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# Regulation of Platinum-Compound Cytotoxicity by the c-Jun N-Terminal Kinase and c-Jun Signaling Pathway in Small-Cell Lung Cancer Cells

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## ABSTRACT

Cytotoxic platinum compounds including cisplatin are standard cancer chemotherapeutics and are also activators of stress-signaling pathways. In this study, we tested the role of the c-Jun N-terminal kinase (JNK) family of mitogen-activated protein kinases and their transcription factor target, c-Jun, in the cytotoxic response of small-cell lung cancer (SCLC) cells to cisplatin and its less effective *trans*-isomer, transplatin. Both agents stimulated JNK activity; the transplatin response was rapid and transient, whereas JNK activation by cisplatin was delayed and sustained. Despite the differential kinetics of JNK activation, expression of nonphosphorylatable JNK mutants sensitized the SCLC cells to killing by cisplatin or transplatin, suggesting that JNK activation in response to these agents signals a protective response. Consistent with this finding, overexpression of the JNK target, c-Jun, significantly protected SCLC cells from platinum compounds, whereas expression of a c-Jun mutant encoding only the DNA binding domain increased the sensitivity of the SCLC cells to these drugs. These findings support the hypothesis that activation of the JNKs by platinum compounds controls c-Jun-dependent transcriptional events that promote a protective response in SCLC cells. Oligonucleotide array analysis identified genes encoding a variety of signaling proteins whose expression was reciprocally changed by c-Jun and c-Jun-DBD (c-Jun-DNA binding domain). It is noteworthy that genes whose products are involved in DNA repair, glutathione synthesis, or drug accumulation did not exhibit altered expression by c-Jun or c-Jun-DBD. The findings indicate that inhibition of the JNK pathway is a potential means to enhance the sensitivity of SCLC cells to platinum compounds.

Small cell lung cancer (SCLC) is an aggressive form of lung cancer that displays rapid proliferation and extensive metastasis, requiring a chemotherapeutic treatment. The platinum compounds including cisplatin represent a widely used family of cytotoxic drugs for the treatment of lung cancers (Bunn and Carney, 1997). Cisplatin reacts with DNA to form adducts leading to the inhibition of DNA replication and transcription, an event essential for its cytotoxic activity (Crul et al., 1997; Jordan and Carmo-Fonseca, 2000). An isomer of cisplatin, transplatin, is significantly less cytotoxic than cisplatin and is almost ineffective as a chemotherapeutic drug despite the ability of transplatin to also form DNA adducts and block DNA replication. The distinct toxicities of cisplatin and transplatin probably relate to their relative ease of repair through the nucleotide excision repair pathway. Repair of cisplatin adducts is highly inefficient, whereas transplatin adducts are much more efficiently repaired (Jordan and Carmo-Fonseca, 2000). Although SCLC frequently responds to cytotoxic platinum compounds, these lung cancers are rarely cured because of intrinsic and acquired resistance of the tumor cells to these cytotoxic insults (Carney et al., 1983; Carmichael et al., 1989).

At the molecular level, platinum-compound DNA adduct formation stimulates signal pathways that induce cell-cycle arrest and apoptosis (Crul et al., 1997; Jordan and Carmo-Fonseca, 2000). Cellular resistance to cisplatin is a major clinical problem and limits the effectiveness of cisplatin as a chemotherapeutic agent. Cellular mechanisms that are proposed to limit the action of cisplatin on tumor cells include decreased cellular accumulation of the drug, increased drug inactivation by intracellular thiols such as glutathione, and increased DNA adduct repair (Crul et al., 1997; Jordan and Carmo-Fonseca, 2000). Although acquired resistance to cisplatin in vivo is likely to be multifactorial, increased DNA repair is considered to be the first and most frequent re-

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**ABBREVIATIONS:** JNK, c-Jun N-terminal kinase; SCLC, small-cell lung cancer; IL2R$\beta$, interleukin-2 receptor $\beta$; ERK, extracellular signal-regulated kinase; c-Jun-DBD, c-Jun DNA binding domain; IGF, insulin-like growth factor; COX-1, cyclooxygenase-1; MAP, mitogen-activated protein; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; PM, perfect match; MM, mismatch; MES, 2-(*N*-morpholino)ethanesulfonic acid; APF, alanine-proline-phenyalanine.



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sponse to cisplatin exposure. Repair of cisplatin DNA adducts occurs primarily by the nucleotide excision repair complex that is composed of multiple proteins, some of which are defective in the inherited disease xeroderma pigmentosum (Crul et al., 1997). Moreover, the induction of nucleotide excision repair pathway components XPA, XPE, and ERCC1 have all been noted to occur in cisplatin-resistant cells.

Another cellular response to cisplatin is activation of the c-Jun N-terminal kinases (JNKs), members of the mitogen-activated protein (MAP) kinase family of enzymes that are widely activated by diverse cell stresses (Kyriakis and Avruch, 1996; Ip and Davis, 1998). JNKs phosphorylate several transcription factors including c-Jun, ATF2, Elk-1, and p53 and stimulate their transcriptional activity (Hazzalin and Mahadevan, 2002; Whitmarsh and Davis, 2000). The strong and prolonged JNK activation in response to a variety of stresses, including UV and ionizing radiation and chemotherapeutic agents, suggest that this pathway may mediate cytotoxic responses of cells to DNA damage. Yet the precise role of JNK activation in cell fate after platinum-compound exposure remains unclear. In the present study, we demonstrate a role for the JNK pathway in promoting a protective response in SCLC cells exposed to cytotoxic platinum compounds and identify genes whose expression is controlled by the JNK and c-Jun pathway that may serve as novel effectors of the protective response.

## Materials and Methods

**Cell Culture and Retrovirus-Mediated Gene Transfer.** SCLC cell line SHP77 exhibits features of both classic and variant SCLC and was cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 50 $\mu$g/ml streptomycin, 50 units/ml penicillin, and 1% tylosin. SHP77 cells stably expressing HA-JNK1-APF and HA-JNK2-APF have been described previously (Butterfield et al., 1997). The cDNAs encoding full-length c-Jun and the c-Jun amino acids 224 through 332 encoding the DNA binding domain (c-Jun-DBD) were ligated between the *Hin*dIII and *Hpa*I sites of the LNCX retroviral vector (Miller and Rosman, 1989). After retroviral packaging of the LNCX vectors in 293T cells (Beekman et al., 1998; Butterfield et al., 1997), SHP77 cells expressing the constructs were generated by retrovirus-mediated gene transfer and selection in medium containing G418. Pooled G418-resistant cell populations were used for all experiments.

**Assay of Cell Viability and Apoptosis.** Cell viability was defined by trypan blue exclusion. Parental or transfected cell lines were seeded in 24-well plates at a density of 25,000 cells per well. Twenty-four hours later, the cells were treated with 0 to 100 $\mu$M cisplatin or transplatin for 24 h. Cisplatin (Sigma Chemical, St. Louis, MO) was initially dissolved in dimethyl sulfoxide at a concentration of 200 mM, whereas transplatin was dissolved in sterile water at 200 mM. Dilution of these solutions was made into media to obtain the desired drug concentrations (10–100 $\mu$M). Control treatments contained 0.1% dimethyl sulfoxide. Equal portions of cell suspensions and 0.4% trypan blue in phosphate-buffered saline were mixed, and the number of cells excluding the dye was counted with use of a hemacytometer.

Morphological detection of apoptosis was performed by May-Grünwald/Giemsa staining of the cells (Levresse et al., 1998). After 24-h exposure to either cisplatin or transplatin, cells were fixed in methanol/acetic acid (3:1) and stained for 15 min with 3% May-Grünwald/Giemsa. At least 2000 cells were examined microscopically, and those presenting blebs, chromatin condensation, or nuclear fragmentation were defined as apoptotic cells.

**Assay of JNK Activity.** After treatments, SCLC cells were collected by centrifugation and lysed at 4°C in 0.5 ml of MAP kinase lysis buffer (50 mM $\beta$-glycerophosphate, pH 7.2, 2 mM $MgCl_2$, 0.1 mM sodium vanadate, 1 mM EGTA, 1 mM dithiothreitol, 0.5% Triton X-100, 2 $\mu$g/ml leupeptin, and 4 $\mu$g/ml aprotinin). After a 5-min microcentrifugation (10,000*g*), aliquots of the extracts containing 200 $\mu$g of proteins were incubated at 4°C for 2 h with GST-c-Jun (1–79) immobilized to glutathione agarose. The GST-c-Jun complexes were washed three times in MAP kinase lysis buffer by repetitive centrifugation (1000*g*) and incubated for 20 min at 30°C in 40 $\mu$l of 50 mM $\beta$-glycerophosphate, pH 7.2, 10 mM $MgCl_2$, 0.1 mM sodium vanadate, 1 mM EGTA, and 20 $\mu$M [$\gamma$-$^{32}$P]ATP (~20,000 cpm/pmol). The reactions were terminated with SDS-PAGE–loading buffer, and the phosphorylated GST-c-Jun polypeptides were resolved by SDS-PAGE, identified by Coomassie blue staining, excised from the gel, and counted in a scintillation counter.

**Immunoblot Analysis.** To prepare whole-cell lysates, cells were collected in the culture medium, washed in ice-cold phosphate-buffered saline, and resuspended in 500 $\mu$l of ice-cold MAP kinase lysis buffer containing 300 mM NaCl. Cells were incubated 30 min at 4°C, mixed vigorously, and clarified at 4°C by microcentrifugation (10,000*g* for 15 min). Aliquots of cell lysates (200 $\mu$g of protein) were mixed with SDS sample buffer, electrophoresed on 10% SDS-PAGE gels, and transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 and 3% nonfat dry milk, then incubated with different antibodies as indicated at 1 $\mu$g/ml for 16 h at 4°C. The filters were extensively washed in Tris-buffered saline containing 0.1% Tween 20, and bound antibodies were visualized with use of alkaline phosphatase–coupled secondary antibodies and Lumi-Phos reagent (Pierce Chemical, Rockford, IL) according to manufacturer's instructions.

**Gene Expression Analysis.** Total RNA was purified from SHP77 cells transfected with LNCX, LNCX-c-Jun, or LNCX-c-Jun-DBD with TRIzol reagent (Invitrogen, Carlsbad, CA), and poly-$A^+$ RNA was further purified from the total RNA with the PolyTract System (Promega, Madison, WI). Two micrograms of poly-$A^+$ RNA was converted to double-stranded cDNA using the Superscript Choice System (Invitrogen) and an oligo-dT primer containing a T7 RNA polymerase promoter (Genset, Paris, France). After second-strand synthesis, the reaction mixture was extracted with phenol-chloroform-isoamyl alcohol, and the cDNA was recovered by ethanol precipitation. Biotin-labeled cRNA was generated by *in vitro* transcription using an ENZO Bioarray High-Yield Kit (Affymetrix, Inc., Santa Clara, CA). A 1.5-$\mu$l aliquot of the cDNA template was transcribed in the presence of a mixture of unlabeled ATP, CTP, GTP, and UTP as well as biotin-labeled CTP and UTP (bio-11-CTP and bio-16-UTP), and the biotin-labeled cRNA was purified using RNeasy affinity columns (QIAGEN, Valencia, CA). To ensure optimal hybridization to the oligonucleotide array, the cRNA was converted to fragments between 35 and 200 bases in length by incubating the cRNA at 94°C for 35 min and then added at a concentration of 0.05 $\mu$g/ml to a hybridization solution containing 100 mM MES, 1 M $Na^+$, and 20 mM EDTA in the presence of 0.01% Tween 20.

With the helpful guidance of the University of Colorado Health Sciences Center Gene Expression core facility, the biotinylated cRNA samples in a volume of 200 $\mu$l were hybridized to Affymetrix GeneChip HuGeneFL arrays for 18 to 20 h and washed and stained with Streptavidin-phycoerythrin according to the manufacturer's specifications. GeneChips were probed with biotinylated cRNA synthesized from two independently prepared poly($A^+$) RNAs from SHP77 cells expressing LNCX, c-Jun, and c-Jun-DBD. The GeneChips were scanned at a resolution of 6 microns with a Gene Array Scanner (Hewlett Packard, Palo Alto, CA), and the data were analyzed with the Affymetrix proprietary software. Each gene on the Affymetrix GeneChip is represented as a probe set composed of 20 distinct perfect match (PM) oligonucleotides as well as 20 corresponding mismatch (MM) oligonucleotides. The difference between PM and MM represents the specific expression signal, and the average of the

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20 separate PM minus MM pairs is the "average difference". In addition to calculating the average difference for each gene, the software provided by Affymetrix calculates statistics to indicate whether a gene transcript is present or absent and whether it is differentially expressed between two chips probed with different samples. Because the probe sets that define certain genes may have one or more MM intensities greater than their corresponding PM intensities caused by nonspecific interactions, a negative average difference is possible.

**Reverse Transcription/Polymerase Chain Reactions.** Total RNA was extracted from SHP77 cells expressing the different c-Jun and JNK-APF constructs with TRIzol reagent, and 0.5 $\mu$g was reverse-transcribed in a volume of 10 $\mu$l with murine leukemia virus reverse transcriptase (PerkinElmer Life Sciences, Boston, MA) and random hexamers according to the manufacturer's specifications. The reverse-transcription products were amplified by PCR in 100-$\mu$l reactions containing AmpliTaq DNA polymerase (PerkinElmer), 1× PCR buffer II (PerkinElmer), 1.7 mM $MgCl_2$, 120 $\mu$M dNTPs, and 1 $\mu$M forward and reverse primers specific for glyceraldehyde-3-phosphate dehydrogenase (forward, 5′-GAAATCCCATCACCATCTTCCAG-3′; reverse, 5′-ATGAGTCCTTCCACGATACCAAAG-3′), semaphorin E (forward, 5′-TGGACTGCGTAGCCTTGTCAAC-3′; reverse, 5′-GAAACCCCTTCATTGGAACTCAC-3′), or gravin (forward, 5′-TTGTCTTCCACCGAGAGCACAG-3′; reverse, 5′-TTGTTCTTGTTTCCCATCTGGC-3′). The glyceraldehyde-3-phosphate dehydrogenase, semaphorin E, and gravin PCR reactions were submitted to 20, 40, and 30 cycles, respectively, of 30 s at 94°C, 30 s at 55°C, and 1.5 min at 72°C. Aliquots of the reactions were resolved on 1.5% agarose gels and stained with ethidium bromide.

## Results

**Relative Cytotoxicity of Platinum Compounds toward SHP77 Cells.** SHP77 cells were treated with different concentrations of cisplatin or transplatin, and viable cells were counted 24 h later. As shown in Fig. 1A, treatment with cisplatin induced a concentration-dependent loss in cell viability ($IC_{50}$ ~40 $\mu$M). Under the same conditions, transplatin was notably less cytotoxic toward SHP77 cells ($IC_{50}$ >100 $\mu$M). In cells exposed to cisplatin for 24 h, the loss of cell viability is accompanied by the induction of apoptosis, reaching 28% of the cells in SHP77 cell cultures exposed to 100 $\mu$M cisplatin (Fig. 1B). Consistent with the weaker cytotoxic effect, transplatin also induced a significantly weaker apoptotic response in SHP77 cells (Fig. 1B). The greater cytotoxicity of cisplatin compared with transplatin in SHP77 cells is consistent with the relative effectiveness of the two drugs as chemotherapeutics despite the ability of both drugs to form DNA adducts. The differential toxicities of the two drugs may relate to the ability of cisplatin, but not transplatin, to form 1,2-intrastrand DNA crosslinks and the greater ease of repair of transplatin-DNA adducts (Crul et al., 1997; Jordan and Carmo-Fonseca, 2000).

**Cisplatin and Transplatin Induce Different Kinetics of JNK Activation.** Cellular JNK activity is stimulated by diverse cytotoxic stimuli (Kyriakis and Avruch, 1996; Ip and Davis, 1998). To assess JNK activity in SHP77 SCLC cells after treatment with cisplatin or transplatin, the endogenous JNKs were adsorbed to glutathione agarose–immobilized GST fusion protein encoding the $NH_2$-terminal 79 amino acids of the transcription factor c-Jun and assayed for their phosphotransferase activity toward the GST-c-Jun polypeptide (see *Materials and Methods*). JNK activity was increased by both cisplatin and the less toxic isomer transplatin, although the kinetics of activation differed (Fig. 2A). Cisplatin measurably increased JNK activity after 4 h of exposure, with maximal activation after 24 h of exposure. In contrast, transplatin induced a rapid and transient increase in JNK activity that was detectable within 1 h and was maximal after 4 h of exposure, with a return to basal activity after longer times of treatment. Analysis of the dose-response for

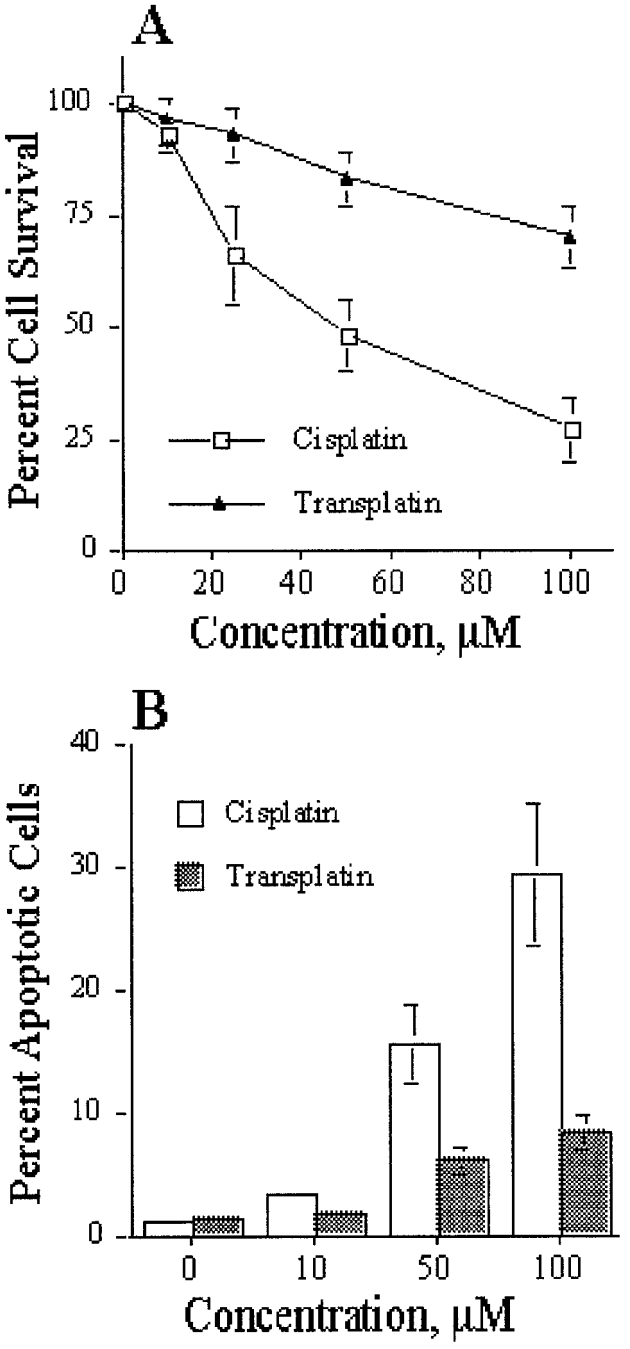


**Fig. 1.** Characterization of the cytotoxic action of platinum compounds in SHP77 cells. A, cells were treated with the indicated concentrations of cisplatin or transplatin for 24 h. Cell viability was assessed by trypan blue exclusion and expressed as the percentage of the number of viable cells counted in the control cultures. The data given are the mean ± S.E.M. of three independent experiments. B, cells were treated with the indicated concentrations of platinum compounds for 24 h. After methanol/acetic acid fixation and May-Grünwald/Giemsa staining, a total of 2000 cells per slide were scored for membrane blebs, chromatin condensation, or nuclear fragmentation. The results are expressed as the percentage of apoptotic cells and are the mean and S.E.M. of three experiments.

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JNK activation by each compound (24 h of exposure for cisplatin and 4 h for transplatin) revealed a concentration-dependent JNK stimulation by both compounds, although cisplatin was a stronger JNK activator than transplatin (Fig. 2B).

These findings are consistent with those from a previous study demonstrating different kinetics of JNK activation by cisplatin and transplatin in mouse keratinocytes (Sanchez-Perez et al., 1998) and suggests that the time course of JNK activation is related to the specific platinum-compound DNA adduct formed by cisplatin or transplatin. In this regard, the level of unrepaired DNA damage could influence the duration of JNK stimulation (Sanchez-Perez et al., 1998). As previously noted, the DNA lesions produced by transplatin are generally considered to be more easily repaired than the damage induced by cisplatin (Lippert, 1996). Thus, the prolonged JNK activation observed in cisplatin-exposed cells may relate to unrepaired DNA damage.

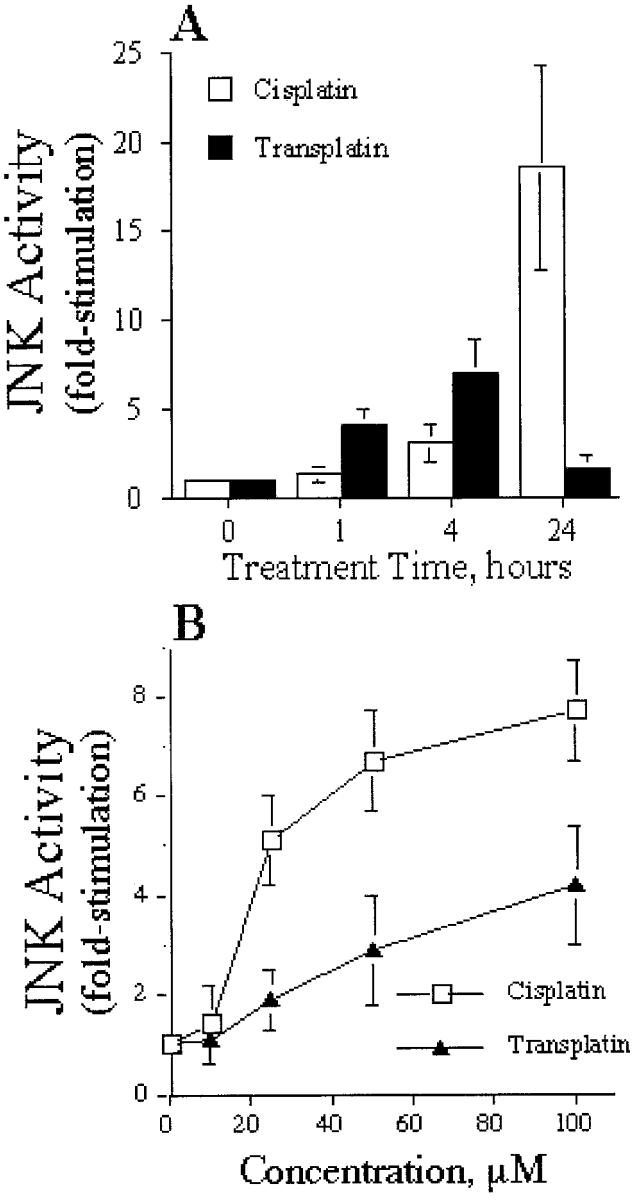


**Fig. 2.** JNK regulation by cisplatin and transplatin in SHP77 cells. A, suspensions of SHP77 cells were exposed to 100 $\mu$M concentrations of each compound for the indicated times, and JNK activity was measured with the GST-c-Jun adsorption assay. The data are expressed as the fold-stimulation over JNK activity measured in untreated controls and are the mean ± S.E.M. of three independent experiments. B, suspensions of SHP77 cells were exposed to the indicated concentrations of cisplatin or transplatin for 24 and 4 h, respectively, and then extracts were prepared and assayed for JNK activity. The data expressed are the mean and S.E.M. fold-stimulation of JNK activity over that activity measured in extracts from control cells.

**Influence of Inhibitory JNK Mutants on JNK Activation and Cell Killing by Platinum Compounds.** The more pronounced JNK activation stimulated by cisplatin correlates with enhanced cell killing, suggesting that the JNK pathway may promote apoptosis in platinum compound–treated SCLC cells. Indeed, JNK activity has previously been invoked as pro-apoptotic in cells treated with cisplatin (Zanke et al., 1996). To directly test the role of JNK activation in the cellular response to cisplatin and transplatin, inhibitory JNK1 and JNK2 mutants were stably expressed in SHP77 cells. Specifically, mutant JNK polypeptides were used in which the phosphorylated threonine and tyrosine within the threonine-proline-tyrosine phosphorylation motif were mutated to alanine and phenylalanine, producing a nonphosphorylatable JNK-APF polypeptide. When sufficiently overexpressed in cells, the JNK-APF polypeptide acts as a competitive inhibitor of JNK signaling (Butterfield et al., 1997; Wojtaszek et al., 1998). We verified the ability of JNK1-APF and JNK2-APF to inhibit JNK activation in response to platinum drugs (Fig. 3). In control cells infected with an empty LNCX retroviral vector, cisplatin and transplatin induced a dose-dependent JNK activation that was similar to the results observed in parental SHP77 cells (Fig. 2B). In JNK-APF–expressing cells, JNK activation by lower concentrations of cisplatin and transplatin was inhibited, whereas the JNK activation stimulated by 50 $\mu$M cisplatin or 100 $\mu$M transplatin was nearly equal to the JNK activation observed in the Neo control cells (Fig. 3), suggesting that the inhibitory action of JNK-APFs can be overcome at higher levels of stress-pathway activation.

We next investigated the influence of these constructs on cell viability after 24 h of exposure to different concentrations of cisplatin and transplatin. Figure 4 shows that SHP77 cells expressing JNK1-APF or JNK2-APF constructs were more sensitive to killing by both platinum compounds compared with the LNCX control cells, although the degree of sensitization was greater for transplatin. A reduced sensitivity of the LNCX controls to cisplatin relative to the parental SHP77 cells (Fig. 1A) was observed, although the transplatin sensitivities of the parental and LNCX SHP77 cells were similar. Note that a significantly enhanced sensitivity of cells expressing JNK1-APF or JNK2-APF cells to platinum compounds was observed at the lower concentrations tested (10 and 25 $\mu$M cisplatin, 25 and 50 $\mu$M transplatin) but not at the highest concentrations (50 $\mu$M cisplatin and 100 $\mu$M transplatin). This finding is consistent with the weak inhibition of JNK activation by JNK-APFs observed at the higher platinum-compound concentrations (Fig. 3). We also performed a limited analysis of the action of the JNK-APF constructs on the platinum-compound sensitivity of the SCLC cell line H345 (data not shown). Compared with SHP77 cells, H345 cells are more sensitive to cisplatin ($IC_{50}$ ~20 $\mu$M) and transplatin ($IC_{50}$ ~80 $\mu$M). Expression of the JNK-APF constructs did not further increase the sensitivity of H345 cells to cisplatin, but JNK1-APF and JNK2-APF reduced the $IC_{50}$ for

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transplatin to 30 and 50 $\mu$M, respectively, relative to an $IC_{50}$ of ~80 $\mu$M for the H345 LNCX line.

**Role of c-Jun in SHP77 Sensitivity to Platinum Compounds.** The findings in Fig. 4 support the hypothesis that the JNK pathway regulates a protective response to cisplatin and transplatin in SCLC cells. The c-Jun protein, a defined transcription factor target of the JNK pathway (Hazzalin and Mahadevan, 2002), is of interest in this regard because recent studies have highlighted the involvement of c-Jun in the cellular response to DNA-damaging agents (Delmastro et al., 1997; Li et al., 1998). To investigate the role of c-Jun as an effector of the JNK pathway in SCLC cells, SHP77 cells overexpressing c-Jun or an inhibitory c-Jun mutant were generated by retrovirus-mediated gene transfer (see *Materials and Methods*). The $NH_2$-terminal transcriptional activation domain of the c-Jun protein is critical for JNK regulation because it encodes the JNK binding domain and the specific sites phosphorylated by the JNKs (Ser 63 and Ser 73). An inhibitory mutant of c-Jun in which the transcriptional activation domain was deleted, leaving only the DNA-binding domain (c-Jun-DBD), was expressed in SHP77 cells. This mutant protein retains the DNA binding properties of c-Jun, but is unable to interact with and become phosphorylated by the JNKs or serve as a transcriptional activator. This mutant constitutes, therefore, a useful approach to investigating the potential role of JNK-dependent c-Jun regulation in SCLC cell response to platinum compounds.

The immunoblot in Fig. 5A demonstrates the relative expression of the c-Jun and c-Jun-DBD proteins in SHP77 cells. Cell survival of these transfected cells was determined by trypan blue exclusion after 24 h of treatment with the indicated concentrations of platinum compounds. SHP77 cells overexpressing c-Jun exhibited markedly reduced killing by cisplatin or transplatin compared with the Neo controls, even at the highest drug concentrations tested (Fig. 5B). Conversely, the expression of the inhibitory c-Jun-DBD mutant modestly but significantly increased the killing of SHP77 cells by both platinum compounds. Similar findings showing sensitization of glioblastoma cells and carcinoma cell lines to cisplatin by expression of inhibitory c-Jun constructs have been reported previously (Potapova et al., 1997), suggesting that the c-Jun transcription factor plays an important role in the induction of a protective response against cytotoxic platinum compounds.

**Identification of Gene Expression Changes in SHP77 Cells Transfected with c-Jun and c-Jun-DBD.** From the findings shown in Figs. 4 and 5, we considered the hypothesis that the JNK and c-Jun pathway control the expression of genes that function to inhibit platinum compound–induced cell death in SCLC cells. We used Affymetrix GeneChips to

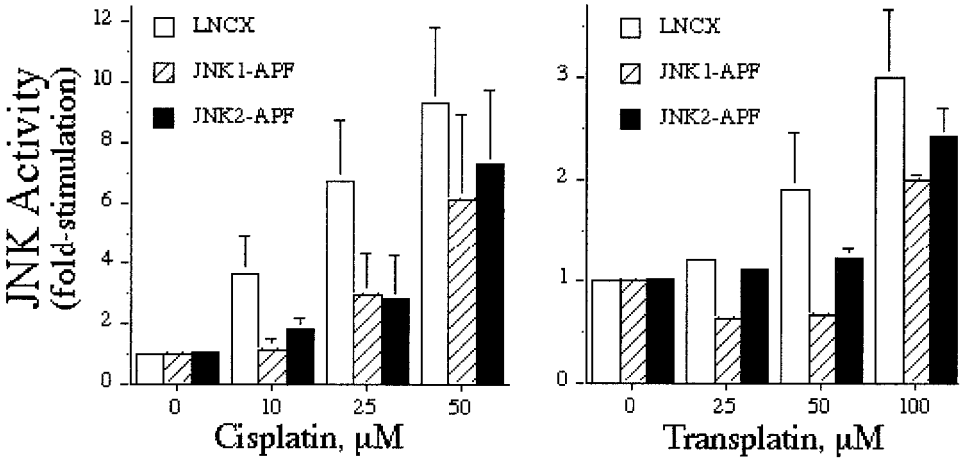


**Fig. 3.** Expression of inhibitory JNK-APF mutants reduce cisplatin- and transplatin-stimulated JNK activity. SHP77 cells expressing JNK1-APF, JNK2-APF, or empty LNCX vector (Neo control) were generated by retrovirus-mediated gene transfer. Cell extracts were prepared from untreated cultures or from cells exposed to either cisplatin or transplatin for 24 or 4 h, respectively. The JNK activity was assayed with the GST-c-Jun binding assay and expressed as the fold-stimulation of activity over that measured in untreated cells. The average fold-stimulation of JNK activity and S.E.M. is shown.

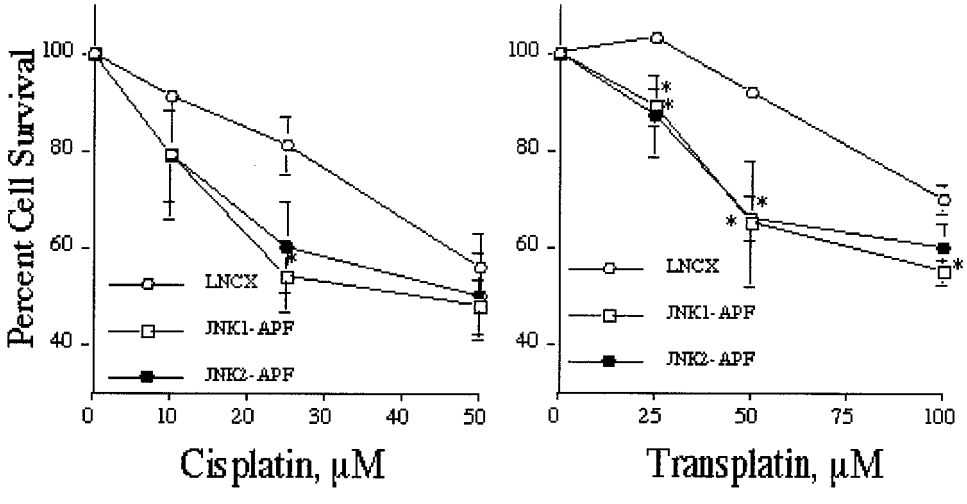


**Fig. 4.** Stable expression of inhibitory JNK-APF mutants in SHP77 cells increases the cytotoxicity of platinum compounds. SHP77 cells stably expressing JNK1-APF, JNK2-APF, or control LNCX cells were exposed for 24 h to the indicated concentrations of cisplatin or transplatin, and viable, trypan blue–excluding cells were counted. Data given are the mean ± S.E.M. of three experiments and are expressed as the percentage of viable cells relative to the viable cells counted in untreated controls. The asterisks indicate a significant difference ($p < 0.05$) from the respective LNCX cell survival value as assessed by two-tailed Student's $t$ test.

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identify genes whose expression was reciprocally changed at least 2-fold in SHP77 cells expressing c-Jun or c-Jun-DBD relative to LNCX-expressing cells. The results from duplicate GeneChips probed with independent biotinylated cRNA are presented in Table 1 and indicate the altered expression of genes whose encoded products largely serve diverse signaling functions within cells. These genes include receptors, transcription factors, low-molecular-weight G-proteins, the SH3-containing protein SH3GL1 (Giachino et al., 1997), gravin, an A-kinase–anchoring protein family member (Nauert et al., 1997), lipocortin/annexin-1 (Wallner et al., 1986), a $Ca^{2+}$/phospholipid binding protein, cyclooxygenase-1 (COX-1), semaphorin E, and phorbolin 1 (Madsen et al., 1999), a protein with a presently undefined function. The reciprocal changes in expression of the insulin-like growth factor-1 receptor (IGF-1R), interleukin-2 receptor $\beta$ (IL2R$\beta$), lipocortin/annexin-1, and COX-1 were confirmed by immunoblot analyses, whereas the changes in semaphorin E and gravin expression were confirmed by RT-PCR (Fig. 6). Note that SHP77 cells transfected with JNK1-APF and JNK2-APF functioned similarly to c-Jun-DBD with regard to the expression of IGF-1R, IL2R$\beta$, lipocortin/annexin-1, and COX-1.

In contrast, expression levels of genes whose products perform defined functions in the recognition or repair of cisplatin-DNA adducts (SSRP1, HMG-1 and -2, XPE, XPA, ERCC1, and MSH2) were not changed among the LNCX, c-Jun, and c-Jun-DBD transfectants (Table 1). Also, the expression of a known antiapoptotic gene, Bcl-2, was not significantly different among the transfected cell lines (Table 1). We performed a series of immunoblot experiments in which the levels of ERCC1, XPA, and MSH2 proteins were measured at 1, 4, 7, and 24 h after the addition of cisplatin or transplatin. Cellular ERCC1, XPA, and MSH2 were readily detected by immunoblot analyses (data not shown), consistent with the GeneChip results. Moreover, none of these proteins were further induced in expression upon treatment of either SHP77 or H345 SCLC cells with cisplatin or transplatin. In addition, SHP77 cells expressing the different c-Jun and JNK-APF constructs, which reciprocally regulate the expression of genes shown in Table 1, exhibited similarly high ERCC1, XPA, or MSH2 expression, again consistent with the negative results observed in the GeneChip experiments. Thus, the results from this experiment strongly suggest that regulation of platinum-compound toxicity in SCLC cells by the JNK and

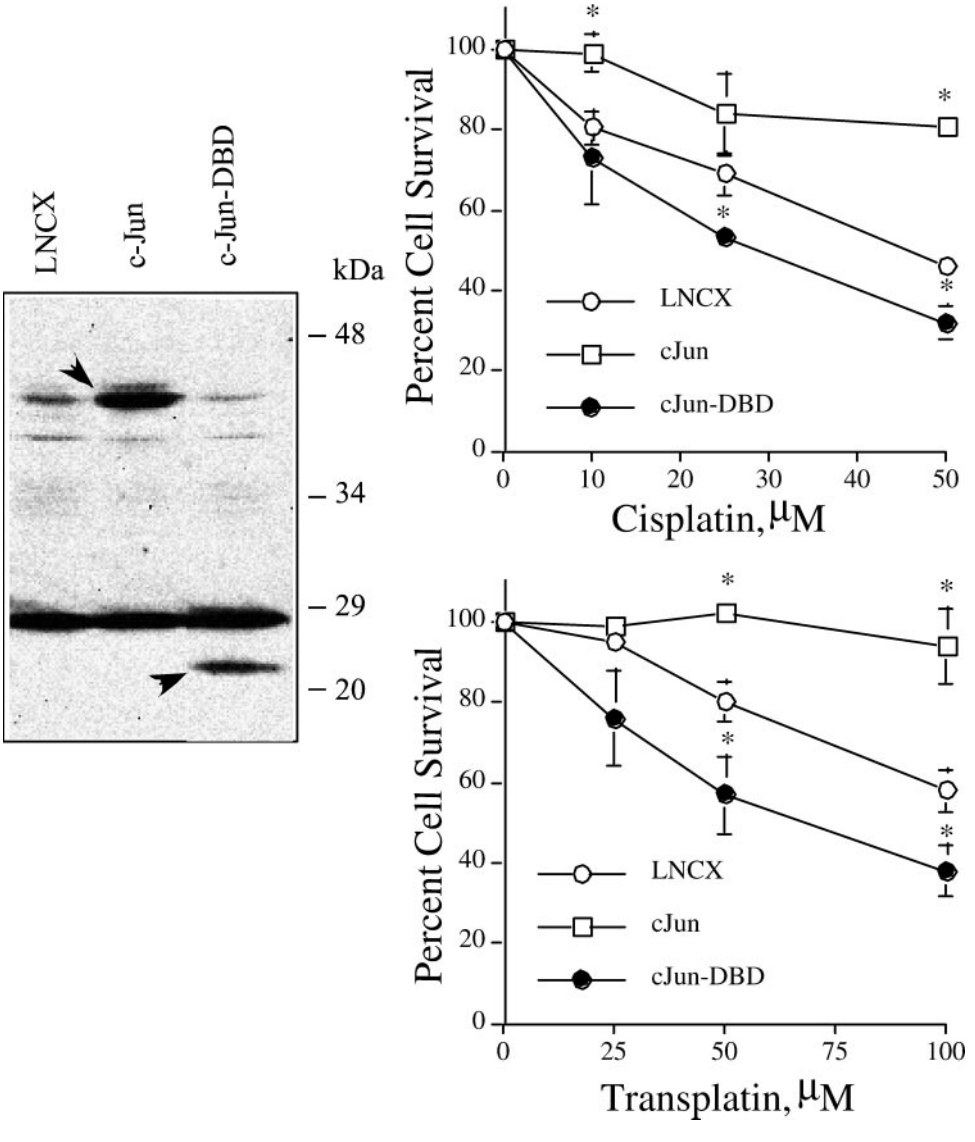


**Fig. 5.** Expression of c-Jun or an inhibitory c-Jun mutant influences the sensitivity of SHP77 cells to platinum compounds. A, SHP77 cells overexpressing c-Jun or expressing the c-Jun-DBD mutant were generated by retrovirus-mediated gene transfer. Total cell extracts were submitted to SDS-PAGE and immunoblotted with an anti-c-Jun antibody directed against the C terminus. B, stably transfected SHP77 cells were treated with the indicated concentrations of cisplatin or transplatin for 24 h, and trypan blue–excluding cells were counted. Data are expressed as the percentage of untreated values and are the mean ± S.E.M. of three independent experiments.

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c-Jun pathway occurs in a manner distinct from the previously defined mechanisms for acquired resistance to cisplatin in tumor cells.

## Discussion

The results presented in this study indicate that the activation of the JNK pathway and c-Jun regulation in response to platinum compounds stimulates a protective response in SCLC cells. Potapova et al. (1997, 2001) have also demonstrated that cisplatin-induced JNK activation and c-Jun regulation promotes cell survival of human glioblastoma and carcinoma cell lines. By contrast, Sanchez-Perez and associates have shown that the JNK and c-Jun pathway is activated after exposure of mouse keratinocytes to platinum compounds and promotes cell death (Sanchez-Perez and Perona, 1999; Sanchez-Perez et al., 1998, 2000). Similarly, Zanke et al. (1996) have demonstrated that the JNK pathway promotes cisplatin-induced cell death in a murine sarcoma cell line. In keeping with these conflicting reports of the role of the JNK pathway in the control of cell death, a deficiency in JNK1 and JNK2 is embryonic lethal in mice and is characterized by a severe deregulation of apoptosis in the developing brain (Kuan et al., 1999; Sabapathy et al., 1999). A reduction in apoptosis is observed in specific hindbrain regions, but increased apoptosis is observed in the forebrain. These discrepancies highlight the presently understood complexity of the cellular roles of the JNK pathway in the responses to cytotoxic stress and the control of apoptosis.

The apparently disparate and opposing actions of the JNK pathway in the response of cells to cytotoxic stresses may reflect differences in the specific cell culture model systems. Our findings and those of Potapova et al. (1997, 2001) which invoke a protective role for the JNK/c-Jun pathway in response to platinum compounds used various human carcinoma and glioblastoma-derived cell lines, whereas the studies showing a proapoptotic action of the JNK pathway used cultured keratinocytes, mouse embryo fibroblasts, human embryonic kidney 293T cells, and a murine sarcoma cell line (Zanke et al., 1996; Sanchez-Perez et al., 1998, 2000; Sanchez-Perez and Perona, 1999). The known JNK polypeptides are encoded by three distinct genes that are alternatively spliced to yield 10 defined polypeptides (Kyriakis et al., 1995; Ip and Davis, 1998). Although the repertoire of JNK isoforms expressed in different cells is only beginning to emerge (Carboni et al., 1998; Dreskin et al., 2001), it is likely that the expression pattern of JNK genes and their splice variants may vary considerably among specific cell types. The findings that mouse embryo fibroblasts lacking JNK1, but not JNK2, are protected from UV-induced DNA fragmentation (Tournier et al., 2000) provides precedent for the specific repertoire of JNK isoform expression influencing the cellular response to cytotoxic stimuli.

In contrast to the protective function of the JNK pathway in SHP77 cells treated with platinum compounds, we previously demonstrated a proapoptotic role for the JNK pathway in SHP77 cells exposed to UV irradiation (Butterfield et al., 1997). Specifically, JNK1-APF inhibited cell death induced by UV, whereas JNK2-APF yielded a null effect. Another possibility for the differing roles of the JNK pathway in the cellular responses to cytotoxic stress such as UV and platinum compounds is a combinatorial activation of the JNKs with other additional signaling pathways. A study in ovarian carcinoma cells demonstrated ERK and JNK regulation in response to cisplatin and observed enhanced cytotoxicity of cisplatin when the ERK pathway was inhibited (Persons et al., 1999). We previously observed that UV irradiation of SCLC cells potently activates the JNKs with little or no activation of the ERK pathway (Butterfield et al., 1997). These results suggest that activation of JNKs in concert with the ERK pathway, for instance, by platinum compounds dictates a cellular response different from JNK activation alone achieved with UV irradiation.

TABLE 1

Genes that were deemed present by the Affymetrix software (at least in the c-Jun samples)

Those genes defined as 'reciprocally regulated genes' are genes whose expression significantly changed 2-fold or greater compared with LNCX. In addition, the tabulated genes are limited to those genes whose expression increased or decreased on both experimental GeneChips compared with both LNCX control GeneChips.

| Accession Number | Gene | Relative Expression | | |
|---|---|---|---|---|
| | | LNCX | c-Jun | c-Jun-DBD |
| M33197 | GAPDH | 44593 | 49088 | 49822 |
| Reciprocally regulated genes | | | | |
| X79780 | Rab11B | 59 | 1770 | −25 |
| S82240 | Rho8 | 426 | 1111 | 180 |
| X99656 | SH3GL1 | 133 | 2627 | −705 |
| U81607 | Gravin | 204 | 1037 | −24 |
| M26062 | IL2R | 321 | 1222 | −130 |
| M25269 | ELK1 | 407 | 1291 | 184 |
| D85131 | Myc-associated zinc finger protein | 276 | 2983 | 538 |
| X04434 | IGF-1R | −37 | 1301 | 92 |
| X05908 | Lipocortin/annexin-1 | 4731 | 8255 | 748 |
| AB000220 | Semaphorin E | 373 | 4568 | 1148 |
| U03891 | Phorbolin 1 | 510 | 2359 | 38 |
| M59979 | COX-1 | 303 | 1704 | −97 |
| DNA repair and antiapoptotic genes | | | | |
| M86737 | SSRP1 | 7861 | 7420 | 6519 |
| D63874 | HMG-1 | 18138 | 15681 | 12913 |
| X62534 | HMG-2 | 5811 | 3136 | 3266 |
| U32986 | XPE | 5804 | 5610 | 6880 |
| D14533 | XPA | 2307 | 912 | 1227 |
| M13194 | ERCC1 | 5351 | 4300 | 4865 |
| U03911 | hMSH2 | 4483 | 2158 | 3267 |
| M14745 | Bcl-2 | 1523 | 904 | 1791 |

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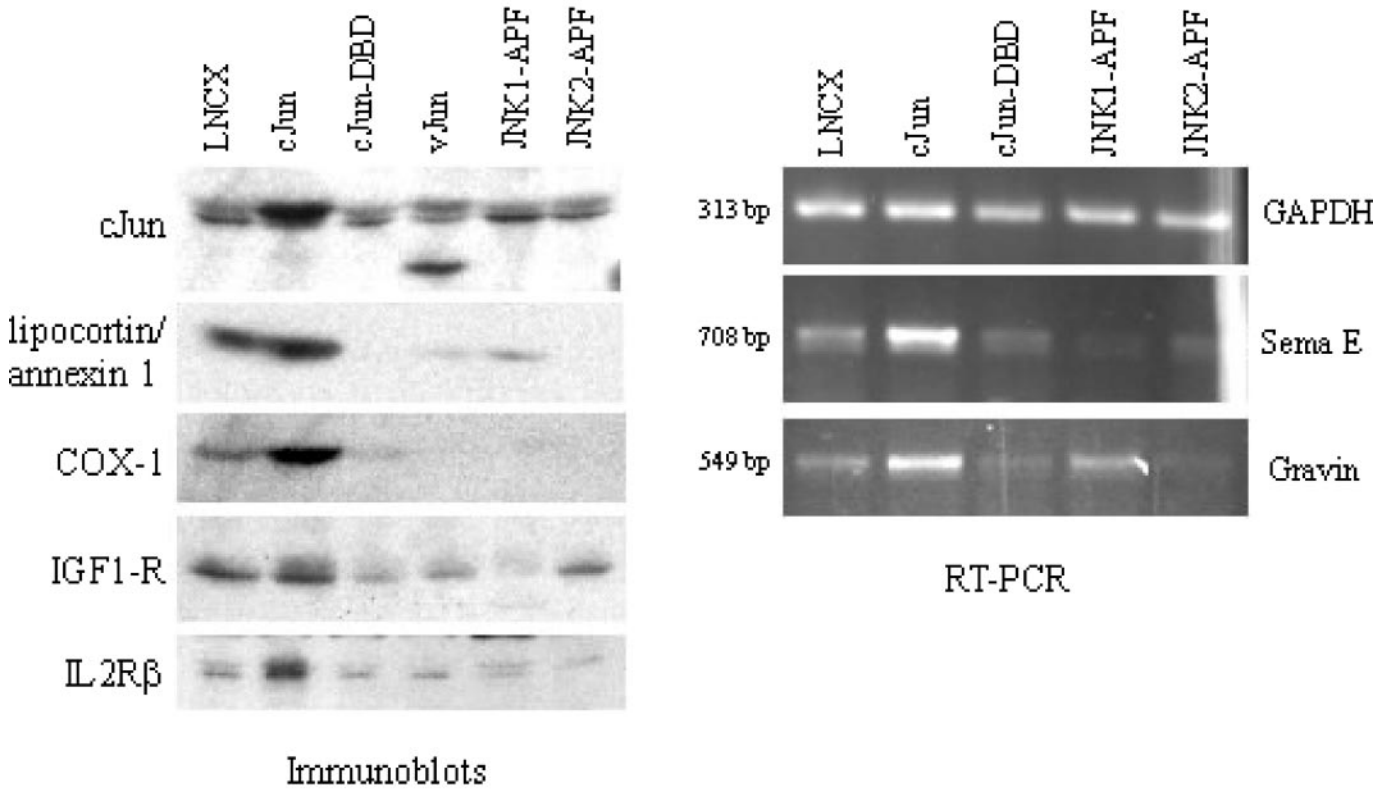


**Fig. 6.** Confirmation of gene expression changes predicted by Affymetrix GeneChip experiments. Left, cell extracts were prepared as described under *Materials and Methods* and immunoblotted with antibodies directed against the indicated proteins. The specific antibodies purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) were sc-44 for c-Jun, sc-12740 for lipocortin/annexin-1, sc-1752 for COX-1, sc-713 for IGF1-R, and sc-671 for IL2Rβ. Right, reverse transcription-PCR analyses were performed on 0.5 μg of total RNA using specific primers and conditions as described under *Materials and Methods*. Portions (25 μl) of the PCR reactions were resolved by electrophoresis on 1.5% agarose gels and stained with ethidium bromide.

Transcriptional regulation by the JNK and c-Jun pathway of genes encoding specific components of the nucleotide excision repair complex as a mechanism for protection of cells from platinum-compound toxicity is an appealing hypothesis. In fact, evidence supports a role for c-Jun in the transcriptional induction of DNA repair (Potapova et al., 1997). Another study has shown that ERCC1, a component of the nucleotide excision repair complex, is induced after cisplatin treatment of ovarian cancer cells in a manner coincident with increased c-Jun expression and phosphorylation as well as c-Jun and c-Fos binding to an AP-1–like site in the 5′-flanking region of the ERCC-1 gene (Li et al., 1998). However, our GeneChip and immunoblotting experiments do not support the induction of DNA repair enzymes as a mechanism for increased platinum-compound resistance in c-Jun-transfected SHP77 cells (Table 1; data not shown). Rather, the reciprocal regulation of diverse genes by c-Jun and c-Jun-DBD with no apparent role in either DNA repair or glutathione metabolism and drug transport was observed. Among the genes regulated by c-Jun and c-Jun-DBD, precedent exists for the potential role of IGF-1R, semaphorin E, and lipocortin/annexin-1 as negative regulators of apoptosis or inducers of drug resistance. IGF-1R signaling leads to protection from apoptosis in many cell types (Adams et al., 2000), and IGF-1 and IGF-2 are widely expressed in SCLC cell lines (Quinn et al., 1996). Thus, changes in the expression of the IGF-1R would be predicted to influence antiapoptotic inputs mediated by IGF autocrine loops in SCLC. Semaphorins comprise a gene family in which prototypic members were found to regulate neural axon guidance. Semaphorin E has been previously identified as a cisplatin-resistance gene in a screen of a drug-resistant ovarian cancer cell line (Yamada et al., 1997). Semaphorin E was overexpressed in a panel of cisplatin-resistant cell lines; its expression was induced by diverse chemotherapeutics, and radiation and transfection of semaphorin E conferred a drug-resistant phenotype to cells previously sensitive to cisplatin (Yamada et al., 1997). Also, enhanced expression of semaphorin E has been noted in lung adenocarcinoma cells with a high metastatic potential (Martin-Satue and Blanco, 1999). Lipocortin/annexin-1 is a $Ca^{2+}$/phospholipid binding protein that was first identified as an inhibitor of phospholipase $A_2$ (Wallner et al., 1986), although its precise function within cells remains controversial. Similar to semaphorin E, induction of annexins has been observed in drug-resistant tumor cell lines (Cole et al., 1992; Sinha et al., 1998). Thus the results from our study indicate that inhibition of platinum compound–induced killing by c-Jun overexpression may be mediated by the IGF-1R, semaphorin E, and lipocortin/annexin-1, which are likely to function distinctly from DNA repair, glutathione metabolism, and decreased drug accumulation.

In conclusion, this study establishes a protective role for the JNK pathway and the transcription factor target, c-Jun, in the setting of platinum-compound treatment of SCLC cells. A noteworthy finding is that transplatin, an ineffective chemotherapeutic in the clinical setting, can be rendered

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significantly more effective toward SCLC cells by inhibition of JNK signaling or c-Jun function. Targeting signal pathways such as the JNKs that mediate inhibition of cell death may be an effective means to enhance the efficacy of existing cancer chemotherapeutics.

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