Resistance to Interleukin 6 in Human Non-Small Cell Lung Carcinoma Cell Lines: Role of Receptor Components

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Abstract
The role of interleukin 6 (IL-6) in regulating the growth of three human non-small cell lung carcinoma (NSCLC) cell lines (NSCLC-3, NSCLC-5, and NSCLC-7, derived from a primary lesion, a brain lesion, and lymph node metastases, respectively) was examined. Although IL-6 alone did not alter the growth of these cells, the addition of soluble IL-6 receptor (sIL-6R) led to the inhibition of proliferation of one of the NSCLC cell lines, NSCLC-5. This antiproliferative effect was neutralized by antibodies to IL-6 and the IL-6R binding and signaling component (gp130). The IL-6-related cytokines, leukemia inhibitory factor and oncostatin M, inhibited proliferation of NSCLC-5 cells but were ineffective in NSCLC-3 and NSCLC-7 cells. NSCLC-7 cells (but not NSCLC-3 or NSCLC-5 cells) secreted biologically active IL-6 and expressed IL-6R. However, antibodies to IL-6 or gp130 failed to alter the proliferation of NSCLC-7 cells. All three cell lines expressed gp130 mRNA and protein. The level of expression of gp130 protein varied in the three cell lines (NSCLC-7 > NSCLC-3 > NSCLC-5). The examination of tyrosine phosphorylation of gp130 (as an early event in IL-6 signal transduction) revealed that gp130 could be phosphorylated in all cell lines after stimulation with IL-6 and/or IL-6 + sIL-6R. These results demonstrate that the mechanisms responsible for IL-6 resistance in different NSCLC cell lines vary and involve defects at either one or more levels of the IL-6 signaling cascade. In the NSCLC-5 cell line, IL-6 resistance (which can be reversed in the presence of sIL-6R) is due to the transcriptional inactivation of the IL-6R gene. In contrast, in the other two cell lines (NSCLC-3 and NSCLC-7), defect(s) in the signaling cascade downstream of gp130 phosphorylation, together with a lack of expression of IL-6R in NSCLC-3 cells, result in IL-6 resistance.

Introduction
Tumor development and progression involve a continual series of complex, heterogeneous changes within the tumor cell and its cellular milieu. These cumulative changes lead to perturbations in the homeostatic mechanisms regulating cell growth that ultimately result in the uncontrolled proliferation of tumor cells (1–4). The mechanisms by which tumor cells acquire growth advantages over normal cells are often due to alterations in the signaling pathways involved in either the production or function of extracellular signals such as growth factors or cytokines. Many of the oncogenes and tumor suppressor genes represent mutated growth factor receptors, key signaling elements, or nuclear transcription factors involved in the signal transduction pathway of these extracellular growth regulatory signals.

IL-6 is an important, pluripotent, growth-regulatory cytokine that is produced by a variety of cell types. IL-6 exerts diverse biological effects and is capable of growth promotion, growth inhibition, and growth differentiation of different cells, including those of the immune system (5–7). IL-6 plays an important role in inflammatory responses and is the key cytokine involved in the induction of most acute-phase proteins (8, 9). Thus, the levels of IL-6 within a given cellular microenvironment, as well as the functional nature of the IL-6 signaling pathway, are critical factors required for regulating normal cell function. The IL-6 signal transduction cascade is initiated after the interaction of IL-6 with its specific cell surface receptor (10), which is a multichain complex comprised of a specific ligand-binding α subunit (IL-6R) and a common signal-transducing β subunit (gp130). The signaling component, gp130, is also used by other cytokine receptor complexes, including LIF, OSM, ciliary neurotropic factor, and IL-11 (10–12).

Aberrant production of IL-6 has been implicated in the pathogenesis of several disease states. For example, in multiple myeloma, IL-6 is thought to act as an autocrine or paracrine growth factor (13, 14). In contrast, in certain other tumors, such as those of leukemia, melanoma, breast, and lung cancer, IL-6 has been shown to exert an inhibitory effect in some in vitro and animal tumor model systems (15–18). Thus, for the latter group of cells, the diminished presence of IL-6 or alterations in its signaling mechanisms could affect

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2 The abbreviations used are: IL-6, interleukin 6; IL-6R, IL-6 receptor α subunit; sIL-6R, soluble IL-6R; NSCLC, non-small cell lung carcinoma; LIF, leukemia inhibitory factor; OSM, oncostatin M; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FBS, fetal bovine serum; RIPA, radioimmunoprecipitation assay.
the negative control of cell growth. Indeed, the expression of IL-6 in some mammary carcinoma cells is reduced compared to that in normal mammary epithelial cells (19). It has also been demonstrated that the response of different-stage cancers to IL-6 varies; e.g., early-stage melanomas are more responsive to the inhibitory effects of IL-6 than late-stage melanomas or metastatic lesions (17). Normal lung cells are more responsive to the inhibitory effect of IL-6 than lung tumors (18). Thus, IL-6 represents a potentially important growth-regulatory cytokine that can directly or indirectly (via stromal and/or immune cells) affect tumor cell proliferation and progression.

The molecular abnormalities responsible for the altered functional role of IL-6 in tumor cells are poorly understood. As a first step in understanding the mechanisms of disruption of IL-6 signal pathways, we examined the role of IL-6 in three different NSCLC cell lines derived from primary and metastatic lesions. We show that although all three cell lines do not respond to IL-6 alone, they differ in the mechanisms by which they acquire resistance to this cytokine.

Results

IL-6 Differentially Regulates Proliferation of NSCLC Cells. IL-6 has been reported to inhibit the growth of lung tumor cells as well as normal bronchial epithelial cells (18). An examination of the effect of varying concentrations of 5–1000 ng/ml of IL-6 on the proliferation of three NSCLC cell lines (NSCLC-3, NSCLC-5, and NSCLC-7) revealed that none of the three cell lines tested responded to IL-6 alone (Fig. 1). However, IL-6 in the presence of sIL-6R (which is capable of augmenting the activity of IL-6 even in cells expressing the receptor) led to a concentration-dependent inhibition of proliferation of one of the NSCLC cell lines, NSCLC-5 (Fig. 2A). A maximum inhibition of 40–60% was observed in the presence of 50 ng/ml of IL-6 and 100 ng/ml of sIL-6R. This effect was neutralized by antibodies to IL-6 (Fig. 2B), IL-6R (data not shown), and gp130 (Fig. 2C). In contrast, the proliferation of NSCLC-3 and NSCLC-7 cells was unaffected by IL-6 in the presence of sIL-6R (Table 1).

We next determined the effect of IL-6-related cytokines LIF, OSM, and IL-11 on the growth of the three NSCLC cell lines. LIF and OSM exerted effects similar to those of IL-6; both were capable of inhibiting proliferation of the NSCLC-5 cells, but not the NSCLC-3 or NSCLC-7 cells (Table 2). However, IL-11 was ineffective in all three cell lines (Table 2).

Production of IL-6 and Expression of IL-6R. Many tumor cells produce IL-6, which could function in an autocrine manner to alter the growth of these cells. We determined whether NSCLC cells produced IL-6 or IL-6R. Neither NSCLC-3 nor NSCLC-5 cells produced IL-6 (Table 3). In contrast, NSCLC-7 cells secreted a significant amount of IL-6 (5–15 ng/10⁶ cells; Table 3). The IL-6 produced by
Table 1  Effect of the combination of IL-6 and IL-6R on proliferation of NSCLC-3 and NSCLC-7 cells

NSCLC-3 and NSCLC-7 cells were treated with IL-6 + sIL-6R as described in the legend to Fig. 1. Data represent the mean of two or three separate experiments, each carried out in quadruplicate (SD < 10%).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[3H]Thymidine incorporation (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (ng/ml)</td>
<td>IL-6R (ng/ml)</td>
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<tr>
<td>-----------</td>
<td>--------</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
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<tr>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>500</td>
</tr>
<tr>
<td>200</td>
<td>400</td>
</tr>
</tbody>
</table>

* ND, not determined.

Table 2  Effect of LIF, OSM, and IL-11 on proliferation of NSCLC cells

NSCLC cells were treated with various concentrations of cytokines as described in the legend to Fig. 1. Data represent the mean of two or three separate experiments, each carried out in quadruplicate (SD < 10%).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Concentration (ng/ml)</th>
<th>[3H]Thymidine incorporation (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>LIF</td>
<td>10</td>
<td>86</td>
</tr>
<tr>
<td>LIF</td>
<td>50</td>
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<tr>
<td>LIF</td>
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<tr>
<td>OSM</td>
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<tr>
<td>IL-11</td>
<td>100</td>
<td>102</td>
</tr>
<tr>
<td>IL-11</td>
<td>200</td>
<td>100</td>
</tr>
</tbody>
</table>

* ND, not determined.

Table 3  Production of IL-6 and IL-6R by NSCLC cells

IL-6 and IL-6R concentrations present in the culture medium (secreted) and in total cell extracts (cellular) were determined by ELISA, as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IL-6 (ng/10^6 cells)</th>
<th>IL-6R (ng/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Secreted</td>
<td>Cellular</td>
</tr>
<tr>
<td>NSCLC-3</td>
<td>ND*</td>
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</tr>
<tr>
<td>NSCLC-5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NSCLC-7</td>
<td>5-15</td>
<td>&lt;0.1</td>
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<td></td>
<td></td>
<td></td>
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</tbody>
</table>

* ND, not detectable.

NSCLC-7 cells was biologically active because it was capable of inducing synthesis of the acute-phase proteins fibrinogen and α1-protease inhibitor in the human hepatoma cell line Hep 3B, and this activity was neutralized by antibodies to IL-6 (data not shown). Similarly, expression of IL-6R mRNA and protein was detected only in the NSCLC-7 cell line (Table 2 and Fig. 3). In the NSCLC-7 cells, IL-6R was present both in the culture medium (0.4-0.6 ng/10^6 cells) and in total cell extracts (0.2-0.3 ng/10^6 cells), indicating that these cells also secreted the soluble form of IL-6R. The amount of IL-6R protein and mRNA present in NSCLC-7 cells was comparable to that present in another IL-6-responsive cell line, Hep 3B (data not shown). Whereas neither NSCLC-3 nor NSCLC-5 cells expressed IL-6R mRNA or protein, the IL-6R gene was transcriptionally inactivated in these cells.

To determine whether IL-6 produced by NSCLC-7 cells was capable of regulating the growth of these cells, we examined the effect of antibodies to IL-6 and gp130 on the proliferation of these cells (Fig. 4A and 4B). Neither anti-IL-6 nor anti-gp130 altered [3H]thymidine incorporation in NSCLC-7 cells in the absence or presence of sIL-6R. This result suggests that the IL-6 secreted by these cells does not regulate growth in an autocrine manner.

Expression of gp130 in NSCLC Cells. After the binding of IL-6 to its receptor, the ligand-receptor complex is associated with gp130, the signal-transducing component of the IL-6 receptor. Determination of the expression of gp130 at the mRNA level by RT-PCR indicated that gp130 mRNA was present in all three NSCLC cell lines (Fig. 5). The expression of gp130 mRNA in an IL-6-responsive cell line (TF1) was used as a control. Immunoblot analyses of gp130 in total cell lysates (Fig. 6) with gp130 antibody directed to the intracellular domain revealed the presence of two forms of gp130.
protein, which were differentially expressed in NSCLC cells. Identification of two forms of gp130 has been reported previously in primary rat hepatocytes (20). The protein was most abundant in the IL-6-nonresponsive cell line (NSCLC-7), whereas it was least abundant in the IL-6-responsive cell line (NSCLC-5). Expression of gp130 in the NSCLC cell lines was also confirmed by fluorescence microscopy of fixed NSCLC cells immunostained with anti-gp130 antibody directed to the intracellular domain and FITC-labeled second antibody (data not shown).

Tyrosine Phosphorylation of gp130. To evaluate whether NSCLC cells are capable of initiating IL-6 signal transduction, we examined tyrosine phosphorylation of gp130 after stimulation of the three cell lines with IL-6 or IL-6 + sIL-6R for 10 min. In the NSCLC-3 and NSCLC-5 cells, which do not express IL-6R, tyrosine phosphorylation of gp130 was observed only after treatment with IL-6 + sIL-6R (Fig. 7). However, in the NSCLC-7 cell line, which expresses IL-6R, tyrosine phosphorylation was observed after both treatments (Fig. 7). The amount of tyrosine phosphorylation was significantly greater in NSCLC-7 cells than in NSCLC-3 or NSCLC-5 cells. This finding is consistent with the observation that NSCLC-7 cells are more abundant in gp130 protein.

Discussion
Tumor progression and metastases are often accompanied by the loss or reversion of the growth-suppressive effects of cytokines such as IL-6, TGF-β, OSM, IL-1, and TNF-α (17, 21, 22). The acquisition of resistance to these inhibitory cytokines that usually occurs in the later stages of tumor progression may, in part, confer tumor cells with a growth advantage by enabling them to escape some of the normal
homeostatic mechanisms regulating negative growth-control. The mechanisms by which tumor cells fail to respond to these cytokines vary and involve loss-of-function mutations in the receptor or components of the signaling pathway (22–24). The present study demonstrates that three different human NSCLC cell lines derived from primary and metastatic lesions display heterogeneity: (a) response to IL-6; (b) production of IL-6 and its receptor components; and (c) mechanisms responsible for IL-6 resistance.

Several studies have demonstrated that IL-6 differentially regulates the growth of various tumor cells. Takizawa et al. (18) reported that IL-6 variably inhibits the growth of established lung tumor cell lines; IL-6 was growth inhibitory in several NSCLC cell lines, but not in small cell lung carcinoma cells. The inhibitory effect of IL-6 in NSCLC cells ranged from 20–50% and was less than that observed in a virally transformed normal bronchial epithelial cell line (BEAS-2B). Differences in IL-6 response reported in melanoma cell lines demonstrated that although early-metastases were growth inhibited by IL-6, advanced-stage metastatic lesions were either resistant to the inhibitory effects of IL-6 or were stimulated by this cytokine (17). The IL-6-resistant cell lines were also resistant to other cytokines, including IL-1, TNF-α, OSM, and TGF-β (17, 21, 23). The NSCLC cell lines in this study, which represent early-stage primary or advanced-stage metastatic tumors, exhibited variable responses to IL-6. Although all three NSCLC cell lines were resistant to IL-6 alone, proliferation of one of these cell lines, NSCLC-5 [derived from an advanced (stage IV) brain metatstatic lesion], was inhibited by IL-6 in the presence of sIL-6R and the cytokines LIF and OSM. In contrast, proliferation of the NSCLC-3 cell line (derived from a stage-I primary lesion) and the NSCLC-7 cell line (derived from a stage-IIIb metastatic lesion of the lymph node) was not affected by IL-6 + sIL-6R, LIF, or OSM. Thus, in the NSCLC model system, resistance to IL-6 and other cytokines, using gp130 as the signaling component of their receptors, does not seem to correlate with tumor progression.

The finding that sIL-6R reverses IL-6 resistance in the NSCLC-5 cell line, which does not express IL-6R protein or mRNA, indicates that the mechanism for IL-6 nonresponsiveness is related to the inability of these cells to express IL-6R and not to defects in downstream events. This conclusion was further substantiated by the observation that two other redundant cytokines, LIF and OSM, which use gp130 and activate signal transduction pathways similar to IL-6, were capable of inhibiting the growth of NSCLC-5 cells. In contrast, in the NSCLC-3 cell line, which also lacks IL-6R, IL-6 resistance was not reversed in the presence of sIL-6R. This result indicates that the mechanism for IL-6 resistance in NSCLC-3 cells involves defects at more than one level: (a) IL-6R expression; and (b) step(s) further downstream in the signal transduction cascade. The resistance of tumor cells to other growth-inhibitory cytokines (such as TGF-β) has also been frequently (but not always) shown to involve reduced receptor expression (24–26). Transfection of the gene for the TGF-β receptor in resistant cells can lead to restoration of TGF-β response in vitro and decreased tumorigenicity in vivo (25, 26).

Altered production of growth signals by tumor cells is often thought to disrupt the balance between negative and positive growth-regulatory mechanisms and to promote tumor progression. In multiple myeloma, increased autocrine or paracrine production of IL-6 is thought to stimulate the growth of tumor cells (13, 14), whereas in some lung and breast tumor cells, diminished production of IL-6 and TGF-β is observed, which is thought to affect the normal negative regulatory mechanisms (18, 19). In addition to the autocrine effect, growth factors also influence tumor growth via paracrine mechanisms by altering the regulatory properties of accessory cells, such as stromal or immune cells (27). The NSCLC cell lines we evaluated also exhibited altered production of IL-6; NSCLC-7 cells produced significantly high levels of IL-6, whereas no IL-6 was detected in NSCLC-3 and NSCLC-5 cells. Because these cells are resistant to IL-6, altered production of this cytokine would seem to influence tumor growth, if any, via paracrine mechanisms. The paracrine influence would also depend on the type of accessory cells present in the tumor microenvironment, which would likely vary at primary and metastatic sites.

An early event in IL-6 signal transduction involves tyrosine phosphorylation of the signal-transducing component of the IL-6 receptor complex, gp130. All three NSCLC cell lines we studied expressed gp130. However, expression of this protein did not correlate with IL-6-responsiveness. The highest levels of gp130 protein were present in the IL-6-nonresponsive cell line (NSCLC-7), whereas the IL-6 + sIL-6R-respon-
sive cell line (NSCLC-5) expressed the least amount of gp130 protein. The gp130 protein present in these cells was capable of being tyrosine-phosphorylated after stimulation. In the NSCLC-3 and NSCLC-5 cells, which lack IL-6R binding subunits, phosphorylation of gp130 was observed only after stimulation with IL-6 + sIL-6R, whereas in the NSCLC-7 cell line, phosphorylation was observed after stimulation with IL-6 and IL-6 + sIL-6R. The amount of phosphorylation in the NSCLC-7 cells was significantly greater than that present in the other two cell lines, which is consistent with the amount of protein present in these cells. Thus, these results suggest that the abnormality responsible for IL-6 resistance in the NSCLC-3 and NSCLC-7 cells does not reside in initial signaling events but involves some step(s) further downstream in the signal transduction cascade.

In conclusion, this study demonstrates that the mechanisms by which tumor cells acquire resistance to IL-6 are heterogeneous and involve defects at different levels in the signaling cascade. Whether acquisition of resistance to IL-6 in NSCLC plays a critical role in tumor progression or metastases remains to be evaluated and may be dependent on the mechanism of IL-6 resistance and the effects of other negative growth-regulatory cytokines. In vitro resistance of tumor cells to the inhibitory effects of IL-6, due to loss of IL-6R expression, may not be sufficient to confer an in vivo growth advantage, because sIL-6R or IL-6-related cytokines (if present in the tumor microenvironment) could restore negative growth-regulation.

Materials and Methods

Cell Culture, Cytokines, and Cytokine Antibodies. NSCLC cell lines were established from tumor specimens of patients undergoing surgery. The three cell lines used in this study represented: (a) undifferentiated large cell carcinoma (stage I) from a lung lesion (NSCLC-3); (b) poorly differentiated carcinomas (stage IV) from a metastatic brain lesion (NSCLC-5); and (c) large cell anaplastic carcinoma (stage IIIb) from lymph node metastases. Cells were maintained in RPMI 1640 supplemented with l-glutamine at 20% FBS at 37°C in a humidified atmosphere of 5% CO2/95% air. Cultures were maintained in vitro for 3 months, and new cultures were subsequently set up from frozen stocks of early passage cells. Recombinant human IL-6 was a gift from Sandoz (East Hanover, NJ). Recombinant human sIL-6R and neutralizing antibodies to human IL-6 and gp130 were purchased from R & D Systems, Inc. (Minneapolis, MN). Anti-human gp130 (directed to the intracellular region) protein A-Agarose were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

Cell Proliferation Assay. Proliferation of NSCLC cells was determined by [3H]thymidine incorporation into DNA. Cells (1–2 × 10^5/well) were plated in 96-well microtiter plates and were incubated for 48 or 72 h at 37°C in the absence or presence of IL-6, sIL-6R, or antibodies to IL-6 or gp130 (either alone or in various combinations) in a total volume of 200 µl of RPMI 1640 supplemented with l-glutamine and 2% FBS. [3H]Thymidine (specific activity of 6.7 Ci/mmol; Dupont New England Nuclear, Boston, MA) was then added at a concentration of 1 µCi/well, and the cells were incubated for an additional 4 h. Cells were then harvested onto glass fiber filters in a cell harvester, and radioactivity incorporated into DNA was determined by liquid scintillation counting.

Determination of IL-6 and IL-6R Levels. NSCLC cells were grown to near confluence and were incubated for an additional 48 h in RPMI 1640 supplemented with l-glutamine and 20% FBS. The culture medium was collected and stored at -20°C, and cells were washed twice with normal saline, were scraped, and were centrifuged. The cell pellet was then solubilized in 20 µl Tris (pH 7.4) containing 1% Triton X-100 and 1 µm phenylmethylsulfonyl fluoride and was sonicated for 30 s. The IL-6 and IL-6R levels in the culture medium and cell extracts were determined by ELISA (R & D Systems, Inc.), according to the manufacturer’s protocol.

RNA Protection Assay for IL-6R. Total RNA was isolated using TRIzol Reagent (Life Technologies Inc., Gaithersburg, MD), which uses the acid-guanidinium isothiocyanate procedure (28). IL-6R cDNA (synthetic 120 oligomer corresponding to nucleotides 89–202, subcloned between Psml and EcoRI in the pGEM 3 vector (gift of Genetics Institute, Boston, MA), was linearized with PvuII, and an anti-sense, high specificity activity (2-3 × 10^6 cpm/µg) RNA probe of 200 nucleotides was transcribed using a[32P]cytidine 5'-triphosphate and SP6 RNA polymerase with the Promega transcription in vitro system (Madison, WI). GAPDH RNA (specific activity of 2-4 × 10^6 cpm/µg) was transcribed from a cDNA cloned in the pT7-2 plasmid kindly provided by Dr. D. Goldthwait, Case Western Reserve University, Cleveland, OH) using T7 RNA polymerase. Total RNA (10–40 µg) was hybridized overnight at 50°C with 1–2 ng of [32P]-labeled IL-6 (100,000–300,000 cpm) and GAPDH (4,000–6,000 cpm) RNA probes in the presence of a 30-µl solution [80% deionized formamide, 1× EDTA, 40 µm PIPES (pH 6.4), and 0.2 M sodium acetate]. The unprotected fragments were digested with 1.8 µg/ml of RNase A and 36 units/ml of RNase T1, for 30 min at 30°C, and the protected fragments were electrophoresed on a 5% polyacrylamide/8 M urea gel and were visualized by autoradiography.

Reverse Transcription-PCR for gp130 mRNA. Reverse transcription was performed on 1 µg of total RNA, followed by PCR amplification. The 5′ oligonucleotide primer corresponded to nucleotides 1767–1791 of the coding strand, and the 3′ oligonucleotide primer corresponded to nucleotides 2490–2556 of the noncoding strand (29). β2-microglobulin primers (30) were included in the reaction mixture to normalize for gp130 PCR products obtained from different NSCLC cells. Thirty-five PCR cycles were performed, and the PCR products were electrophoresed on an 8% polyacrylamide gel. A single amplified product of the predicted size was obtained for gp130 (~690 bp) and β2-microglobulin (~120 bp).

Immunoblot Analyses. Cells grown in T75 flasks were washed twice with normal saline and were lysed in 750 µl of modified RIPA buffer (50 mM Tris (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM Na2VO4, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and 20 µM/mg each of leupeptin, pepstatin, and aprotinin). Cell lysates were rocked at 4°C for 20 min and were centrifuged at 12,000 × g for 15 min. The supernatants were collected and assayed for protein content by the Coomassie Plus assay (Bio-Rad, Hercules, CA), and 40 µg of cell extracts were subjected to SDS-PAGE on 8% polyacrylamide gels and were transferred to nitrocellulose membranes. The membranes were stained with Fastgreen (Bio-Rad), were washed in PBS, were blocked at room temperature for 2 h in 5% nonfat dry milk in PBS containing 0.02% Tween-20, and were incubated overnight at 4°C with anti-gp130 (directed to the intracellular region) or anti-phosphoryrosine (Upstate Biotechnology, Inc.) at concentrations of 1 µg/ml in PBS containing 3% nonfat dry milk. After four washes (15 min each) in PBS containing 0.05% Tween 20, the membrane was incubated at room temperature for 1 h with peroxidase-labeled second antibody (Amersham, Arlington Heights, IL) and was washed as before, and gp130 was detected by enhanced chemiluminescence (Amersham).

Immunoprecipitation. Cells grown in 100-mm tissue culture dishes were stimulated with 50 ng/ml of IL-6 or 50 ng/ml of IL-6 + 100 ng/ml of sIL-6R for 10 min at 37°C. After incubation, the medium was removed immediately, and the cells were washed twice with normal saline and were lysed in 400 µl of modified RIPA buffer. Cell lysates were prepared as described in "Immunoblot Analyses," were incubated with 100 µl of protein A-Agarose (Bio-Rad) at 4°C for 2 h, and were centrifuged, and the supernatant was assayed for total protein. An aliquot (200-µg total protein) of the cell extracts was incubated overnight with 10 µl of anti-human gp130/protein A-Agarose at 4°C. The immunoprecipitates were washed six times with 1 ml of modified RIPA buffer, were boiled in SDS sample buffer, and were subjected to SDS-PAGE on 8% polyacrylamide gels. Immunoblot analyses with anti-phosphothreonine antibodies were carried out as described. The membranes were then stripped in 62.5 µl Tris-HCl (pH 6.7), 100 mM 2-mercaptoethanol, and 2% SDS at 60°C for 30 min and were reprobed with anti-gp130.
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References