The JUN Kinase/Stress-activated Protein Kinase Pathway Is Required for Epidermal Growth Factor Stimulation of Growth of Human A549 Lung Carcinoma Cells*

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Epidermal growth factor (EGF) plays a major role in non-small cell lung cancer cell autocrine growth and has been reported to activate the JUN kinase/stress-activated protein kinase (JNK/SAPK) pathway in model cells. Activation of JNK/SAPK leads to the phosphorylation of c-JUN protooncogene on serines 63 and 73. This mechanism is required for and cooperates in the transformation of the embryonic fibroblasts by Ha-RAS. However, the function of JNK/SAPK in human tumor growth is unknown. We have tested several lung carcinoma cell lines. All exhibited UV-C-inducible JNK/SAPK activity; two exhibited constitutive activity in low serum, and two (M103 and A549) exhibited EGF-inducible JNK/SAPK activity. In A549 cells, EGF induced a rapid and prolonged (up to 24 h) activation of the JNK/SAPK pathway that correlated with a 150–190% growth stimulation. Stably transfected clones of A549 cells expressing c-JUN(S63A,S73A), a transdominant inhibitor of c-JUN, completely blocked the EGF-stimulated proliferation effect but did not alter the basal proliferation rate. Consistent with these results JNK antisense oligonucleotides targeted to JNK1 and JNK2 entirely eliminated the EGF-stimulated JNK/SAPK activity and blocked EGF-stimulated growth but not basal growth. In contrast, specific inhibition of the RAF/ERK pathway by PD98059 (MEK1 inhibitor) completely blocked ERK activation by EGF and basal cell growth but not EGF-stimulated growth, thereby dissociating the growth-promoting roles of each pathway. Our observations indicate, for the first time, that JNK/SAPK may be a preferential effector pathway for the growth properties of EGF in A549 cells.

The JUN kinase/stress-activated protein kinase (JNK/SAPK)1 pathway is known to mediate cellular stress responses.

It is activated by ultraviolet light (UV), γ-irradiation, heat shock (1), proinflammatory cytokines (2–3), and by different DNA damaging agents such as cisplatinum (4) and alkylating agents (5). The activation of this pathway leads to the phosphorylation of the c-JUN protooncogene in its N-terminal activation domain at serine residues 63 and 73. JUN N-terminal kinases (JNKs) phosphorylate c-JUN at these sites (2, 6) leading to enhanced transcriptional activity required for the transformation of primary rat embryonic fibroblasts in cooperation with activated RAS (7–8). By using a nonphosphorylatable c-JUN, c-JUN(S63A,S73A), where the two serine residues at position 63 and 73 are replaced by two alanine residues, it is possible to inhibit the transformation of rat embryonic fibroblasts cells by activated RAS (7). In addition to JNK activation of AP-1, a transcription factor consisting of c-FOS/c-JUN heterodimers or c-JUN/c-JUN homodimers, JNKs also phosphorylate ATF-2 (9–11) and Elk-1 (12). AP-1 and ATF-2 are important transcription factors regulating numerous genes implicated in cell growth, transformation, differentiation, and DNA repair (4, 13–15).

The mitogen-activated protein kinases (MAPK) form a group of three different kinase cascade pathways that includes the cascade leading to the activation of JNK/SAPK, but also the members of the RAF/ERK kinase pathway and finally a cascade leading to the activation of p38 MAPK pathway. UV-C irradiation is the most effective inducer of the JNK/SAPK pathway (6, 16). UV-C and proinflammatory cytokines are also strong inducers of p38 MAPK (17). Phorbol esters and several growth factors preferentially activate the RAF/ERK pathway (14, 18). Thus, the RAF/ERK pathway mediates ligand-activated receptor tyrosine kinase signal transduction leading to the activation of RAS and RAF, two protooncogenes involved in cell proliferation and transformation (19–21). Activated RAS stimulates RAF, which induces MEK1, which in turn activates p42 and p44 kinases, also called extracellular signal regulated kinases 1 and 2 (ERK1 and ERK2).

It was shown that a direct biochemical link exists between growth factor receptors such as epidermal growth factor receptor (EGFr) and the RAF/ERK pathway through growth factor receptor-bound protein 2 (GRB2) (22). Furthermore, it is well known that epidermal growth factor (EGF) activation of RAF/ ERK pathway is an important mitogenic mechanism for many cell types (23–26).

However, recently, it has been shown that RAS mediates cell proliferation and cell transformation not only through RAF/ERK but also through other pathways involving protooncogenes of the Rho family such as CDC42, Rac1, and p120GAP GTPase-activating protein. For example, the activation of Rac1 (27–29) as well as p120GAP (30) is required for RAS transformation of Rat-1 fibroblast and NIH3T3 cells. Furthermore, the activation
of JNK/SAPK by EGFR was shown to be mediated by Rac1 and CDC42 following RAS stimulation (31–32). Thus, although the RAF/ERK pathway is implicated in EGF-induced proliferation, the role of JNK/SAPK pathway as a mediator of EGF-stimulated growth remains unknown.

Autocrine and paracrine growth stimulation by EGFR and related molecules have been implicated in the transformation of a variety of human tumors. EGFR stimulates the proliferation of several kinds of human tumor cells such as gliomas (33), mammary tumor cells (34), and keratinocytes (35–36). EGFR as well as transforming growth factor-α, amphiregulin, and heparin-binding EGFR bind to EGFR. EGFR and other EBBR proto-oncogenes are frequently overexpressed in several human cancer cells including glioblastoma (37–39) and breast cancer (40–42). Moreover, a recent study shows that alteration of the EGFR results in a truncated mutant receptor with constitutive activity confers enhanced tumorigenicity and increased cell proliferation in human glioblastomas (43). Several studies have shown that EGFR also plays a major role in autocrine growth of human non-small cell lung cancer cells (NSCLC) (35, 44–45). Furthermore, numerous investigations describe an abundant expression of EGFR in NSCLC lines in culture (46–49) including A459 NSCLC and in primary human non-small cell lung cancers (45, 50). For example, recently it has been shown that 82 of 88 NSCLC tumors express EGFR and 45% overexpress the receptor (51). Taken together, these studies suggest an association of EGFR overexpression with cell proliferation and tumorigenicity.

However, the components of the signal transduction pathway responsible for proliferation and transformation of human tumor cells by ligand-activated EGFR are poorly understood. Here, we show that EGFR is a potent activator of proliferation of A549 NSCLC. Moreover, EGFR is a strong and preferential inducer of prolonged (up to 24 h) activation of JNK/SAPK pathway in these cells. Stable expression of a nonphosphorylatable c-JUN, c-JUN(S63A,S73A) (4, 7), leads to complete inhibition of growth stimulation induced by EGFR but does not alter basal growth. Furthermore, inhibition of JNK/SAPK pathway using specific phospho-orthioate antisense oligonucleotides against JNK1 and JNK2 specifically inhibits EGF activation of JNK/SAPK and blocks EGFR-stimulated proliferation. Conversely, inhibition of the RAF/ERK pathway decreases basal growth but does not interfere with EGFR stimulation of growth. These results indicate an essential role for JNK/SAPK pathway in growth stimulation by EGF in human A549 lung carcinoma cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatment**—Non-small cell lung cancer cells A549 (gift of N. Dean), NCI-H292 (gift of X. K. Zhang), NCI-H101, M103 (gift of L. Defos), and immortalized normal lung fibroblast CCD16Lu (gift of X-K. Zhang) were cultured in Dulbecco's modified Eagle's Medium (DMEM) (Life Technologies, Inc.) supplemented with 20% FBS (fetal bovine serum) (v/v). For treatment with recombinant human EGF (rEGF), UV-C, 12-O-tetradecanoylphorbol-13-acetate (TPA) and insulin, cells were seeded 2 days before treatment and were maintained in 0.5% FBS for 17 h prior to treatment.

For ERK inhibition studies, cells were treated with PD98059 (New England Biolabs), a specific inhibitor of RAF/ERK pathway (52–53) at 2–50 μM for 90 min prior addition of rEGF.

**Protein Kinase Assays**—For kinase assay, cells were seeded into 6-well tissue culture plates 2 days prior the analysis to provide ~80% confluent preparations. For treatment with different agents, cells were washed twice with ice-cold phosphate-buffered saline and suspended in lysis buffer (25 mM HEPES, pH 7.7, 0.3 mM NaCl, 1.5 mM MgCl2, 0.1% Triton X-100, 100 μg/ml phenylmethylsulfonyl fluoride, 100 μg/ml 1-tosylamido-2-phenyltetrazolium chloride, 1 mM EDTA, 2 μg/ml leupeptin, and 2 μg/ml aprotinin, 20 μM β-glycerophosphate, 0.1 mM Na3VO4). The protein concentration of the cell extracts was determined by the Bradford dye method (Bio-Rad). Typically all preparations yielded very similar protein concentrations and were adjusted to provide equal amounts of cellular protein in all samples prior to analysis. The kinase assay was performed as described by Hibi et al. (1). Briefly, 50 μg of whole cell extract was mixed with 10 μg of glutathione S-transferase-c-JUN-(1–223) (GST-c-JUN-(1–223)) for 3 h at 4 °C. GST-c-JUN fusion proteins were previously purified from Escherichia coli and bound to agarose beads (Sigma). After four washes, beads were incubated with 30 μl of kinase reaction buffer (20 mM HEPES, pH 7.7, 20 mM MgCl2, 20 mM β-glycerophosphate, 20 mM p-nitrophenol phosphate, 0.1 mM Na3VO4, 2 mM dithiothreitol, 20 μM ATP, and 5 μl of [γ-32P]ATP) for 20 min at 30 °C. After the reaction was stopped by addition of 20 μl of Laemmli sample buffer (54). The phosphorylated GST-c-JUN assay protein was eluted by boiling the sample for 5 min and resolved by a 10% SDS-polyacrylamide gel electrophoresis. For ERK/MAPK assay 50 μg of whole cell extract was immunoprecipitated with anti-ERK2 antibodies which exhibit cross-reactivity with ERK1 (Santa Cruz Biotechnology Inc.) for 2 h at 4 °C. Immunoprecipitates were then washed three times and assayed for kinase activity in kinase buffer using 4 μg of myelin basic protein (MBP, Sigma) as substrate (16). The reaction was stopped by addition of Laemmli sample buffer. The phosphorylated MBP protein was resolved by 15% SDS-polyacrylamide gel electrophoresis, dried, and exposed to film X-OMat 228 (Kodak).

The quantification of 32P-phosphorylated GST-c-JUN and MBP was carried via an Amalgram-1000 (LKB Inc.) for dried SDS-polyacrylamide gel electrophoresis or using an Ultrascan XL (LKB Inc.) for autoradiographs. Background values were subtracted in all cases.

**Proliferation Assay**—Cells were seeded in 24-well tissue culture plates in the presence of DMEM supplemented with 0.5% FBS. One day later, the desired volume of rEGF was added to the cells to a final concentration of 10–100 mM, and the media were not changed during the experiment. Cells were then counted at different days using a Coulter counter. For each experiment wells were counted in triplicate. For experiments with PD98059, the compound was added 1 day after the seeding and 90 min prior to the addition of rEGF and remained in the media throughout the experiment.

**A549 Clone Selection**—The dnJUN expression plasmid pLHCc-JUN(S63A,S73A) was constructed as described previously (55). A549 cells were transfected for 12 h in 6-well tissue culture plates with 2 μg of pLHCc-JUN(S63A,S73A) or the empty vector, pLHCX. In both cases the cells were cotransfected with 200 ng of pSV2Neo (bearing the Geneticin® resistance gene), using Lipofectin (Life Technologies, Inc.) at 20 μl/ml media (56). Clone selection was performed by adding G418 (Life Technologies, Inc.) to the media at 1 mg/ml for 3 days after the transfection. After 3 weeks several clones were isolated using cloning rings. The expression of c-JUN(S63A,S73A) by the clones was determined by Western analysis using anti-c-JUN antibodies (Santa Cruz Biotechnology Inc.). Selected clones were then maintained in media supplemented with G418 (0.5 mg/ml), and only low passage cells (p ≤ 10) were used for the experiments described here.

**Inhibition of JNK Activity by JNK Antisense Oligonucleotides**—All oligonucleotides used in this study were phosphorothioate oligonucleotide prepared following the same procedure described by Dean et al. (57, 58) and Monia et al. (59) and were a gift from ISIS Pharmaceuticals, Inc. In this method antisense sequences are chosen based on their ability to eliminate steady state target RNA of A549 cells >95% following a 4.5 h lipofection at 0.4 μM (57–59). Two antisense oligonucleotides complementary to the two major forms of human JNK (10), termed antisense JNK1 and antisense JNK2, were chosen with sequences 5’-CTCTCTGTAGGCCCGCTTGG-3’ and 5’-GTCCGCCGCCAGGGCAATC-3’, respectively. For JNK and growth assays, the oligonucleotides were used as described (57–58). Briefly, 0.2 μM JNK1 plus 0.2 μM JNK2 antisense or scrambled sequence oligonucleotides were added to MEM (minimum essential media) containing 10 μg/ml Lipofectin (Life Technologies, Inc.) at 1 mg/ml. This preparation was added to 80% confluent A549 cells cultured in 10 cm plates. After 4.5 h of the transfection medium was replaced with DMEM with 5% FBS. Cell extracts were prepared 48 h after the transfection. For JNK assays, the cells to be stimulated with rEGF were maintained overnight in 0.5% FBS and then were treated by addition of rEGF to 100 nM for 20 min prior to lysis.

Proliferation assays were carried out in 24 well-plates as described above (“Proliferation Assay”). After the lipofection, cells were main-
these cells, maintained in 0.5% FBS, with 10 nM rhEGF or presses high levels of EGF receptors (48). To determine if EGF been shown previously that the NSCLC cell line, A549, ex-
mixed in 0.5% FBS overnight prior to analysis. Similar obser-
ations hold for cells maintained in (5%) FBS medium (data not shown). These observations suggest that lung carci-
oma cells exhibit a variety of states of JNK activity including substantial constitutive activity in low serum. In some cases such as M103, JNK activity can be stimulated further by ad-
dition of rhEGF (Fig. 3). A549 cells exhibited the polar phenot-
type of maximum induction combined with nearly undetectable
basal activity. Therefore, these cells were chosen for further
study.

RESULTS

EGF Activates JNK/SAPK Pathway in A549 Cells—It has been shown previously that the NSCLC cell line, A549, ex-
presses high levels of EGF receptors (48). To determine if EGF activates the JNK/SAPK pathway in A549 cells, we treated these cells, maintained in 0.5% FBS, with 10 nM rhEGF or other agents known to activate JNK (20% FBS, TPA, insulin, and UV-C). JNK activity was determined by examination of the phosphorylation of GST-c-JUN 20 min after the treatment. The addition of FBS, TPA, or insulin failed to significantly activate the JNK/SAPK pathway (Fig. 1). Several other agents such as transplatinum and PD98059 (MEK1 inhibitor used later in this study) also failed to activate JNK (data not shown). However, addition of rhEGF or treatment with UV-C strongly and rap-
idly activated JNK in A549 cells (Fig. 1).

To determine whether this activation is dose-dependent, we examined JNK activity as a function of rhEGF concentration. The enzyme activity increased markedly upon addition of 10 nM rhEGF with only small increases upon further addition of rhEGF up to 200 nM (Fig. 2). Thus, near maximal values are observed within the physiological concentration range of EGF consistent with a high affinity and saturable receptor-mediated process. The maximum JNK activity observed following stim-
ulation by rhEGF was half that obtained with UV-C, the strongest observed inducer of the pathway (60) suggesting that rhEGF is a potent inducer of JNK activity.

To investigate the generality of JNK activity in other lung cells, we treated different lung cancer cell lines (NCI-H1011, M103, and NCI-H292) and an immortalized normal lung fibro-
blast cell line (CCD16Lu) with 10 nM rhEGF. Among the cells tested, rhEGF consistently activated JNK in M103 up to 3-fold (e.g. Fig. 3), whereas in the other cell lines, only UV-C induced a response. Moreover, M103 and NCI-H1011 cells exhibited substantial JNK activity in the absence of stimulation (Fig. 3). For example, this activity is readily apparent for cells main-
tained in 0.5% FBS overnight prior to analysis. Similar observa-
tions hold for cells maintained in (5%) FBS medium (data not shown). These observations suggest that lung carci-
noma cells exhibit a variety of states of JNK activity including substantial constitutive activity in low serum. In some cases such as M103, JNK activity can be stimulated further by ad-
dition of rhEGF (Fig. 3).

EGF-stimulated Proliferation Correlates with JNK Activaton—To examine whether the EGF-stimulated JNK activity is related to a functional effect in A549 cells, we added rhEGF every 2 days to cells, previously seeded in 0.5% FBS, in amounts that elevated the concentration by 10 nM for each addition. We then followed the course of proliferation over the succeeding 5 days by direct cell counting. After 4 days the EGF-treated cells showed a significant increase in proliferation (p < 0.05) and by day 5 reached a density of 150% that obtained by control cells (Fig. 4). This EGF-dependent proliferation experiment has been repeated 3 times each in triplicate, with similar results each time. Plots of the log of growth versus time are linear over the course of the first 4 days and yield a doubling time of 60 h for A549 cells in low serum and 40 h upon the presence of rhEGF. Similar experiments have been carried out using a larger amount of rhEGF provided as a single addition of 100 nM on day 1, which yielded comparable or greater stimulation of proliferation up to 190% untreated cells (Fig. 9B, below). Furthermore, similar results hold for variants of A549 cells such as PHCXR-empty vector transfected cells (Fig. 6 B). These observations indicate that EGF is a potent mitogen of A549 lung carcinoma cells.

The JNK/SAPK pathway is known to be rapidly activated (30 min after stimulation) by EGF. However, the effect is com-
monly transient in nature (16, 61), whereas serum growth factor-dependent G1 stage of the cell cycle is thought to require 7–10 h exposure (62–65). To determine the duration of the JNK activation after EGF treatment, we carried out a time course assay. Fig. 5 shows that JNK is highly induced 30 min after the addition of the growth factor and remains activated up to 24 h. The activation is maximal at 5–8 h post-stimulation and thereafter decreases slowly. Furthermore, JNK activity remains readily detectable at 24 h. Five independent time course replicates of this experiment have been carried out, and all exhibit very similar characteristics. Thus, these observations indicate that the EGF-prolonged activation of the JNK/SAPK pathway is in agreement with the observed effects of EGF on cell proliferation.

Expression of the Transdominant Inhibitor of c-JUN, c-JUN(S63A,S73A) Blocks the Effect of EGF on Cell Growth—To determine whether there is a causal relationship between JNK activity and the EGF stimulation of proliferation, we have developed stable clones expressing the transdominant inhibitor of c-JUN, c-JUN(S63A,S73A), termed dnJUN (4). Several clones were isolated after G418 selection. These clones exhibited a range of increased expression of immunoreactive c-JUN between 2- and 15-fold greater than the steady state value of endogenous c-JUN observed in A549 parental cells or cells transfected with pLHCX empty vector (Fig. 6A). Two clones, dnJUN14 and dnJUN18, were chosen for further study owing to their high expression of dnJUN protein. The effect of dnJUN expression was examined by carrying out proliferation studies with and without rhEGF similar to that shown in Fig. 4. No difference in basal growth was observed for clones expressing the maximum amount of dnJUN compared with parental or empty vector control cells (Fig. 9, compare B and C). Indeed, none of dnJUN expressing clones exhibited altered basal growth properties (data not shown).

In the presence of 100 nM rhEGF, parental and empty vector control cells exhibited an EGF-dependent increase in growth of 150–200% that of untreated cells by day 4 (Fig. 6B). Furthermore, dnJUN18, which stably expresses moderate amounts of

FIG. 4. rhEGF promotes proliferation of A549 cells. Cells were seeded at 12,000 cells/cm² on day 0, and 10 nM rhEGF was added every 2 days from day 1. * indicates time points where the number of cells + EGF (●) is significantly different than the number of the untreated cells (○) with p < 0.05.

FIG. 5. rhEGF activates JNK pathway up to 24 h in A549 cells. Cells seeded 48 h prior to treatment were stimulated by addition of 100 nM rhEGF. Cell extracts were prepared at the indicated times after stimulation and used for JNK activity as described in Fig. 1. The entire experiment has been repeated 5 times with similar results, and the peak of JNK activity has a standard deviation of 20%.

dnJUN (Fig. 6A), responded poorly to EGF. Even in the presence of 100 nM rhEGF, these cells only increased in number by 118% of untreated cells. However, dnJUN14, which expresses maximum level of dnJUN protein (Fig. 6A), failed to exhibit any EGF-stimulated increase in proliferation (Fig. 6B). The inhibition of EGF-dependent growth by dnJUN leads to a large and significant (p < 0.01 for comparison to either parental or pLHCX control cells) difference in growth which is readily apparent by day 4 (Fig. 6B). Moreover, other clones (dnJUN3 and dnJUN16, Fig. 6A) that exhibit low but detectable levels of expression of dnJUN also exhibit decreased and reproducible inhibition of EGF-dependent proliferation. This result has been observed in numerous variations of the experiment (e.g. Fig. 9C). Thus, while expression of dnJUN does not disrupt normal growth, dnJUN-expressing cells are inhibited in their ability to respond to exogenous EGF in proportion to the level of expression of dnJUN.

An unusual feature of the JNK family of kinase is the ability of the enzyme to bind the N-terminal portion of the c-JUN protein (10, 66). To rule out the possibility that overexpression of the dnJUN protein affects growth by binding to and inhibiting some JNK isotypes or other upstream components, the EGF dependence of JNK activity in dnJUN 14 and dnJUN 18 was examined. The results reveal that addition of rhEGF activates JNK in dnJUN clones 14 and 18 to levels that are 20–48% that of UV-C stimulation or comparable to the 43–45% levels observed for the parental and empty vector control cells (Fig. 7). Thus, the sum of results suggests that overexpression of a transdominant inhibitor of c-JUN blocks EGF stimulation of proliferation in A549 in a manner consistent with a dominant negative inhibitor of c-JUN, a downstream effector of the JNK/SAPK pathway.

JNK Antisense Oligonucleotides Alter EGF Activation of JNK/SAPK Pathway and Inhibit EGF-stimulated Growth—To confirm the direct role of JNK/SAPK pathway in EGF-stimulated growth in A549 cells, we used phosphorothioate oligonucleotides targeted to JNK1 and JNK2 mRNA to block JNK/SAPK pathway. A549 cells were transfected with 0.2 μM each antisense oligonucleotides (JNK1 + 2AS). As a control, two oligonucleotides corresponding to a scrambled sequence of antisense JNK1 and JNK2 oligonucleotides, respectively (JNK1 + 2Scr), were used at the same concentrations. Forty hours after the lipofection a JNK assay was performed on cells treated or not with 100 nM rhEGF. Fig. 8A shows that rhEGF strongly activates JNK in both parental A549 cells and in cells treated with JNK1 + 2-scrambled sequence oligonucleotides; in the antisense-treated cells the activation is dramatically reduced by more than 11-fold. Similar results have been observed in variations of this analysis using other control sequences such as a mixture of sense sequence oligonucleotides or lipofection alone and support the observation that suppression of
EGF-mediated Growth Requires JNK/SAPK

Fig. 6. Expression of dnJUN suppresses the EGF-dependent proliferation of A549 cells. A, Western analysis of A549 cells clones isolated after selection with G418. Clones were analyzed at passage 4 following transfection. B, proliferation assay performed as described in Fig. 4, in the presence or absence of 100 nM rhEGF added on day 1. The histogram shows the ratio of number of cells + EGF/number of cells −EGF throughout the 8 days of the proliferation assay. All cell numbers were determined in triplicate, and the same results were obtained in three independent experiments. In general, A549 cells and derivative cell lines exhibit maximum cell number at the end of log phase growth which variably occurs on days 4–6 and may be followed by a decline in counted cells owing to detachment (cf. A549pLHCX versus A549 parental cells).

Fig. 7. Stable expression of dnJUN does not interfere with EGF and UV-C stimulation of JNK activity. Cell extracts of dnJUN14, dnJUN18 clones, pLHCX empty vector control cells, and A549 parental cells were assayed following no treatment (control) or treatment with 100 nM rhEGF for 20 min or with UV-C (100 J/m²). The kinase activity was carried out as described in Fig. 1.

JNK activity is specific to treatment with JNK1 + 2AS. These observations also indicate that the optimized lipofection procedure used here is highly efficient. Moreover, treatment of cells at higher concentrations, 0.4 μM, leads to suppression that lasts up to 72 h (data not shown). The combined effects of prolonged inhibition by more than 11-fold of JNK activity suggests that the effects of suppressed JNK on phenotypic properties such as proliferation can be assessed.

To determine whether the antisense-induced reduction in JNK activation is related to an effect on proliferation, we performed a proliferation assay using the same combination of oligonucleotides at the same concentration (0.2 μM JNK1 + 0.2 μM JNK2 AS or Scr) as for kinase assays. Twenty four hours after the lipofection, we added 100 nM rhEGF to the cells. Cells were counted 6 days later. A549 cells that have been treated with 0.2 μM JNK1 + 2AS exhibit basal growth levels very similar to cells treated with scrambled sequence or Lipofectin reagent (Fig. 8B, open bars). Moreover, these values are comparable to the basal growth of dnJUN expressing cells (Fig. 8B, dashed line; cf. Fig. 9C). This comparison shows that the proliferation of oligonucleotide-treated cells (Fig. 8B, JNK1 + 2AS, open bars) is essentially the same as observed for dnJUN-expressing cells. Thus, inhibition of JNK activity by the antisense oligonucleotides appears to have very little specific or toxic effect on the normal proliferative properties of A549 cells.

This conclusion is reinforced by the 165% EGF stimulation of growth of cells transfected with the scrambled oligonucleotides (p < 0.001) (Fig. 8B, JNK1 + 2Scr, filled bars).

Addition of 100 nM rhEGF stimulated proliferation by 160–180% in A549 parental cells, in cells treated with Lipofectin alone, or in cells treated with the scrambled sequence control oligonucleotides. In all cases the difference is significant (p < 0.0001). However, A549 cells transfected with JNK1 + 2 anti-sense oligonucleotides failed to grow better in the presence of rhEGF (Fig. 8B, JNK1 + 2AS, solid bars). Thus the maximum proliferation obtained is very similar to that of dnJUN-expressing cells in the presence or absence of rhEGF (cf. Fig. 9C). Similar experiments using different combinations of controls such as sense sequence control oligonucleotides or individual control oligonucleotides at varying concentrations yielded very similar results. The sum of these results indicates that specific inhibition of JNK/SAPK pathway blocks EGF stimulation of proliferation in A549 cells but does not alter basal growth. PD98059 a RAF/ERK Inhibitor Does Not Block EGF-stimulated Growth in A549 Cells—In many cell types, the mitogenic effects of EGF are mediated at least in part through the RAF/MEK1/ERK pathway suggesting the possibility that this pathway and JNK/SAPK may cooperate in mediating the EGF-dependent growth stimulation (23–26). To test this hypothesis we have used PD98059, a specific inhibitor of MEK1, the enzyme responsible for ERK activation (52–53, 67–68).

Fig. 9A shows that PD98059 considerably reduced the phosphorylation of MBP at a concentration of 2 μM and that the activity is nearly completely inhibited at 30 μM. However, PD98059 at 50 μM did not affect EGF activation of JNK in A549 cells (data not shown). PD98059 was previously shown to inhibit cell growth of cells transformed with RAS and RAF protooncogenes (52). Therefore, we tested the effect of PD98059 on A549 growth at different concentrations: 5, 10, and 20 μM with or without 100 nM rhEGF. In the absence of rhEGF and after 6 days of culture, the number of A549 cells decreased with increasing amounts of PD98059 to values less than half compared with untreated cells (Fig. 9B). This shows that PD98059 affects basal cell growth in a dose-dependent manner as described in previous studies (52). As before (Fig. 6), addition of rhEGF to A549 cells leads to the stimulation of proliferation by nearly 200% (Fig. 9B, 0 μM PD98059). Moreover, when rhEGF was added to cells in the presence of increasing concentrations of PD98059, the EGF-stimulated proliferation remains at about 200% in all cases (Fig. 9B). The EGF-stimulated doubling of proliferation was observed in the presence of 20 μM PD98059 where basal growth is reduced by over 60% of control values (Fig. 9B). The EGF-stimulated doubling of growth at each concentration of PD98059 is significant with p = 0.03 in all cases.
growth is not affected by inhibition of the RAF/ERK pathway. Therefore, the ability of cells to respond to EGF appears to depend entirely on the JNK/SAPK pathway to the exclusion of the RAF/ERK pathway. If the RAF/ERK pathway mediates basal growth and not EGF-stimulated growth while the JNK/SAPK pathway is required for EGF-stimulated growth and not basal growth, it would be expected that the inhibitory effects of PD98059 and dnJUN expression would be entirely additive. This effect is shown in Fig. 9C. The proliferation of dnJUN14 cells was examined in the presence and absence of rhEGF with increasing concentrations of PD98059. As for the parental cells, basal growth is inhibited by PD98059 in a dose-dependent manner. However, unlike parental cells (Fig. 9B), dnJUN14 cells that express the highest amount of dominant negative inhibitor of c-JUN protein failed to respond to rhEGF even in the absence of PD98059. Moreover, the proliferation rate of dnJUN14 is diminished with increasing concentrations of PD98059 very similar to the decrease in basal growth of the parental cells in the presence and absence of rhEGF (Fig. 9C). The proliferation at 20 μM PD98059 is significantly reduced (p < 0.03) both in presence and absence of rhEGF. Thus, the addition of PD98059 to A549 cells provides a complete dissociation of the role of the RAF/ERK and JNK/SAPK pathways. Taken together, these results indicate that for A549 cells the JNK/SAPK pathway is required for EGF stimulation of growth but is not required for proliferation in low serum, whereas RAF/ERK pathway does not mediate EGF-dependent proliferation but plays a role in basal growth of A549 cells.

DISCUSSION

EGF is an important growth factor of numerous human tumor cell lines (see Introduction). A major intracellular effector of EGF is RAS protooncogene which activates MAPK pathways (2, 16, 62). Considerable evidence suggests that EGF is mediated through RAF/ERK and JNK/SAPK pathways. Taken together, these results indicate that for A549 cells the JNK/SAPK pathway is required for EGF stimulation of growth but is not required for proliferation in low serum, whereas RAF/ERK pathway does not mediate EGF-dependent proliferation but plays a role in basal growth of A549 cells.
These two protooncogenes have been reported to be essential for JNK/SAPK activation by EGF and are also implicated in transformation (27–28, 30, 69) and cell cycle progression through G1 (70). Little is known regarding a role for JNK/SAPK activation in cell proliferation and transformation of human tumor cells. In this study we have used a well defined transdominant inhibitor of c-JUN, specific antisense oligonucleotides targeted to JNK1 and JNK2, and a well characterized cancer cell line expressing EGF receptors to address a novel function for JNK/SAPK in EGF-induced cell proliferation.

Several reports have shown the important role that EGF plays in autocrine growth of NSCLC cells. We have chosen A549 (NSCLC, adenocarcinoma) as a model. EGF strongly activates JNK/SAPK in these cells. We also observed EGF-induced stimulation of JNK activity in another lung cancer line, M103.

In many studies the activation of JNK by rhEGF, UV-C, or other agents is transient and occurs 30 min after the treatment of the cells. Such transient effects have been argued to be unlikely to maintain prolonged proliferation (62–65). Recently, it has been shown that UV-C can activate JNK in a prolonged way in HeLa cells and NIH3T3 (3, 71). This long effect according to Chen et al. (72) mediates programmed cell death. In the case of A549 cells, the prolonged activation of JNK is correlated with cell proliferation and is consistent with a role of JNK in mediating the 7–10-h long exposure required for effective stimulation of mammalian cells by serum growth factors (62–65). Therefore, it is possible that a different mechanism exists depending on the agent that activates JNK/SAPK, thereby leading to different physiological responses.

The first evidence that JNK/SAPK is required for transformation was obtained using primary mouse fibroblasts, with the use of a transdominant inhibitor protein, c-JUN(S63A,S73A) (7). C-JUN(S63A,S73A) blocks the pathway by overexpressing a protein mutated on the phosphorylation sites of c-JUN. The extension that the JNK/SAPK pathway is a major growth-promoting mechanism in human A549 lung carcinoma cells is reinforced by recent studies showing that the Rho proteins, Rac1 and CDC42, are required for RAS-transformed NIH3T3 cells (27, 69). These proteins stimulate the activity of JNK/SAPK pathway (31) and mediate RAS transformation. Moreover, recently Clark et al. (30) demonstrated inhibition of focus formation by RAS-transformed NIH3T3 cells upon expression of a dominant negative inhibitor of JNK kinase. These observations provide further evidence that activation of the JNK/SAPK pathway mediates transformation by activated RAS. The issue of whether or not the JNK/SAPK pathway plays a similar role in human cancer cells has not been addressed. Our study clearly suggests a role for JNK/SAPK pathway in EGF-stimulated proliferation of A549 NSCLC. This is consistent with recent reports showing that EGF is important for cell growth in this type of cancer and that EGF is commonly an autocrine proliferation factor of NSCLC (73). Moreover, it correlates with the fact that increased expression of c-JUN is an early event in human lung carcinogenesis and that c-JUN has a role in mediating the effects of growth factors during tumor development (74–75).

We have used a dominant negative of c-JUN, dnJUN, to dissect the JUN kinase pathway. This derivative has been characterized and successfully used in a number of previous studies (4, 7–8). In addition, overexpression of dnJUN even for the clones with the highest expression observed here has no effect on the basal growth of A549 cells in low serum or complete medium showing that this derivative does not have nonspecific growth inhibitory properties. Similarly, overexpression of dnJUN does not alter the enzyme activity of JUN kinase (cf. Fig. 7) showing that the derivative acts at a point distal to JUN kinase in the JNK/SAPK signal transduction pathway consistent with inhibition of AP-1 transactivation function as previously shown (7–8). Moreover, the interference in the JNK/SAPK pathway by dnJUN and sensitization of PC3 cells to genotoxic damage by cisplatin is very similar to sensitization by another well characterized dominant negative c-JUN inhibitor, TAM-67 (4).

To specifically block JNK/SAPK pathway we have used antisense oligonucleotides. Antisense oligonucleotides have already been used in several studies in A549 cells to inhibit protein kinase C-α (57, 58) and c-RAF kinase expression (59).

Recently, oligonucleotides directed against JNK1 were used in U937 cells to block JNK1 expression (76). In our study we used a combination of oligonucleotides targeted to JNK1 and JNK2 to achieve complete inhibition of JNK activity. We demonstrated here that EGF stimulation of JNK/SAPK pathway is completely eliminated by using these oligonucleotides. We also showed that this reduction correlates with the inhibition of EGF-stimulated growth in A549 cells. The same effect is observed with dnJUN showing that blocking phosphorylation of the transactivating domain of c-JUN and JNK activity resulted in the same effect in cells stimulated by EGF. Thus, the use of specific antisense oligonucleotides strongly reinforces the conclusions based on the dnJUN expressing cells that JNK mediates EGF-stimulated growth in A549 lung carcinoma cells.

The potential role that RAF/ERK pathway plays in transformation and growth cannot be ruled out. Minden et al. (16) have shown that a differential activation of RAF/ERK and JNK/SAPK exists in HeLa cells and MRC5 (human fibroblasts). For example UV-C is a very strong activator of JNK/SAPK and a poor inducer of RAF/ERK, whereas TPA and EGF preferentially activate the RAF/ERK pathway. EGF also activates JNK/SAPK pathway to various extents depending on cell type. We used a specific inhibitor of RAF/ERK pathway (PD98059) to block the activation of RAF/ERK by EGF (52–53). This compound inhibits differentiation (66) and cell growth of NIH3T3 but not NIH3T3 cells expressing EGF receptor (52). In our case, A459 basal growth is inhibited by this agent, thereby showing that this pathway is important for basal proliferation of these cells. However, the EGF effect on growth was not blocked by this inhibitor even though we found it inhibits ERK enzyme activity. This demonstrates that the RAF/ERK pathway is not important for EGF stimulation of growth in A459 cells. In contrast, the JNK/SAPK pathway is essential to EGF-stimulated proliferation in A549 cells. We have not examined the p38 MAPK pathway that shares many of the same stimuli as the JNK/SAPK pathway and, therefore, cannot rule out a role of this pathway in EGF-stimulation of growth.

This study provides the first evidence for a significant growth-promoting role of JNK/SAPK in human cancer cells. The observation that additional cell lines such as M103 exhibit EGF activation of JNK while other human lung carcinoma lines such as H1011 exhibit constitutive activation of this kinase indicates that JNK/SAPK may be an important growth-promoting pathway in NSCLC. These observations suggest several readily testable predictions that may be helpful in evaluating the generality of the conclusions.

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REFERENCES
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