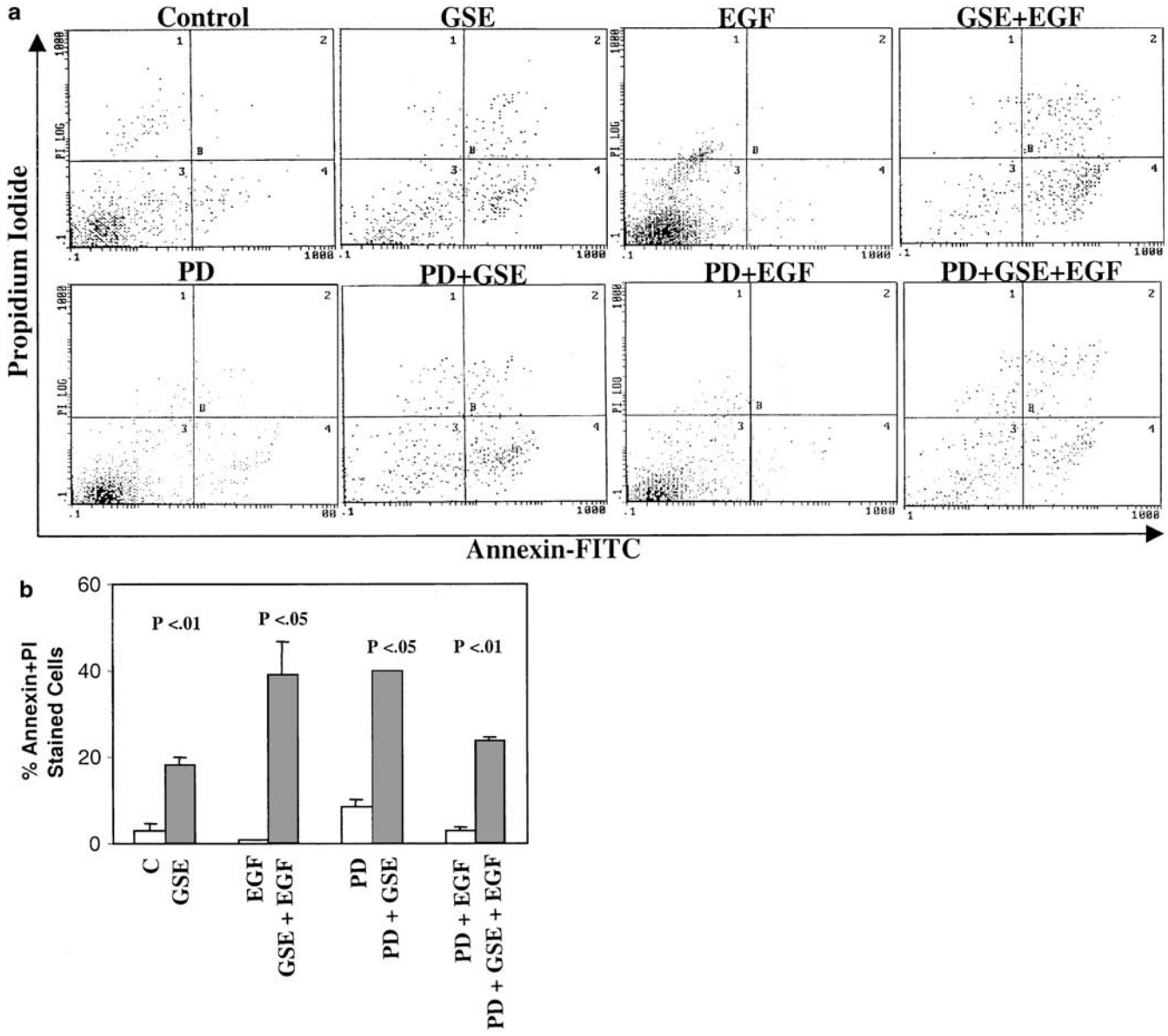


**Figure 7** Effect of GSE on apoptosis induction in DU145 cells. Cells were grown in 60 mm dishes, and at 50–55% confluency, serum starved for 24 h followed by treatment with PD98059 (50 $\mu$M), GSE (50 $\mu$g/ml) and/or EGF (50 ng/ml) as described in Materials and methods. After desired treatments, both attached and floating cells were collected by trypsinization and processed for FACS analysis of Annexin V–PI-stained cells detailed in Materials and methods. The representative dot plots of Annexin V/PI staining followed by FACS analysis (**a**) were analysed for quantification of apoptosis response (**b**). The data shown are representative of three independent experiments with similar results

in part be because of a cellular stress, and since MEK1 inhibitor inhibited GSE-caused increase in phospho-ERK1/2 but failed to reverse the apoptosis induction, we next assessed the question whether GSE activates stress-associated MAPKs, the JNK and p38 phosphorylation under serum-starved conditions as well as that stimulated by EGF, and that such an activation is involved in GSE-caused apoptotic death of DU145 cells. Compared to DMSO-treated control starved cells, treatment with GSE (50 $\mu$g/ml) caused a very strong activation of both JNK1 and JNK2 as evidenced by a 6–7-fold increase in phospho-JNK1/2 protein levels (Figure 8a, upper panel) without any noticeable changes in their total protein levels (Figure 8a, lower panel). Comparable activation of JNK1/2 by GSE was also observed in the treatment conditions where serum-starved cells were stimulated with EGF suggesting that the observed effect of GSE on JNK1/2 activation is independent of an activation of EGFR–ERK1/2-mediated mitogenic signaling (Figure 8a). To further confirm this observation, serum-starved cells were pretreated with MEK1 inhibitor PD98059 alone, PD98059 + GSE, PD98059 + EGF or PD98059 + GSE + EGF, and assessed for JNK1/2 activation. As shown in

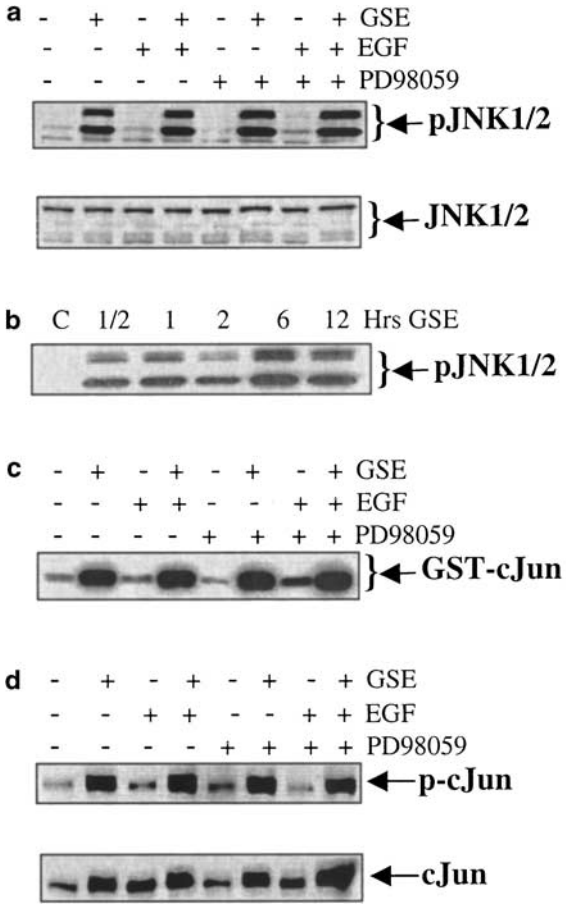


**Figure 8** Effect of GSE on JNK activation, JNK kinase activity and phosphorylation of cJun in DU145 cells. Cells were grown to 70% confluency and serum starved for 24 h followed by treatment with PD98059 (50 $\mu$M), GSE (50 $\mu$g/ml) and/or EGF (50 ng/ml) as described in Materials and methods. Equal amounts of protein from cell lysates were resolved on 12% SDS–PAGE followed by Western blotting, and membranes probed with antiphospho-JNK1/2 (**a**, upper panel) or anti-JNK1/2 antibody (**a**, lower panel). JNK kinase activity (**c**) was determined by pulling down JNK using N-terminal c-*jun* fusion protein bound to glutathione sepharose beads, and then kinase reaction was carried out in the presence of ATP and kinase buffer. Phosphorylation of cJun was measured by Western blotting as detailed in Materials and methods. Phospho- and total cJun (**d**) levels were determined by SDS–PAGE and Western blotting followed by detection with appropriate primary and secondary antibody and ECL detection. To analyse the time kinetics of GSE-induced JNK1/2 phosphorylation, starved cells were treated with 50 $\mu$g/ml GSE, and total cell lysates were analysed for phospho-JNK1/2 (**b**) levels at the indicated times following GSE treatments by immunoblotting as detailed in Materials and methods.

Figure 8a (upper panel), PD98059 did not result in a decrease in GSE-caused activation of JNK1/2 in both serum-starved EGF-stimulated conditions, which further suggests a lack of EGFR-MEK1-ERK1/2 activation cascade in GSE-caused JNK1/2 activation. In further studies assessing whether GSE-caused robust activation of JNK1/2 is transient or sustained, a time kinetics study was done. As shown in Figure 8b, GSE treatment at 50 $\mu$g/ml dose resulted in a very strong activation of JNK1/2 within 30 min that sustained at all time points examined up to 12 h. The observed activation of JNK1/2 by GSE was not due to an increase in total JNK1/2 protein levels (data not shown). Minimal, if any, effect of GSE was evident on p38 activation (data not shown).

### *Effect of GSE on JNK kinase activity and phosphorylation of cJun*

JNK is known to induce phosphorylation of cJun at Ser63, a site important for cJun-dependent transcriptional activity (Pulverer *et al*., 1991; Dai *et al*., 1995). Accordingly, next we examined whether GSE-caused JNK1/2 activation is associated with an increase in JNK kinase activity leading to an increase in phosphorylation of cJun. Following different treatments, JNK proteins were immunoprecipitated from whole cell extracts, and N-terminal cJun fusion protein bound to glutathione sepharose beads was used as a substrate to measure immunoprecipitated JNK activity. As shown in Figure 8c, consistent with a strong activation of JNK1/2, similar treatment of starved cells with GSE resulted in a strong increase (eightfold) in JNK kinase activity compared to DMSO-treated starved cells. Similar effect of GSE on an increase in JNK kinase activity was also evidenced in EGF-stimulated cells, which was independent of MEK1 inhibitor PD98059 pretreatment of either starved or EGF-stimulated cultures (Figure 8c). In other studies assessing the effect of GSE on phosphorylation of cJun, similar treatment of starved cells with GSE resulted in a fivefold induction of phospho-cJun (Figure 8d, upper panel) and a twofold increase in total cJun (Figure 8d, lower panel) protein levels as compared to DMSO control. A comparable pattern to those for JNK1/2 activation and JNK kinase activity was evidenced in other treatment conditions involving EGF-stimulated starved cells and those also treated with PD98059 (Figure 8d).

### *Involvement of GSE-caused JNK1/2 activation in apoptotic death of DU145 cells*

As discussed earlier, based on a lack of GSE-caused increase in ERK1/2 phosphorylation in the apoptotic response of GSE and the fact that JNK activation has been shown to be a key regulator of apoptosis induction, we performed several additional studies to answer the question whether GSE-caused JNK1/2 activation is involved in the apoptotic effect of GSE. For these studies, serum-starved cells were pretreated with a novel JNK inhibitor SP600125 (Bennett *et al*., 2001) for 15 min followed by GSE for 2 h, and cell lysates were analysed for JNK1/2 activation and kinase activity as well as phospho-cJun levels. As shown in Figure 9, pretreatment of cells with JNK inhibitor at 20 or 50 $\mu$M dose resulted in a strong to almost complete inhibition of GSE-caused induction of: (a) JNK1/2 activation without an alteration in total protein (Figure 9a), (b) JNK kinase activity (Figure 9b), and (c) phospho-cJun levels (Figure 9c). However, similar JNK inhibitor pretreatment did not inhibit GSE-caused apoptotic

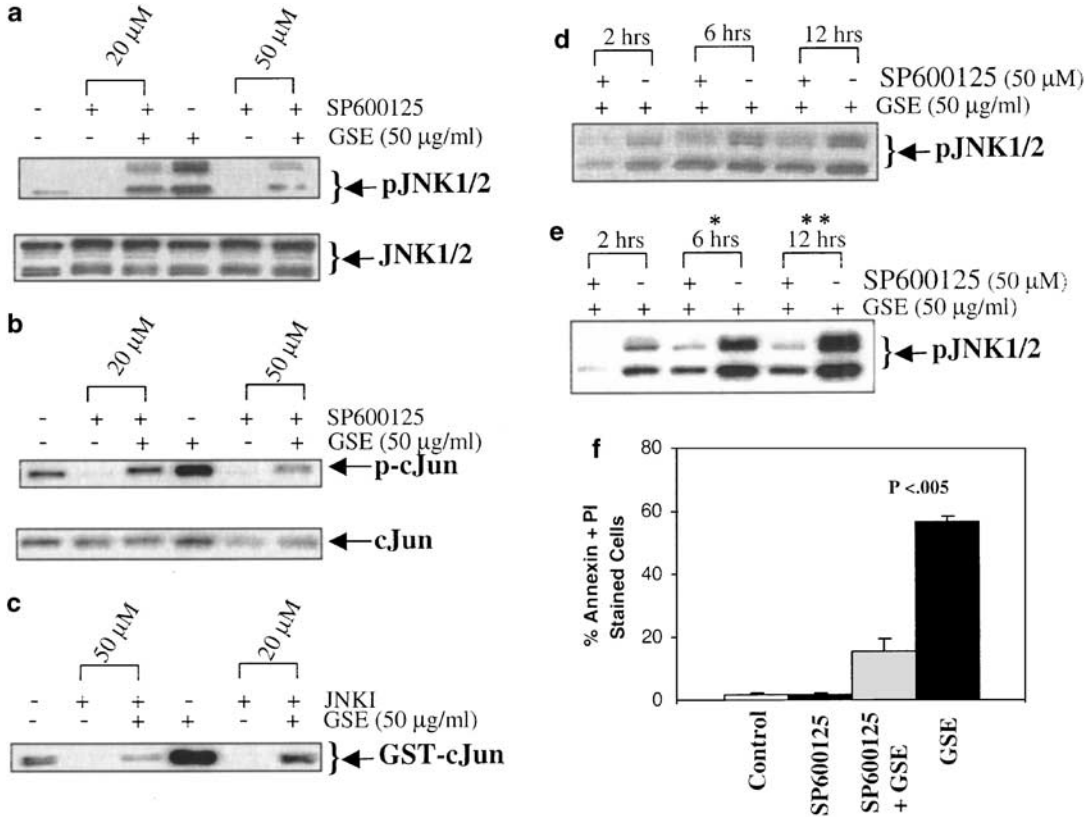


**Figure 9** GSE-caused JNK1/2 activation leads to apoptotic death of DU145 cells. Cells were grown to 70% confluency and serum starved for 24 h followed by treatment with SP600125 (20 or 50 $\mu$M) for 15 min followed by without or with GSE treatment at 50 $\mu$g/ml dose for 2 h as described in Materials and methods. Equal amounts of protein from cell lysates were resolved on 12% SDS–PAGE followed by Western blotting, and membranes probed with antiphospho-JNK1/2 (**a**, upper panel) or anti-JNK1/2 antibody (**a**, lower panel), and antiphospho-cJun (**b**, upper panel) or anti-cJun antibody (**b**, lower panel). JNK kinase activity (**c**) was determined as detailed in Materials and methods. To analyse whether single JNK inhibitor pretreatment has sustained inhibitory effect on GSE-caused JNK1/2 activation, 24 h serum-starved cells were treated with SP600125 (50 $\mu$M) for 15 min followed by GSE treatment at 50 $\mu$g/ml dose for 2, 6 or 12 h as described in Method. Equal amounts of protein from cell lysates were resolved on 12% SDS–PAGE followed by Western blotting, and membranes were probed with antiphospho-JNK1/2 (**d**). To inhibit sustained GSE-caused JNK1/2 activation and reversal of associated apoptosis, 24 h starved cells were first pretreated with SP600125 at 50 $\mu$M dose for 15 min and then with GSE at 50 $\mu$g/ml dose for 2, 6 or 12 h. In the total 6 h GSE treatment study, one additional SP600125 treatment (50 $\mu$M) was done to the same culture at 2 h after initial GSE treatment (*), and in the total 12 h GSE treatment study, two additional SP600125 treatments (each at 50 $\mu$M) were done to the same culture at 2 and 6 h after initial GSE treatment (**). Total cell lysates from all these studies were analysed for JNK1/2 activation by immunoblotting (**e**), and both attached and floating cells from 12 h studies were analysed for apoptotic cells using Annexin V/PI staining (**f**) as described in Materials and methods

death (data not shown). This observation was at odds with what we anticipated and is known in the literature regarding the role of JNK activation in apoptosis induction. Additional studies were next performed to address this issue. Since we observed that GSE causes sustained JNK1/2 activation up to 12 h after its treatment, a time point when apoptotic cell death was analysed, we first assessed whether a single JNK inhibitor pretreatment 15 min prior to GSE remains effective in inhibiting GSE-caused JNK1/2 activation at later time points. As shown in Figure 9d, this turned out to be the case where although JNK inhibitor effectively inhibited GSE-caused JNK1/2 activation following 2 h of GSE treatment, it was not effective at 6 and 12 h time points. Based on this result, we made additional JNK inhibitor treatments where starved cells were first pretreated with SP600125 at 50 $\mu$M dose for 15 min and then with GSE at 50 $\mu$g/ml dose. Two additional SP600125 treatments (each at 50 $\mu$M) were done to the previously treated culture at 2 and 6 h after initial GSE treatment, and different samples from this treatment conditions were analysed for JNK1/2 activation. As shown in Figure 9e, additional JNK inhibitor treatments indeed resulted in a strong inhibition of GSE-caused sustained JNK1/2 activation at both 6 and 12 h after single GSE treatment. We next assessed whether these multiple JNK inhibitor treatments also reverse GSE-caused apoptotic death at 12 h after single GSE treatment. As shown in Figure 9f, indeed a multiple JNK inhibitor treatment that produced a sustained inhibition of GSE-caused JNK1/2 activation also almost completely reversed the GSE-induced apoptotic death of DU145 cells. Together these results provide convincing evidence that GSE causes a robust and sustained JNK1/2 activation, which is involved in GSE-caused apoptosis in DU145 cells.

## Discussion

The present study elucidates the biological effects of GSE on human PCA DU145 cells, and based on the data reported here, supports the notion that GSE could be an effective cancer preventive and therapeutic agent against PCA with defined mechanism of action. As illustrated in Figure 10, the central finding of the present study is that GSE has at least three defined mechanisms of action on signaling pathways associated with cell proliferation and apoptotic cell death. One of these mechanisms involves the inhibitory effect of GSE on both EGF-induced and constitutively active classical EGFR–Shc–ERK1/2–Elk1–AP1 activation (Figure 10). The second mechanism of action of GSE involves a stress response signal(s), yet to be identified and its role defined, that causes a robust phosphorylation of ERK1/2 (via MEK1) and JNK1/2 (Figure 10). Whereas GSE-caused robust increase in ERK1/2 phosphorylation possibly does not contribute significantly to a biological response because of a direct (third mechanism) inhibitory effect of GSE on the kinase activity of ERK1/2 to Elk1, GSE-caused robust and sustained JNK1/2 activation leads to a strong apoptotic death of DU145 cells (Figure 10).

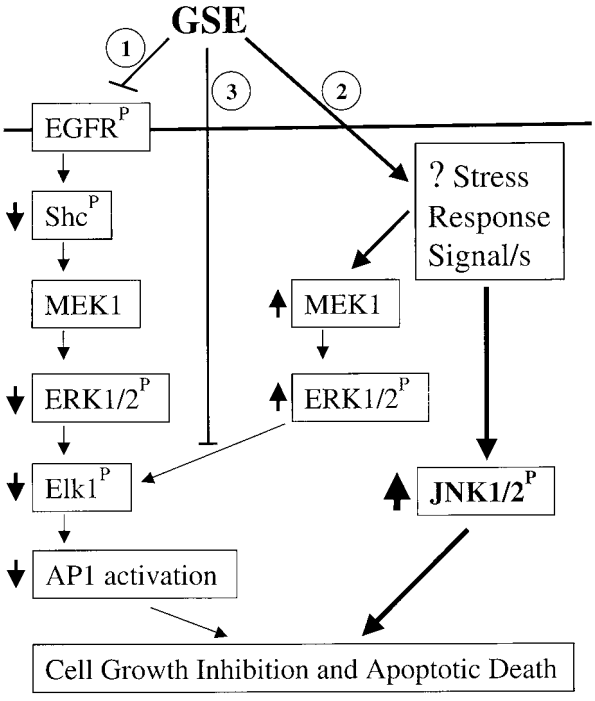


**Figure 10** Proposed mechanisms of GSE action on mitogenic and stress signaling in DU145 cells. Based on the results shown in Figures 1–9, we propose that GSE has at least three defined mechanisms of action on signaling pathways associated with cell proliferation and apoptotic cell death. The mechanism #**1** involves the inhibitory effect of GSE on both EGF-induced and constitutively active classical EGFR–Shc–ERK1/2–Elk1–AP1 activation. The mechanism #**2** of GSE action involves a yet-to-be-identified (?) stress response signal/s that causes a robust phosphorylation of ERK1/2 (via MEK1) and JNK1/2. Whereas GSE-caused robust increase in ERK1/2 phosphorylation possibly does not contribute significantly to a biological response because of a direct (mechanism #**3**) inhibitory effect of GSE on the kinase activity of ERK1/2 to Elk1, GSE-caused robust and sustained JNK1/2 activation leads to a strong apoptotic death of DU145 cells

Several studies in recent years have shown that genetic alterations resulting in an enhanced expression of receptor and associated ligand lead to an epigenetic mechanism of autocrine growth loop via ligand–receptor interaction in advanced and androgen-independent PCA (Putz *et al*., 1999). For example, high levels of EGFR expression and autocrine secretion of its ligand have been reported in DU145 cells (Connolly and Rose, 1989; Tillotson and Rose, 1991). Furthermore, it has also been shown that mitogenic (e.g. ERK1/2–Elk1–AP1) and cell survival and antiapoptotic (IGF-1/IGFBP3 and NF-$\kappa$B) signaling pathways are constitutively active in prostate cancer tissues as well as derived cell lines (Gioeli *et al*., 1999; Palayoor *et al*., 1999; Agarwal, 2000; Zi *et al*., 2000; Mukhopadhyay *et al*., 2001; Dhanalakshmi *et al*., 2002). These studies raise the argument that inhibiting one particular signaling pathway is possibly not sufficient to stop proliferation and induce apoptotic death of human PCA cells; they are resistant to apoptosis induction by most of the chemotherapy agents as well as TNF$\alpha$ (Yu *et al*., 2000). In view of these caveats, the results of our present study showing multiple mechanisms of action of GSE in inhibiting mitogenic and inducing apoptotic signaling pathways could be of high significance in controlling the growth and causing apoptotic death of advanced human PCA cells.

Ligand–receptor interaction causing an autocrine growth loop is a predominant epigenetic event in advanced and androgen-independent human PCA tissue and derived cell lines (Robinson *et al*., 1996; Agarwal, 2000). Consistent with this, there is enormous amount of renewed interest in targeting EGFR activation in both laboratory and clinical studies for the treatment of cancers with activated EGFR including human prostate cancer (Lin *et al*., 1999; Putz *et al*., 1999). Accordingly, the strong inhibitory effect of GSE on EGF-induced EGFR and Shc activation as well as EGFR–Shc binding is an important finding to be further explored in future studies. It has been well characterized that EGFR activation ultimately activates MAPK/ERK1/2, which, following activation, translocates to the nucleus followed by activating transcription factors that promote cell division and/or differentiation. It has also been established that it is the level of MAPK/ERK1/2 stimulation that decides activation of transcription factors for cell proliferation (Seger and Krebs, 1995). Consistent with these studies, we observed that GSE inhibits ERK1/2 kinase activity to Elk1 in both cellular and cell-free systems, as well as Elk1 phosphorylation levels and AP1 activation. In other studies, though we found that GSE strongly induces phospho-ERK1/2 levels in serum-starved DU145 cells, the level of increase in kinase activity to Elk1 was only 1.6–1.9-fold, and that in the same experimental conditions GSE decreased constitutively phosphorylated levels of Elk1 and AP1. Additional studies employing cell-free system showed

that GSE directly impairs the kinase activity of ERK1/2 to Elk1, which clearly explains a lack of similar response of GSE, to a robust increase in ERK1/2 phosphorylation, on ERK1/2 kinase activity and Elk1 phosphorylation. This result also identifies an additional mechanism of action of GSE on ERK1/2–Elk1–AP1 activation, and explains why GSE-caused robust ERK1/2 phosphorylation is not a mitogenic response as supported by our DNA synthesis data showing strong inhibition by GSE.

The data obtained in DNA synthesis studies also suggest that possibly there are more than one mitogenic response operational even in starved DU145 cells where a selective inhibition of MEK1 by PD98059 is not sufficient to exert a strong inhibitory effect on DNA synthesis, and that GSE presumably produces additive inhibitory effect by inhibiting as not yet identified other mitogenic responses. One possibility could be the constitutively active nature of ERK1/2–Elk1–AP1 in DU145 cells reported earlier (Putz *et al*., 1999) and also found by us in the present study. Under that situation, one could argue that an inhibition of MEK1 by PD98059 would be ineffective because downstream mitogenic signals are sufficiently activated independent of MEK1 for DNA synthesis. This argument could also be supported by the data obtained with GSE, which show a much stronger inhibition in DNA synthesis that corroborate with a strong to complete inhibition of ERK1/2 kinase activity, and Elk1 and AP1 activation by GSE. The other possibility could be that GSE activates JNK as an apoptotic response, and that the possible inhibition of other mitogenic responses is just removal of cells by apoptosis. Whereas cell viability data partially support this possibility, conversely to this argument, when starved cells were treated with MEK1 inhibitor + EGF, a strong increase in DNA synthesis was observed which was comparable to that in starved cells. This finding points to the argument that EGF is stimulating a mitogenic response in starved DU145 cells that is independent of MEK1 involvement. The other interesting finding was that when cells were also treated with GSE in PD98059 + EGF study, they showed comparable DNA synthesis inhibition (82%) as observed in PD98059 + GSE treatment, again suggesting an additional inhibitory effect of GSE on DNA synthesis possibly by inhibiting EGF-induced activation of an unidentified MEK1-independent mitogenic response. With regard to an additional mitogenic signaling by EGF independent of MEK1, in a recent study, it has been shown that EGFR activation through redox mechanism activates NF-$\kappa$B independent of MEK1 (Hirota *et al*., 2001). It is also important to emphasize that NF-$\kappa$B is constitutively active in DU145 PCA cells (Palayoor *et al*., 1999), and so does EGFR–ligand autocrine growth loop (Hirota *et al*., 2001). Whether the observed increase in DNA synthesis by EGF in the presence of MEK1 inhibitor is because of NF-$\kappa$B activation, remained to be established; however, in our ongoing studies, we observed strong inhibition of constitutively active NF-$\kappa$B in DU145 cells by GSE (unpublished data). We are currently studying in detail a possible link between these effects of GSE with the observed inhibition of DNA synthesis in PD98059 + EGF-treated cells.

The JNK pathway is activated rapidly by distinct extracellular stimuli, such as ultraviolet irradiation, oxidative stress, DNA-damaging agents, inflammatory cytokines, growth factors, and is activated more slowly by the initiation of the apoptotic cell death response by events such as ligation of the Fas protein (Deak *et al*., 1998; Ichijo, 1999; Leppa and Bohmann, 1999). Although there have been several reports showing that JNK and/or p38 activation occurs without influencing cell death (Liu *et al*., 1996; Lenczowski *et al*., 1997), high levels of JNK and p38 activities have been correlated with the induction of apoptosis in many instances (Goillot *et al*., 1997; Rodrigues *et al*., 1997; Yang *et al*., 1997). Activation of JNK has been shown to phosphorylate N-terminal domain of the transcription factor cJun thereby inducing its transactivation potency, and that cJun activation leads to apoptosis (Zanke *et al*., 1996; Tournier *et al*., 2000). Several reports, however, also indicate that under certain circumstances, cJun activation inhibits apoptosis and promotes cell proliferation, transformation, or differentiation (Nishina *et al*., 1997; Smith *et al*., 1997). In our studies, we found that GSE strongly increases JNK1/2 phosphorylation, the kinase activity as well as the levels of phosphorylated cJun, and that these effects of GSE were independent of EGF stimulation and the presence of MEK1 inhibitor. The time kinetics studies showed that GSE-caused JNK1/2 activation occurs early and is a sustained response. In terms of a direct involvement of GSE-caused JNK1/2 activation in its apoptosis response, using selective JNK inhibitor, we observed almost complete reversal of GSE-caused apoptosis induction that convincingly establishes a cause- and -effect relation between JNK1/2 activation and apoptosis induction by GSE. The other apoptosis results showing that MEK1 inhibitor PD98059 + GSE show a fivefold higher apoptotic death (40% apoptotic cells) compared with PD98059 alone showing around 8% apoptotic death suggest that inhibiting MEK1 pathway is not sufficient for strong apoptosis induction in DU145 cells, and that GSE inhibits additional antiapoptotic pathways and/or activates proapoptotic signals in DU145 cells. The other important observation in apoptosis studies was the strong effect of GSE + EGF in inducing apoptotic death. How this combination sensitizes DU145 cells to strong apoptotic death also remains to be established.

In summary, we conclude that GSE has at least three defined mechanisms of action on signaling pathways associated with cell proliferation and apoptotic cell death of DU145 cells. Since one mechanism-based approach to control PCA growth and metastatic potential is to identify the agents that could inhibit proliferation and induce apoptosis by multiple pathways, we suggest that the dual action of GSE needs further investigation to develop this dietary agent for PCA prevention and therapy.

## Materials and methods

### *Cell line and reagents*

Human prostate carcinoma DU145 cells were from American Type Culture Collection (Manassas, VA, USA). RPMI 1640 medium and all other culture materials were from Life Technologies, Inc. (Gaithersburg, MD, USA). GSE used in the present study was from Traco Labs (Champaign, IL, USA). The primary antibodies for Phospho-cJun, cJun, rabbit anti-mouse immunoglobulin and goat anti-rabbit immunoglobulin-horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies for phosphotyrosine, phospho- and total ERK1/2, JNK1/2, p38 and nonradioactive kits for MAPK and JNK kinase activities were purchased from New England Biolabs, Inc. (Beverly, MA, USA). Antibody to EGFR and Shc were from Upstate Biotechnology (Waltham, MA, USA). The ECL detection system was from Amersham (Arlington Heights, IL, USA). Cell proliferation ELISA BrdU kit was from Roche Diagnostics (Indianapolis, IN, USA). Annexin V-Vybrant apoptosis kit was from Molecular Probes (Eugene, OR, USA).

### *Cell culture and treatments*

DU145 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin–streptomycin under standard culture conditions. At 70% confluency, cultures were switched to serum-free medium for 24 h, and then treated with desired doses of GSE (10, 25, 50 and 75 $\mu$g/ml) in DMSO or DMSO alone for 2.5 h, or 2 h with GSE (10, 25, 50 and 75 $\mu$g/ml) and then stimulated with EGF (50 ng/ml) for 0.5 h. In PD98059 (Alexis, San Diego, CA, USA), GSE and/or EGF combination studies, serum-starved cells were either untreated, or treated with PD98059 (50 $\mu$M) alone for 4.5 h, GSE (50 $\mu$g/ml) alone for 2.5 h, first with PD98059 for 2 h followed by GSE for 2.5 h, EGF (50 ng/ml) for 0.5 h, GSE for 2 h followed by EGF for 0.5 h, PD98059 for 4 h followed by EGF for 0.5 h, or PD98059 for 2 h followed by GSE for 2 h and then stimulated with EGF for 0.5 h. Similarly, in JNK inhibitor studies starved cells were treated with SP600125 (Biomol, Plymouth Meeting, PA, USA) at 20 or 50 $\mu$M dose and 15 min later without or with 50 $\mu$g/ml dose of GSE in DMSO for 2, 6 or 12 h. In another study, we made additional JNK inhibitor treatments where starved cells were first pretreated with SP600125 at 50 $\mu$M dose for 15 min and then with GSE at 50 $\mu$g/ml dose. Two additional SP600125 treatments (each at 50 $\mu$M) were done to the same culture at 2 and 6 h after initial GSE treatment, and different samples from this treatment conditions were analysed for JNK1/2 activation. In each GSE treatment, the final concentration of DMSO in culture medium did not exceed 0.1% (v/v) and the same concentration of DMSO was present in control dishes. After desired treatments, medium was aspirated, cells were washed two times with cold PBS, and total cell lysates were prepared as described earlier (Zi *et al*., 1998), and cytosolic and nuclear extracts were prepared as described by Dhanalakshmi *et al*. (2002).

### *Immunoprecipitation and immunoblotting*

Cell lysates (200–400 $\mu$g protein/sample) were diluted to 1 ml with lysis buffer and precleared with protein A/G + agarose for 1 h, then incubated overnight with primary antibody against EGFR or Shc + protein A/G + agarose beads, and immunocomplexes were collected and washed three times with lysis buffer. For immunoblotting, immunocomplexes, and total cell lysates or nuclear extracts (40–60 $\mu$g protein per sample) were denatured with 2 × sample buffer, samples were subjected to SDS–PAGE on 8 or 12% gel, and separated proteins were transferred onto membrane by Western blotting. Membranes were blocked with blocking buffer for 1 h at room temperature and, as desired, probed with primary antibody against phosphotyrosine, EGFR, Shc, and phospho- or total ERK1/2, Elk1, JNK1/2, p38 and cJun overnight at 4°C followed by peroxidase-conjugated appropriate secondary antibody and ECL detection.

### *Electrophoretic mobility shift assay (EMSA)*

EMSA was carried out using nuclear extracts prepared from treated cells. AP1 consensus oligonucleotides were radiolabeled with [$\gamma$-$^{32}$P]ATP in the presence of $T_4$ polynucleotide kinase in 10 × kinase buffer as per the manufacturer's protocol (Promega, Madison, WI, USA). Protein (~8 $\mu$g) from the nuclear extract was first incubated with 5 × gel shift binding buffer and then with $^{32}$P-end-labeled AP1 consensus oligonucleotide for 20 min at 37°C. DNA–protein complex thus formed was resolved on 6% DNA retardation gels. The gels were dried and bands were visualized by autoradiography. In super shift assay, the nuclear extract was first incubated with anti-cJun or anti-cFos antibody before the addition of $^{32}$P-end-labeled AP1 oligo. DNA–protein complex thus was resolved on 6% DNA retardation gel followed by gel drying and autoradiography.

### *ERK1/2 kinase activity assay*

To assess ERK1/2 kinase activity to Elk1, a nonradioactive assay was used employing a commercial kit and protocol provided by the vendor. Briefly, to immunoprecipitate active MAP kinase, 200–250 $\mu$g protein/cell lysates was incubated with monoclonal phospho-p44/42 MAP kinase antibody. The resulting immunoprecipitates were then incubated with an Elk1 fusion protein substrate in the presence of ATP and kinase buffer for 30 min at 37°C. The reaction was stopped by adding sample buffer and boiling the samples. Phosphorylation of Elk1 is then measured by Western blotting using a phospho-Elk1 antibody. In order to address optimum assay conditions, we carried out several additional kinase assays where varying total protein lysates, amount of primary antibody, amount of GST-Elk1 substrate, amount of ATP in kinase assay, or the exposure times of the blot to film were varied.

### *JNK kinase activity assay*

The JNK kinase activity was also determined using nonradioactive assay kit. Briefly, 200–250 $\mu$g protein/cell lysate was incubated with N-terminal c-*jun* fusion protein bound to glutathione sepharose beads to selectively pull down SAPK/JNK. The beads were washed three times with kinase buffer, and kinase reaction was carried out in the presence of cold ATP for 30 min at 37°C. The reaction was stopped by adding sample buffer and boiling the sample. Phosphorylation of c-*jun* is measured by Western blotting using a phospho-cJun antibody.

### *DNA synthesis and cell viability assays*

The effect of GSE on DNA synthesis was assessed by BrdU incorporation employing colorimetric ELISA. Briefly, cells were cultured in 96-well plates at 37°C for 24 h, and then starved for 24 h in serum-free medium. After 24 h of starvation, cells were treated with DMSO alone or GSE in DMSO for 14 h, EGF alone for 12 h or 2 h GSE followed by EGF for 12 h, PD98059 alone for 16 h or 2 h PD98059

followed by GSE for 14 h, and PD98059 for 4 h followed by EGF for 12 h or PD98059 for 2 h followed by 2 h GSE and then EGF for 12 h. After these treatments, BrdU was added with subsequent incubation for another 2–3 h at 37°C. Thereafter, DNA was denatured and cells were incubated with anti-BrdU antibody followed by addition of substrate. The reaction product was quantified by measuring absorbance at 450 nm wavelength using a scanning multiwell spectrophotometer (ELISA reader).

For cell viability, MTT assay was employed. Briefly, following identical treatments as detailed above for cell proliferation in 96-well plate, cells were washed twice with $1 \times$ PBS and incubated with 1 mg MTT/ml of serum-free media for 2 h. Finally, treatment medium was aspirated out and DMSO was added in each plate, and absorbance of developed color was taken at 590 nm against DMSO using a 96-well microplate reader.

*Quantitative apoptotic cell death assay*

To quantify GSE-induced apoptotic death of DU145 cells, Annexin V and PI staining was performed, followed by flow cytometry. After 24 h of serum starvation, cells were treated identically as described above for DNA synthesis study. In other studies assessing whether JNK1/2 activation by GSE is responsible for apoptosis induction, 24 h starved cells were treated with SP600125 at 50 $\mu$M dose and 15 min later without or with 50 $\mu$g/ml dose of GSE in DMSO for 12 h. In another study, we made additional JNK inhibitor treatments where starved cells were first pretreated with SP600125 at 50 $\mu$M dose for 15 min and then with GSE at 50 $\mu$g/ml dose for 12 h. Two additional SP600125 treatments (each at 50 $\mu$M) were done to the same culture at 2 and 6 h after initial GSE treatment. After desired treatments, both floating and attached cells were collected and subjected to Annexin V and PI staining using Vybrant Apoptosis Assay Kit2 (Molecular Probes, Inc., Eugene, OR, USA) and following the step-by-step protocol provided by the manufacturer.

*Densitometry and statistical analysis*

Autoradiograms of the immunoblots were scanned using Adobe Photoshop, Adobe System Incorporated, (San Jose, CA, USA). The mean density for each band was analysed using Scion Image program, National Institutes of Health (Bethesda, MD, USA), and adjusted for background subtraction. In each case, the numerical data shown under the blots are arbitrary units where GSE treatments are either compared with EGF alone control (Figures 1–3) or with DMSO control (Figures 4 and 5). In both cases, the control densitometry value is employed as '1', and a comparison is then made for densitometry values obtained following GSE treatments. The comparative data are presented as 'fold change' that are over respective control for treatments in each case. The gel data and the corresponding densitometric values shown below them, in all cases, are representative of at least 2–3 independent studies with reproducible results. Statistical significance of differences between control and treated samples were calculated by Student's *t*-test (SigmaStat 2.0, Jandel Scientific). *P*-values of less than 0.05 were considered significant.

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