c-Jun N-terminal Kinase Is Essential for Growth of Human T98G Glioblastoma Cells*

The c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway is activated by numerous cellular stresses. Although it has been implicated in mediating apoptosis and growth factor signaling, its role in regulating cell growth is not yet clear. Here, the influence of JNK on basal (unstimulated) growth of human tumor glioblastoma T98G cells was investigated using highly specific JNK antisense oligonucleotides to inhibit JNK expression. Transient depletion of either JNK1 or JNK2 suppressed cell growth associated with an inhibition of DNA synthesis and cell cycle arrest in S phase. The growth-inhibitory potency of JNK2 antisense (IC50 = 0.14 μM) was greater than that of JNK1 antisense (IC50 = 0.37 μM), suggesting that JNK2 plays a dominant role in regulating growth of T98G cells. Indeed, JNK2 antisense-treated populations exhibited greater inhibition of DNA synthesis and accumulation of S-phase cells than did the JNK1 antisense-treated cultures, with a significant proportion of these cells detaching from the tissue culture plate. JNK2 (but not JNK1) antisense-treated cultures exhibited marked elevation in the expression of the cyclin-dependent kinase inhibitor p21cip1/waf1 accompanied by inhibition of Cdk2/Cdc2 kinase activities. Taken together, these results indicate that JNK is required for growth of T98G cells in nonstress conditions and that p21cip1/waf1 may contribute to the sustained growth arrest of JNK2-depleted T98G cultures.

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The abbreviations used: JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; JNKAS, JNK antisense oligonucleotides; JNK1AS and JNK2AS, antisense oligonucleotides targeting expression of JNK1 or JNK2, respectively; DAPI, 4′,6-diamidino-2-phenylindole; MTS, (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; PI, propidium iodide; UV-C, ultraviolet light C band.
out as an exception in that we failed to see any apoptosis following treatment with JNK antisense oligonucleotides (JNKA). The present study was initiated to further investigate the role of JNK in regulating basal growth (normal culture conditions) of human glioblastoma T98G cells. We demonstrate that targeted inhibition of JNK expression, accomplished through use of specific antisense oligonucleotides, does not result in significant apoptosis of T98G cells but rather results in marked growth suppression that is associated with inhibition of DNA synthesis, cell cycle arrest in G1 phase, and p53-independent induction of the cyclin-dependent kinase inhibitor p21Cip1/Waf1. These findings indicate that JNK is essential for normal growth of T98G cells and suggest that the JNK pathway regulates molecules vital for replication of these tumor cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Metabolic Labeling—Human glioblastoma T98G cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), supplemented with 10% fetal bovine serum (Irvine Scientific). For metabolic protein labeling, cells were incubated in methionine-free, cysteine-free, and serum-free medium for 4 h in the presence of 200 µCi of Translabel™ (ICN Chemical) containing carrier-free [35S]methionine/cysteine. Immediately after incubation, cells were washed with cold phosphate-buffered saline and lysed in 0.5 ml radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors (0.15 mM NaCl, 50 mM Tris-HCl, pH 7.2, 1% deoxycholic acid, 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 10 mM NaF and 10 mM sodium pyrophosphate).

Treatment of Cells with Oligonucleotides—All phosphorothioate oligonucleotides used in this study were prepared and characterized by Isis Pharmaceuticals, Inc. as described previously (18, 37). Two oligonucleotides, ISIS12359 (5'-CTTCGCTGAGCGCCCTTGGG-3') and ISIS12560 (5'-GTGCAGCCAGGCCCAGTG-3'), termed JNK1AS and JNK2AS, respectively, specifically eliminate the expression of respective JNK proteins (17-19). To control for nonspecific events, “sense” sequence oligonucleotides (JNKS1 (ISIS14320) and JNKS2 (ISIS14318), and “scrambled” sequence oligonucleotides (JNK1Scr (ISIS14321) and JNK2Scr (ISIS14319)) with the same base composition as the antisense oligonucleotides, but in arbitrary order, were employed. Oligonucleotides were delivered to cells by lipofection as described below. Briefly, solutions of lipofectin were prepared by mixing 10 µg/ml Lipofectin™ (Life Technologies, Inc.) reagent in minimal essential medium (Life Technologies, Inc.) with an equal volume of oligonucleotide solution, incubating this mixture at room temperature for 15 min and diluting it with Lipofectin™ solution to a final oligonucleotide concentration of 0.4 µM. Cells were incubated with the Lipofectin™-oligonucleotide solution at 37 °C in 10% CO2 for 12–16 h, after which they were washed once with serum-free medium and cultured in complete medium.

Cell Growth and DNA Synthesis Assays—To determine the proliferation rates, cells were seeded at 25 × 104 cells/ml in six-well cluster plates, and growth was assessed by daily cell counting ( Coulter counter) in triplicate. For determination of cell viability and assessment of DNA synthesis, cells were seeded at 1–5 × 105 cells/ml in 6-well tissue culture plates. The viable cell mass was determined by the method of Church and Gilbert (38), and hybridization signals were visualized and quantified using a PhosphorImager (Molecular Dynamics). To monitor the quality of sample loading and transfer, membranes were hybridized to a 24-base oligonucleotide 3'-ACCGATATCT-GATCGTTCTCGAACC-5' complementary to 18 S RNA.

For Western analyses, 50 µg of cell–whole-cell lysates (18) were size-fractionated in 12% SDS-PAGE and transferred onto poly(vinylidene difluoride) membranes. Proteins were detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech) following incubation of the polyvinylidene difluoride membranes with specific antibodies (Santa Cruz Biotechnology).

Flow Cytometric Analyses—Cell cycle distribution was analyzed using propidium iodide (PI)-stained cells. Briefly, 2–5 × 106 cells were fixed in 70% ethanol, incubated with 1 µg/ml RNase A, stained with 1 wash propidium iodide (Roche Molecular Biochemicals), and analyzed on a FACSscaltor flow cytometer (Becton Dickinson). The percentages of cells in the various stages of the cell cycle were determined using the ModFitLT software program.

S-phase cells were detected using 5-bromo-2′-deoxyuridine (BrdUrd) Labeling and Detection Kit 1 (Roche Molecular Biochemicals) as described by the manufacturer with some modifications. Briefly, the cells were treated with oligonucleotides and 24 h later pulsed with 10 µCi of BrdUrd labeling reagent to allow incorporation of BrdUrd into DNA in cells. The cells were fixed for 24 h with 70% ethanol diluted in glycine buffer (50 mM glycine, 0.01% Triton X-100, 0.15 mM NaCl, pH 2.0) and incubated with anti-BrdUrd monoclonal antibodies. After incubation with anti-mouse Ig-fluorescein, bound anti-BrdUrd monoclonal antibodies were visualized by fluorescence-activated cell sorting analysis.

The number of apoptotic cells following treatment with JNKA was determined using an APO-BrdUrd™ kit (Pharmingen) following the manufacturer’s protocol. Briefly, 1–2 × 106 cells were fixed in 1% methanol-free formaldehyde following incubation in 70% ethanol at −20 °C. To detect genomic DNA degradation, bromodeoxyuridine triphosphate was incorporated into DNA breaks by terminal deoxynucleotidyl transferase enzyme. Finally, cells were stained with fluorescein-labeled anti-BrdUrd antibody to detect DNA breaks and with PI/RNase A solution for counterstaining of the total DNA. Dual parameter display was used to assess the number of apoptotic cells following analysis on FACSscaltor flow cytometer.

In Vitro Kinase Activity Assays—To detect JNK activation following UV-C exposure, JNKA-treated and control cultures were irradiated with 40 J/m2 UV-C 24 h postlipofection, washed with cold phosphate-buffered saline 30 min later, and suspended in whole-cell extract buffer (18). An in vitro JNK kinase assay was performed using a fusion protein containing glutathione linked to the 1–222 fragment of human c-Jun (GST-c-Jun) as substrate, as described (6). Briefly, 50 µg of cell lysate was incubated for 3 h at 4 °C with 10 µg of GST-c-Jun bound to glutathione-Sepharose-4B (Amersham Pharmacia Biotech). After washes three times in whole-cell extract buffer and once in kinase reaction buffer (20 mM HEPES, pH 7.7, 20 mM MgCl2, 20 mM β-glycerophosphate, 20 mM β-mercaptoethanol, 0.1 mM sodium vanadate, 2 mM dithiothreitol, the beads were incubated with 30 µl of kinase reaction buffer containing 20 µl ATP and 5 µCi of [α-32P]ATP for 30 min at 30 °C. The reaction was stopped by the addition of Laemmli sample buffer. Samples were boiled for 5 min and resolved by electrophoresis through 12% polyacrylamide-SDS gels (SDS-PAGE).

Northern and Western Analyses—Total RNA was isolated from treated cells using RNA Stat-60 (Tel-Test B) according to the manufacturer’s instructions. RNA (30 µg/ml) was size-separated in agarose-formaldehyde gels and transferred to GeneScreen Plus nylon membranes (NEN Life Science Products). JNK1 and JNK2 cDNAs, isolated from pBSJNK2–1×HA and 3×HA-JNK1SR3, respectively, were labeled with [α-32P]dATP using a random primer labeling kit (Roche Molecular Biochemicals). Hybridization and washes were performed by

the method of Church and Gilbert (38), and hybridization signals were visualized and quantified using a PhosphorImager (Molecular Dynamics). To monitor the quality of sample loading and transfer, membranes were hybridized to a 24-base oligonucleotide 3'-ACCGATATCT-GATCGTTCTCGAACC-5' complementary to 18 S RNA. For Western analyses, 50 µg of cell–whole-cell lysates (18) were size-fractionated in 12% SDS-PAGE and transferred onto poly(vinylidene difluoride) membranes. Proteins were detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech) following incubation of the polyvinylidene difluoride membranes with specific antibodies (Santa Cruz Biotechnology).
Fig. 1. Efficiency of phosphorothioate oligonucleotide uptake in T98G cells. T98G cells at 50% confluence were incubated with 0.4 μM of 3'-fluorescein isothiocyanate-labeled control phosphorothioate oligonucleotide in the presence of 10 μg/ml Lipofectin™ reagent (Life Technologies, Inc.) as described under “Experimental Procedures.” The cells were fixed 24 h following treatment (~80% confluent culture) and subjected to confocal fluorescence microscopy. Nearly 100% of cells showed uptake of the fluorescent oligonucleotide. Image was taken at × 800 magnification.

RESULTS

Specific Inhibition of JNK1 and JNK2 Expression by JNKAS—To examine a role for the JNK pathway in mediating basal growth of T98G cells and to test whether there is a preferential role for JNK1 or JNK2, we employed specific JNK1 and JNK2 antisense oligonucleotides (18–20). Preliminary experiments using a control fluorescein isothiocyanate-labeled oligonucleotide (c-rafISIS1315S) revealed that the efficiency of uptake of the phosphorothioate oligonucleotide, delivered as described under “Experimental Procedures,” was nearly 100% (Fig. 1). The specificity of the elimination of JNK1 and JNK2 mRNAs by JNKAS in T98G cells is shown in Fig. 2. While neither mock treatment nor treatment with control “scrambled” (JNKScr) oligonucleotides had any effect on JNK mRNA levels in T98G cells, treatment with JNK1AS and JNK2AS resulted in >95% elimination of the respective mRNAs 24 h postlipofection (Fig. 2A). Although the JNK1 and JNK2 mRNA levels have largely recovered by 72 h, an effective “window of observation” was provided by the antisense treatment as indicated in Fig. 2B (shaded area). The efficiency of inhibition of JNK expression was confirmed by Western analysis (Fig. 3A). Major JNK isoforms migrate with apparent molecular masses of 46 and 54 kDa. The slower migrating proteins (p54JNK) are largely, but not exclusively, composed of the JNK2 isoforms, whereas the faster migrating proteins (p46JNK) are largely, but not exclusively, composed of JNK1 isoforms (1). Treatment with JNK1AS and JNK2AS markedly attenuated the 46- and 54-kDa components, respectively (Fig. 3A), consistent with the known distribution of JNK isoforms. Time course studies of 35S-metabolically labeled T98G cells indicated that the half-lives for degradation of JNK1 and JNK2 proteins are 14 and 3.4 h, respectively (Fig. 3B). This is consistent with the marked reduction in steady-state levels of both JNK proteins (a reflection of both reduced synthesis and degradation of existing proteins) between 30 and 50 h post-treatment (data not shown). Determination of kinase activity in cells irradiated with UV-C (40 J/m²) 24 h postlipofection revealed a markedly lower level of JNK activation in JNKAS-treated cells relative to control cells (Fig. 3C).

Growth Inhibition of T98G Cells Treated with JNKAS—To determine whether inhibition of JNK has an effect on the growth of T98G cells, we assessed the viability of T98G cells over a 24–120-h time period following JNKAS treatment. Viable cell mass was measured both using an MTS-based colorimetric assay and by direct cell counts. The viability of mock- and JNKScr-treated cultures was very similar to that of untreated cells over the entire time course (Fig. 4, A and B). In contrast, treatment with either JNK1AS or JNK2AS led to a marked reduction in viable cell mass. Growth suppression following treatment with JNK2AS was more pronounced than seen with JNK1AS treatment (~40% versus 65% that of controls at 24 h postlipofection, respectively), especially at later time points (~15% versus 75%, respectively, at 120 h postlipofection), although statistically significant differences in growth inhibition by both JNKAS were observed as early as 24 h postlipofection. As determined by direct counting from 24–72 h postlipofection, the number of viable cells in attached JNK1AS- and JNK2AS-treated cultures was significantly diminished relative to that of control cultures (Fig. 4C). Consistent with the transient loss in JNK expression, JNK1AS-treated cells resumed growth at later times with cell numbers recovering to 70% of that seen in control cultures by day 5 (Fig. 4B). JNK2AS-treated cells exhibited a greater loss in cell mass than their JNK1AS-treated counterparts at all times, and, unlike that seen with JNK1AS, their growth did not recover with time. Five days post-lipofection, the viable cell mass of the JNK2AS-treated population was 15% of that seen for control groups (Fig. 4B). In summary, although the magnitude of the growth inhibition achieved by JNKAS differed somewhat depending on the method used to assess growth (MTS assay versus cell counting), the general observation of a growth inhibition in response to JNKAS treatment was apparent regardless of the method employed.

Fig. 2. Transient elimination of JNK mRNA expression following JNKAS treatment. A, Northern blot analysis of T98G cells after lipofection with a 0.4 μM concentration of the indicated oligonucleotides (0.2 μM concentration of each oligonucleotide when used in combination). The cells were collected for analysis at the designated times, and RNA was prepared using Stat-60 reagent as described under “Experimental Procedures.” Hybridization with a probe recognizing 18 S RNA was used to assess the differences in loading and transfer among RNA samples. B, kinetics of suppression and reappearance of mRNA. The shaded area indicates the period of time when the significant suppression of the corresponding mRNA was achieved as determined in Fig. 2A.
To examine the dose dependence for growth inhibition by JNK1AS and JNK2AS treatment, cells were incubated with various amounts of antisense oligonucleotides ranging from 0 to 0.4 μM. The total amount of oligonucleotides added to cells was kept constant by the addition of appropriate concentrations of corresponding JNKScr oligonucleotides. Both JNK1AS and JNK2AS exhibited their growth-inhibitory effects in a dose-dependent manner (Fig. 5). Cells treated with JNK2AS exhibited a markedly steeper decline in viable cell mass (JNK2 IC50 = 0.14 μM) compared with cells treated with JNK1AS (JNK1 IC50 = 0.37 μM). These effects were not apparent in cells treated with similar doses of JNK1Scr and JNK2Scr oligonucleotides or in cells receiving 0.4 μM JNKScr. Thus, the inhibitory properties of JNKAS are manifested in a dose-dependent manner and do not reflect cytotoxicity of oligonucleotide treatment per se but rather the susceptibility of T98G cells to perturbations in JNK expression.

FIG. 3. Depletion of JNK1 and JNK2 proteins with JNKAS treatment. A, attenuation of JNK protein levels 24 h postlipofection. T98G cells were treated with 0.4 μM oligonucleotides (0.2 μM each in combinations). One hundred-μg aliquots of whole-cell lysates were processed for Western blot analysis, and JNK levels were detected using SC-571 JNK antibodies (Santa Cruz Biotechnology). B, half-life determination of JNK1 and JNK2. T98G cells were metabolically labeled for 4 h with [35S]methionine as described under “Experimental Procedures,” and the amount of labeled JNK proteins at designated times was determined by immunoprecipitation, separation by SDS-PAGE, and visualization by autoradiography (lower panel). JNK1-specific antibodies SC-479 (Santa Cruz Biotechnology) were used to detect JNK1 levels, and SC-571 antibodies were used for detection of JNK2 levels in JNK1-depleted lysates. The log function of normalized intensities of the resulting bands for each protein was plotted as a function of the time after the end of the metabolic labeling period. Slopes (least squares analysis) were used to calculate the half-lives. This experiment was repeated three times, and data from a representative experiment are shown. C, inhibition of JNK activation in JNK-depleted T98G cells. Cells treated with a combination of 0.2 μM each JNK1AS and JNK2AS (labeled JNKAS) or 0.2 μM each JNK1Scr and JNK2Scr (labeled JNKScr) were exposed to 40 J/m² UV-C (lanes labeled +) or left untreated (–) 24 h postlipofection. JNK activity was measured 30 min later by in vitro kinase assay as described under “Experimental Procedures.”

FIG. 4. Depletion of JNK results in growth inhibition of T98G cells. A and B, assessment of the viable cell mass by MTS tetrazolium dye conversion at 24 h (A) and 120 h (B) following treatment with oligonucleotides. T98G cells were seeded in 96-well tissue culture plates at a density of 1000 cells/well and transfected with 0.4 μM of the indicated oligonucleotides the following day. The MTS dye reduction was determined by measurement of optical density at 490 nm. Cell viability was expressed as a percentage of viable cell mass in mock-treated cultures. Cells treated with JNK1AS and JNK2AS exhibit statistically significant inhibition of growth compared with all controls (p < 0.001, Student’s t test) and differ among themselves as early as 24 h postlipofection (*, p < 0.001). C, effect of JNKAS treatment on cell number and portion of attached cells. Cells were seeded in six-well cluster plates at 25 × 10³ cells/cm² and treated with 0.4 μM oligonucleotides. Attached cells (open portion of bars) and cells in medium (closed portion of bars) were counted in triplicate (Coulter counter) 24, 48, and 72 h after lipofection. S.E. values were <10% in all cases.

The Growth Inhibition in JNK-depleted T98G Cultures Is Associated with Cell Cycle Arrest and Inhibition of DNA Synthesis—At least two possible cellular responses could account for the reduction in viable cell mass seen following treatment with JNKAS: decreased DNA synthesis and/or selective death of JNKAS-treated cells. First, we examined the treated cells for evidence of apoptosis. JNKAS-treated cultures, particularly JNK2AS-treated cultures, exhibited some morphological alterations frequently seen in cells undergoing apoptosis including fewer mitoses, rounded up cells (Fig. 6), and detachment of many cells (up to 30%) from the plate surface (Fig. 4C). Detached cells were examined for viability. Up to 90% of detached cells in JNKAS-treated cultures excluded trypan blue and were therefore living cells, although they did not reattach or grow when placed in fresh medium in new dishes (data not shown).

Four additional methods were used to assess apoptosis in JNKAS-treated cells including detection of apoptotic bodies in DAPI-stained cells and various types of flow cytometric analy-
sis: i.e. cell cycle distribution of PI-stained cells, detection of BrdUrd incorporation into cellular DNA breaks (APO-BRDU™), and detection of annexin V-stained populations. Detection of DNA fragmentation by DAPI staining revealed no evidence of apoptotic cells in any treatment groups through 72 h postlipofection (Figs. 7 and 8, data not shown). Interestingly, the structure of nuclei in JNKAS-treated cells differed from that of either mock-treated cultures or cells treated with JNK-Scr oligonucleotides (Fig. 7A). The cause of these alterations is not known, but they may reflect the presence of cells with doubled DNA content that are unable to finish progression through the S phase and mitosis following JNKAS treatment.

No cells with condensed or fragmented nuclei indicative of apoptosis were evident in any treatment group. More sensitive methods for assessing apoptosis based on detection of BrdUrd incorporation into DNA breaks (APO-BRDU) (Fig. 7B) and detection of specific membrane alterations using annexin V (data not shown) also showed no evidence of apoptosis in any of the treatment groups up to 48 h. Although a small fraction of JNK2AS-treated cells (2.9%) did appear to undergo apoptosis at 48–72 h (data not shown), this percentage of apoptotic cells is similar to that seen when assaying the negative control provided with the kit (3.3%). Thus, while JNK2-depletion might result in a low level of apoptosis in T98G cells, our results indicate that apoptosis cannot account for the magnitude of the reduction in viable cell mass seen in JNK2AS-treated cultures.

While no evidence for apoptosis of JNKAS-treated cultures was found, JNKAS treatment resulted in profound alterations in the distribution of cells throughout the cell cycle, indicative of cell cycle arrest (Fig. 8). The percentages of cells in G1, S, and G2/M phases for untreated and control cultures subjected to mock-lipofection or treatment with control oligonucleotides (JNKScr) were similar: G1 = 63 ± 7%; S = 23 ± 6%; G2/M = 15 ± 6% (average data from three independent experiments; Fig. 8, A and C). In contrast, lipofection with either JNK1AS or JNK2AS resulted in a 2-fold reduction in the number of cells in the G1 compartment and an increase in the number of cells in other cell cycle compartments. Interestingly, while JNK1AS-treated cells accumulated in both S and G2/M phases (35 ± 5.5%; 31 ± 4%, respectively), JNK2AS-treated cultures displayed an accumulation of cells exclusively in S phase (50 ± 2%). Additional analysis of distribution of cells through the cell cycle was done using a BrdUrd pulse incorporation assay, which allows detection of S-phase cells by the presence of BrdUrd in their cellular DNA (Fig. 8B). For control populations (mock- and JNKScr-treated cells) and for JNK1AS-treated cultures, the percentage of S-phase cells determined with this method (23 and 35%, respectively) were similar to those obtained with PI staining (22 and 34.5%, respectively; data were analyzed using ModFitLT software). However, in the case of JNK2AS-treated cells, BrdUrd incorporation analysis revealed much lower number of S-phase cells than PI staining (28 versus 50%). This discrepancy is likely to reflect the fact that T98G cells are not actively progressing though S phase and thus are not incorporating BrdUrd due to JNK2AS inhibitory influence.

Fig. 5. Growth inhibition by JNK1AS and JNK2AS is dose-dependent. T98G cells were seeded at a density of 1000 cells/well in 96-well tissue culture plates and treated 24 h later with the indicated amounts of JNKAS and JNKS oligonucleotides. The total amount of oligonucleotides added to each well was kept constant (0.4 μM) by the addition of either JNK1Scr or JNK2Scr. Three days later, the viable cell mass was determined by the addition of MTS as described under “Experimental Procedures.” Broken lines indicate trend lines for growth inhibition by a corresponding oligonucleotide.

Fig. 6. Morphology of JNKAS-treated cells. Microscopic (phase-contrast) appearance of T98G cells is shown at various times following their lipofection with 0.4 μM of indicated oligonucleotides. Untreated, mock-treated, and JNK-Scr-treated control cultures were indistinguishable at all times tested (24 h postlipofection data are shown in the left panels). JNKAS-treated cultures (right panels), particularly JNK2AS-treated cultures, exhibited morphological alterations accentuated with time. Photographs were taken at a × 400 magnification.
on DNA replication (see below).

To further explore the nature of the growth arrest by JNKAS, we directly examined DNA synthesis in JNKAS-treated cultures. As assessed by uptake of [3H]thymidine, DNA synthesis was greatly inhibited in both JNK1AS- and JNK2AS-treated populations (Fig. 9). The effect was greater for JNK2AS-treated cultures, which exhibited nearly complete inhibition of DNA synthesis by 24 h postlipofection (Fig. 9A).

Interestingly, while DNA synthesis in JNK1AS-treated cells started to recover by 72 h postlipofection (consistent with the return of JNK1 expression and transient reduction in viable cell mass; Figs. 1 and 4), no such recovery was observed in JNK2AS-treated cultures (Fig. 9B). This is consistent with the lack of recovery in viable cell mass of JNK2AS-treated cultures noted above (Fig. 4). Thus, while transient depletion of JNK1 led to a transient inhibition of DNA synthesis and reduction in growth of T98G cells, similar elimination of JNK2 expression led to sustained inhibition of DNA synthesis and permanent growth arrest of T98G cells.

Inhibition of Cell Growth by JNKAS Is Associated with In-
FIG. 10. Induction of p21cip1/waf1 expression in JNK2AS-treated cells and associated inhibition of cyclin-dependent kinase activities. A, Northern analysis of p21cip1/waf1 gene expression 24 h post-treatment with 0.4 μM of either JNK1AS, JNK2AS, JNK1Scr, or JNK2Scr oligonucleotides. RNA was prepared using Stat-60 reagent as described under “Experimental Procedures.” Hybridization with a probe recognizing 18 S RNA was used to assess the quality of loading and transfer among samples. B, Western blot analysis for expression of cell cycle regulatory proteins in JNK1AS- and JNK2AS-treated cultures. Cells were harvested 24 h after lipofection, and 100 μg of whole-cell extracts were analyzed. C, inhibition of Cdk2/Cdc2 kinase activities in JNK1AS- and JNK2AS-treated cultures. In vitro kinase assays were performed as described under “Experimental Procedures” after immunoprecipitating complexes containing Cdk2 and Cdc2 and using Histone H1 as a substrate. The sample labeled −H1 depicts a control reaction without substrate.

**Discussion**

The role of the JNK pathway in mediating cellular responses to extracellular stimuli has been studied extensively over the past several years, and the proapoptotic function of activated JNK is supported by numerous studies (1–3, 39). However, evidence supportive of a prosurvival role for the JNK pathway has also been documented (21, 30, 40). One plausible explanation for these seemingly disparate effects is that JNK serves different functions under normal growth conditions and during stress. Support for this view has come from findings that, in contrast to activated (phosphorylated) JNK, which activates and stabilizes its substrates during stress, nonactivated (non-phosphorylated) JNK targets them to degradation (reviewed in Ref. 4). Regulation of normal cell growth may also require basal JNK kinase activity. Indeed, N-terminal phosphorylation of c-Jun has been implicated in the regulation of cell growth both in vitro and in vivo (41).

Using high affinity and high specificity phosphorothioate antisense oligonucleotides targeting JNK1 and JNK2 (18, 19), we have demonstrated that inhibition of JNK1 and JNK2 expression in otherwise unstressed human T98G glioblastoma cells results in marked suppression of growth. The inhibitory effect seen with JNK2AS was much greater than that observed with JNK1AS (Figs. 4 and 5), possibly reflecting specific functions of JNK2 not shared by JNK1. However, physical properties of JNK proteins such as their half-lives could also influence the outcome. The half-life of JNK1 is considerably longer than that of JNK2 (Fig. 3B), and significant elimination of JNK1 protein (<20% of steady-state level) is not achieved until approximately 40 h after JNKAS treatment. Thus, we cannot completely exclude the possibility that a more prolonged depletion of JNK1 in T98G cells would lead to effects comparable with those observed here with depletion of JNK2. In any event, examination of several other human tumor lines has revealed a similarly critical role of JNK2 for cell growth. For instance, JNK2 is required for epidermal growth factor-stimulated growth of A549 cells in soft agar (18, 19), and JNK2AS, but not JNK1AS, treatment of mice bearing established xenografts of human PC3 cells was found to inhibit tumor growth (35).

Our findings reported here with T98G cells differ from our recent observations in several other tumor cell models in which we found that cells deficient of p53 function undergo apoptosis in response to JNK2AS treatment (36). The survival of cells with wild type p53 status was associated with elevated levels of p21cip1/waf1 expression, which was not seen in p53-deficient cells. The HCT116 cells lacking p21cip1/waf1 (51) underwent extensive apoptosis in response to JNK2AS treatment (36). These findings support the hypothesis that p21cip1/waf1 plays an important role in p53-mediated protection of tumor cells from JNK2AS effects. Although exhibiting marked growth arrest, T98G did not undergo apoptosis following JNK2AS treatment (Figs. 7 and 8) despite the absence of functional p53 in these cells. Moreover, only JNK2AS-treated T98G cells were found to express elevated levels of p21cip1/waf1 (Fig. 10), suggesting that induction of p21cip1/waf1 may be executed by a p53-independent mechanism resulting in cell cycle arrest and protection of T98G cells from apoptosis. While the mechanisms(s) contributing to the induction of p21cip1/waf1 and downstream effects remains to be determined, it is clear that these effects are p53-independent.

Although we found that about 10% of JNK2AS-treated T98G cells detached from the tissue culture plate 24 h postlipofection (Fig. 4C), no apoptosis was detected in the detached populations. The vast majority of these cells were found to be viable based on the criterion of trypan blue dye exclusion (data not shown). The percentage of detached cells further increased
with time (30% by 72 h postlipofection), suggesting a possible role for JNK2 in regulating cell adhesion. Indeed, using the same JNKAS, others have reported JNK2AS-specific inhibition of tumor necrosis factor-α-mediated induction in the expression of E-selectin (17), a protein involved in cell adhesion. The role of the JNK pathway in anoikis, apopotic cell death resulting from detachment, has been postulated but remains controversial (42, 43). Although we cannot exclude the possibility that the majority of JNK2AS-treated cells may eventually die by apoptosis subsequent to their detachment from the extracellular matrix, this effect was not detected at the times examined in this study.

We have shown that JNK depletion in T98G cells results in a dramatic inhibition of DNA synthesis (Fig. 9) associated with accumulation of cells in the S and G2 phases of the cell cycle (Fig. 8). In previous studies, we observed that inhibition of c-Jun N-terminal phosphorylation in T98G cells results in impaired DNA repair (40), suggesting that an intact JNK pathway is required for normal DNA repair. Together with our current findings, these observations indicate a possible role for JNK in DNA repair during progression of cells through late S phase. Other components of the DNA replication and/or cell cycle machinery could also be downstream targets of JNK. The Cdk inhibitor p21cip1/

waf1,

which was specifically elevated only in JNK2AS-treated cells, is an attractive target of JNK2 signaling. However, it is likely to be an indirect target, since we have noted that p21cip1/waf1 expression remains elevated up to 72 h after JNKAS treatment associated with sustained growth arrest, although JNK expression has nearly returned to pretreatment levels (data not shown; Fig. 2). It is worth noting that a p21cip1/waf1 expression has been linked to S-phase arrest in several other model systems including hypoxia (44) and treatment of cells with the retinoid CD437 (45).

The phenotype of JNK2AS-treated cells shares certain characteristics with the “permanent” cycle arrest observed following γ-irradiation of human fibroblasts (46, 47). Like JNK2AS-treated cells, these fibroblasts express elevated levels of p21cip1/waf1 and are unable to synthesize DNA or divide for extended time, but they remain viable by the criterion of trypan blue exclusion. Induction of p21cip1/waf1 plays an important role in growth arrest of p53-deficient cells such as T98G (48), as was previously observed in human astrocytoma cells (49). Whether p21cip1/waf1 induction is required for the growth-inhibitory effects seen with JNK2AS remains to be proven. We have found that transient overexpression of p21cip1/waf1 in T98G cells in the absence of JNKAS treatment results in G1-phase arrest rather than S-phase arrest (data not shown). This observation strongly suggests that the growth-inhibitory effects of JNK2AS treatment are not limited to induction of p21cip1/waf1 but must act in collaboration with other JNK2AS-regulated factors to cause S-phase arrest. Further studies are under way to identify these components.

Unlike T98G cells and several other human cancer cell lines we have examined, JNKAS treatment is not growth-inhibitory for normal prostate epithelial cells (data not shown). Likewise, although double knockouts of JNK1 and JNK2 are not viable, cells derived from either JNK1 or JNK2 knockout mice grow normally (50, 51). These observations suggest that JNK1 and JNK2 possess redundant functions (and can therefore compensate for each other) and that neither is crucial for the growth of nontransformed cells. Thus, the requirement of JNK2 for growth may be accentuated in tumor cells. The recent development of procedures to generate somatic gene knockouts in human tumor cells (52, 53) provides the tools to investigate the effects of abrogating JNK expression in human cancer cells. While further investigations are necessary to better understand the role of JNK in regulating tumor cell growth and to identify the specific JNK targets involved, our findings highlight the importance of JNK for maintaining tumor cell homeostasis in the absence of overt stress and suggest that strategies aimed at eliminating JNK could have a therapeutic benefit.

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(1997) Cell 90, 315–323
43. Khwaja, A., and Downward, J. (1997) J. Cell Biol. 139, 1017–1023
44. Shenberger, J. S., and Dixon, P. S. (1999) Am. J. Respir. Cell Mol. Biol. 21, 395–402
45. Zhang, Y., Rishi, A. K., Dawson, M. I., Tschang, R., Farhana, L., Boyanapalli, M., Reichert, U., Shroot, B., Van Buren, E. C., Fontana, J. A. (2000) Cancer Res. 60, 2025–2032
46. Di Leonardo, A., Linke, S. P., Clarkin, K., and Wahl, G. M. (1994) Genes Dev. 8, 2540–2551
47. Noda, A., Ning, Y., Venable, S. F., Pereira-Smith, O. M., and Smith, J. R. (1994) Exp. Cell Res. 211, 90–98
48. Mercer, W. E., Shields, M. T., Amin, M., Sauve, G. J., Appella, E., Romano, J. W., and Ullrich, S. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6166–6170
49. Toms, S. A., Herbergs, A., Liu, J., Kondo, S., Barnett, G. H., Casey, G., and Barna, B. P. (1998) Anticancer Res. 18, 289–293
50. Dong, C., Yang, D. D., Wysh, M., Whitmarsh, A. J., Davis, R. J., and Flavell, R. A. (1998) Science 282, 2092–2095
51. Yang, D. D., Conze, D., Whitmarsh, A. J., Barrett, T., Davis, R. J., and Rinein, M. (1998) Cell 9, 575–585
52. Waldman, T., Kindler, K. W., and Vogelstein, B. (1995) Cancer Res. 55, 5187–5190
53. Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kindler, K. W., and Vogelstein, B. (1998) Science 282, 1497–1501