Overexpression of PIAS3 Suppresses Cell Growth and Restores the Drug Sensitivity of Human Lung Cancer Cells in Association with PI3-K/Akt Inactivation

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Abstract

Constitutively activated signal transducers and activators of transcription (STAT) are reported to cause uncontrolled transmission of growth signals. In this study, we analyzed the roles of an inhibitor of STAT, protein inhibitor of activated STAT (PIAS) 3, in the development of lung cancer. Treatment with an inhibitor of phosphatidylinositol 3-kinase, LY294002, retarded the growth of human lung cancer cells and rendered them more sensitive to chemotherapeutic agents. However, the inhibition of JAK/STAT by AG490 significantly suppressed cell growth but did not increase drug sensitivity at all. Overexpression of PIAS3 not only significantly inhibited cell growth but also rendered cancer cells up to 12.0-fold more sensitive to the above drugs, which was associated with the suppression of Akt phosphorylation. Inhibition of PIAS3 with small interfering RNA, nevertheless, led cancer cells to accelerate cell proliferation, deteriorate chemosensitivity, and augment Akt phosphorylation. Although the overexpression of suppressors of cytokine signaling 3 in cancer cells also inhibited cell growth and STAT3 phosphorylation, it neither increased sensitivity to chemotherapeutic drugs nor affected the phosphorylation of Akt. These results indicate that PIAS3 may be an attractive candidate for targeting the JAK/STAT and PI3-K/Akt signaling pathways in cancer treatment.

Keywords: Protein inhibitor of activated STAT (PIAS) 3, lung cancer, drug resistance, suppressors of cytokine signaling (SOCS) 3, PI3-K/Akt.

Introduction

Lung cancer is the leading cause of death in adult men in Europe, the United States, and Japan. In 2004, approximately 160,440 Americans died of lung cancer from among 563,700 cancer deaths [1]. About 75% of lung cancers are locally advanced or have metastases. Clinical trials have demonstrated median survival times between 35 and 40 weeks, and 1-year survival rates of 20% to 25% [2–4].

Tyrosine kinase (TK) growth factor receptors on the cell surface are good targets in cancer therapy because signals from these receptors promote cell growth and survival. A TK inhibitor, erlotinib, was shown to be superior to placebo in terms of survival and tumor response rate in epidermal growth factor receptor (EGFR)–positive patients with locally advanced or metastatic non–small cell lung cancer (NSCLC) [5]. Specific mutations in the EGFR gene have been reported to correlate with clinical responsiveness to another EGFR TK inhibitor, gefitinib [6]. The binding of TK growth factors or cytokines to their corresponding receptors leads to conformational changes of the receptors that initiate the activation of JAK. Then, JAK activates signal transducers and activators of transcription (STAT) factors to dimerize and translocate into the nucleus to initiate the transactivation of target genes. This pathway is crucial to hematopoiesis, immune response, and oncogenesis [7]. In many cancers, STAT are constitutively activated to upregulate genes encoding apoptosis inhibitors and cell cycle regulators such as Bcl-xL, Mcl-1, cyclins D1/D2, and c-Myc [7–9].

Dysfunction of the regulatory system for the JAK/STAT pathway has been demonstrated in the development of cancer. Three families of proteins, the protein inhibitors of activated STAT (PIAS) [10], the suppressors of cytokine signaling (SOCS) [11–13], and the Src homology 2 containing phosphatase (SHP) [14], participate in the negative regulation of this signal transduction pathway [15]. SOCS proteins become inducible inhibitors of this signaling pathway by competing with STAT for phosphorylated binding sites on receptors or by targeting bound signaling proteins for proteasomal degradation. SHP and PIAS family members are constitutively expressed and can attenuate signal transduction [16]. Recently, He et al. reported that the activity of SOCS3 was silenced due to hypermethylation in its promoter region in seven of eight human lung cancer samples tested. They restored SOCS3 expression in those cancer cells and thereby successfully suppressed tumorigenicity [17]. Wu et al. [18] reported that the expression of
SHP1 proteins or mRNA was dramatically decreased in most leukemia and lymphoma cell lines. Overexpression of SHP1 suppressed the growth of cancer cells. Because STAT3 is frequently activated in a wide variety of human malignancies [19], PIAS3, a specific regulator of STAT3, is possibly an ideal candidate for controlling cancer. However, only a small number of reports on the involvement of PIAS3 in cancer development are available so far. Zhang et al. [20] reported that they could not detect PIAS3 mRNA in most samples of lymphoma cells, and they speculated that loss of PIAS3 expression is partly responsible for the activation of STAT3. Wible et al. [21] reported that ectopic expression of PIAS3 suppressed the survival of prostate cancer cells by inducing the apoptosis of cancer cells.

We demonstrated here that overexpression of PIAS3 in lung cancer cells contributed to growth suppression and restored the drug sensitivity of the cells. The anticancer effects of PIAS3 were associated with the suppression of antiapoptotic molecules, including Akt and Bcl-xL. However, overexpression of SOCS3 induced growth suppression but affected neither the drug sensitivity nor the phosphorylation of Akt. PIAS3 may be a good target for interference with cell signaling for potential therapeutic intervention in lung cancer.

Materials and Methods

Materials

The following materials were used: anti–phospho-STAT3, anti–phospho-Erk1/2, and anti–phospho-Akt antibodies (Cell Signaling Technology, Beverly, MA); antibody to PIAS3 (Santa Cruz Biotechnology, Santa Cruz, CA); anti–β-actin antibody (Sigma, St. Louis, MO); and AG490 and LY294002 (Calbiochem, San Diego, CA). Recombinant human interleukin (IL) 6 protein was purchased from PeproTech EC (London, UK). Carboplatin (CBDCA) was kindly provided by Bristol-Myers Squibb Japan (Tokyo, Japan), and vinorelbine (VNR) was a gift from Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan).

Cell Lines

The human lung cancer cell lines A549, VMRC-LCD (LCD), EBC1, and RERF-LCMS were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). These cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. The human PIAS3 cDNA cloned in a pCI-neo-Myc expression vector (Promega, Palo Alto, CA) was kindly provided by Dr. Tadashi Matsuda (Hokkaido University, Sapporo, Japan). PIAS3-transfected cells were established after stable transfection with the PIAS3 expression vector or pSV2neo (mock) using Lipofectamine Plus (Invitrogen, Tokyo, Japan). These cells were cultured in RPMI supplemented with 10% FBS and 500 µg/ml Geneticin (Gibco, Grand Island, NY), and were maintained under standard cell culture conditions at 37°C and 5% CO2 in a humidified environment. We confirmed an ectopic PIAS3 gene expression in established clones by immunoblotting using an Myc tag. Representative clones expressing high levels of PIAS3 were used in the following experiments.

Adenoviral Vectors

Recombinant replication-defective adenoviral vectors were constructed by the cosmids–adenoviral DNA terminal protein complex method [22]. Adenoviral vectors AdCATK and AdSOCS3 were designed to express the thymidine kinase gene and the human SOCS3 gene, respectively, under the control of the CAG promoter (a modified chicken β-actin promoter with a cytomegalovirus immediate early enhancer) [23]. Solutions of these adenoviral vectors were prepared as described previously and stored at −80°C until use [24]. After seeding 1 × 105 cells in a six-well plate and culturing them in RPMI 1640 supplemented with 10% FBS for 24 hours, the infection of adenoviral vectors was performed by distributing suspensions of AdSOCS3 or AdCATK onto cells at a multiplicity of infection (MOI) of 20, followed by incubation at 37°C for an additional 24 hours.

Cellular Proliferation Assay

Cell growth curves were determined by plating 1 × 105 cells in a six-well plate and by culturing the cells in RPMI 1640 supplemented with 10% FBS for 24 hours. Then, after the cells had been deprived of serum for 20 hours, 60 μM AG490, 10 μM LY294002, or dimethyl sulfoxide (DMSO) alone was added to the cells, followed by incubation at 37°C in RPMI 1640 supplemented with 5% FBS. The cells were stained with trypan blue and counted at indicated intervals.

In Vitro Sensitivity of Tumor Cells to Chemotherapeutic Drugs

Five thousand A549, LCD, EBC1, and LCMS cells were seeded in triplicate onto 96-well culture plates and were cultured in RPMI 1640 supplemented with 10% FBS for 24 hours. After the cells had been deprived of serum for 20 hours, the medium was replaced with a medium containing 10% FBS with DMSO alone, 60 μM AG490 dissolved in DMSO alone, 60 μM AG490 dissoluted in DMSO, or 10 μM LY294002 solubilized in DMSO. After a 24-hour incubation with these inhibitors, various concentrations of CBDCA or VNR were added to the wells. After cultivation for an additional 72 hours, the 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was carried out as described previously [25]. The 50% growth-inhibitory concentration (IC50) of CBDCA and VNR for each cell line was calculated using a curve-fitting parameter. The fold increase of drug sensitivity was calculated as the IC50 values of mock-transfected cells divided by those of PIAS3-transfected or SOCS3-transfected cells, and the findings were presented as mean ± SD from three independent experiments.

Immunoblotting Analysis

After subconfluent tumor cells had been cultured in a medium without serum for 20 hours, 100 ng/ml IL-6 was added to each well. The cells were harvested at indicated intervals and analyzed for the phosphorylation of Akt, STAT,
and Erk1/2. For Bcl-xL, after subconfluent tumor cells had been cultured in a medium without serum for 20 hours, an indicated concentration of CBDCA was added to each well, and the cells were harvested 24 hours later. Protein extraction, immunoprecipitation, Western blot analysis, and immunoblotting were performed as previously described [26].

Small Interfering RNA (siRNA) against PIAS3

We purchased an siRNA cocktail against human PIAS3 from B-Bridge International, Inc. (Sunnyvale, CA), which contains three siRNA: first sequence (sense 5'-cuacaaaaacuca-gaccaTT-3' and antisense 5'-uugucucaguuuuugauTT-3'), second sequence (sense 5'-cauccacguuuagauTT-3' and antisense 5'-uuaacuccacguuugTT-3'), and third sequence (sense 5'-caaacagacagguggaaaaTT-3' and antisense 5'-uuuuccacuccuuggTT-3'). A negative control cocktail was also purchased from B-Bridge International, Inc. (cat. no. C6A-0126). Transfection of siRNA was performed according to the manufacturer's instruction. After a 48-hour incubation, cells were subjected to chemosensitivity tests and immunoblotting analyses.

Statistical Analysis

Data are indicated as mean ± SD for the number of experiments indicated. Fisher’s protected least significant difference (PLSD) test was used for the statistical analysis of cell numbers and IC50 under individual conditions. Differences were considered significant at \( P < .05 \).

Results

Inhibition of Phosphatidylinositol 3-Kinase (PI3-K)

by LY294002 Induced Growth Inhibition and Increased

the Cytotoxic Drug Sensitivity of Human Lung Cancer

Cells, Whereas Inhibition of JAK by AG490 Only

Suppressed Cell Growth

Lung cancer cells are relatively resistant to conventional chemotherapeutic drugs but undergo extensive apoptosis after treatment with pharmacological inhibitors of PI3-K/Akt or JAK/STAT signaling [27]. To analyze if the growth rate and drug sensitivity of human lung cancer A549 and LCD cells are dependent on the activities of these signaling pathways, both cells were treated with the JAK inhibitor AG490 and the PI3-K inhibitor LY294002. Cells were grown in an RPMI medium containing 5% FBS with 60 \( \mu M \) AG490, 10 \( \mu M \) LY294002, or DMSO alone. The concentration of each agent has been confirmed to suppress the expression of phosphor-ylated (p)STAT or pAkt, respectively (data not shown). Treatment with AG490 or LY294002 significantly suppressed the growth of both of these cell lines in comparison with the respective DMSO-treated cells [Figure 1A, untreated A549 vs A549 treated with AG490 (\( P = .0007 \) on day 6); Figure 1B, untreated LCD versus LCD treated with AG490 (\( P = .0012 \); on day 7); Figure 1C, untreated A549 cells versus A549 treated with LY294002 (\( P = .0009 \) on day 5)].

Because both PI3-K/Akt and JAK/STAT signaling pathways have antiapoptotic activities and possibly have mediated

Figure 1. JAK inhibition by AG490 and PI3-K inhibition by LY294002 suppressed cell growth and increased the drug sensitivity of human lung cancer cells. Growth curves of A549 (A) and LCD (B) cells treated with the JAK inhibitor AG490. Cells were grown in an RPMI medium containing 5% FBS with 60 \( \mu M \) AG490 or DMSO (untreated). (C) Growth curves of A549 cells treated with the PI-3K inhibitor LY294002. Cells were cultured in an RPMI medium containing 5% FBS with 10 \( \mu M \) LY294002 or DMSO (untreated). Viable cell numbers were counted using trypan blue dye exclusion at the indicated times after seeding. Each value is the average (points) of triplicate dishes ± SD (bars). (D) LY294002 treatment increased the drug sensitivity of lung cancer cells. The fold increase in drug sensitivity was calculated as the IC50 of DMSO-treated cells divided by those of LY294002-treated or AG490-treated cells. The values are expressed as mean ± SD from three individual experiments. CBDCA, carboplatin; VNR, vinorelbine.
resistance to a wide variety of chemotherapeutic agents in lung cancer cells, we next studied whether treatment with AG490 and LY294002 affected the sensitivity of lung cancer cells to CBDCA and VNR, which are frequently used for lung cancer treatment. The number of viable cells was determined using the MTT assay and graphed as the percentage of untreated cells at different doses of CBDCA and VNR in the absence or in the presence of AG490 or LY294002. Drug sensitivity was expressed as IC50 using a curve-fitting parameter. Fold increase in drug sensitivity was calculated as the IC50 of DMSO-treated cells divided by those of LY294002-treated or AG490-treated cells. Treatment with LY294002 rendered A549 cells 2.85-fold and 7.00-fold more sensitive to CBDCA and VNR, respectively (Figure 1D, LY294002). In contrast, AG490-treated A549 cells were still resistant to both CBDCA and VNR (Figure 1D, AG490).

Establishment of PIAS3-Overexpressing Lung Cancer Cells

To analyze the effects of PIAS3 on cell growth and drug sensitivity, we transfected A549, LCD, and EBC1 cells with a plasmid vector encoding human PIAS3 and established clones expressing high levels of PIAS3 by culturing them in a medium containing Geneticin. We confirmed an ectopic expression of PIAS3 as Myc tag expression by immunoblotting analysis with Myc-specific antibodies and was indicated as the PIAS3–cMyc fusion protein. (B) High levels of PIAS3 expression in established clones are indicated as 68-kDa PIAS3 bands by immunoblotting. Clones stably expressing PIAS3 were established by transfecting a plasmid vector encoding PIAS3, followed by culturing in a medium containing Geneticin. (C) Downregulation of pSTAT3 in PIAS3-overexpressing A549/PIAS3 (upper panel), LCD/PIAS3 (middle panel), and EBC1/PIAS3 (lower panel) cells. The phosphorylation of STAT3 in PIAS3-overexpressing cells was serially analyzed after IL-6 stimulation using phosphospecific antibody.

Overexpression of PIAS3 Increased Drug Sensitivity and Retarded the Growth of Human Lung Cancer Cells, Whereas Overexpression of SOCS3 Only Suppressed Cell Growth

We analyzed the effects of the JAK/STAT signaling pathway inhibitory molecules PIAS3 and SOCS3 on the cell growth and drug sensitivity of lung cancer cells. We transduced SOCS3 using an adenoviral vector. The growth curves of A549 cells were studied after the cells had been transfected with PIAS3, alone or combined with SOCS3. The growth rate of A549 cells was remarkably suppressed when the cells were transfected with either PIAS3 or SOCS3 [Figure 3A, AdCATK versus PIAS3 AdCATK (P = .004 on day 7); AdCATK versus AdSOCS3 (P = .0001 on day 7)]. The magnitude of growth suppression was greater in SOCS3-transfected cells than in PIAS3-transfected cells. Overexpression of PIAS3, together with SOCS3, showed additive inhibitory effects on cellular proliferation [Figure 3A, PIAS3 AdCATK versus PIAS3 AdSOCS3 (P = .043 on day 7)]. Growth suppression by PIAS3 was confirmed in another
A549 clone [Figure 3B, A549/mock versus A549/PIAS3 clone 2 (P = .030 on day 7)]. The growth rate of PIAS3-transfected LCD cells was also lower than that of mock-transfected LCD cells, and representative results are shown [Figure 3C, mock versus PIAS3 clone 1 (P = .0007 on day 6)]. Growth inhibition by PIAS3 was also observed in the other lung cancer cell line EBC1, and the results of a representative clone are shown [Figure 3D, mock versus PIAS3 clone 1 (P = .0005 on day 6)].

We next studied the effects of PIAS3 on the sensitivity of lung cancer cells to chemotherapeutic agents. The fold increase in drug sensitivity was calculated as the IC_{50} values of mock-transfected cells divided by those of PIAS3-transfected or SOCS-transfected cells. The sensitivities of PIAS3-transfected A549 clones to CBDCA were from 1.9 ± 0.2-fold to 5.5 ± 2.8-fold higher (Figure 4, A549, CBDCA/PIAS3 clones 1, 2, and 3). PIAS3-transfected LCD cells were also 1.2-fold and 2.4-fold more sensitive to CBDCA (Figure 4, LCD, CBDCA/PIAS3 clones 1 and 2). The relative sensitivity of PIAS3-transfected A549 cells to VNR was from 11.0 ± 2.9 to 12.0 ± 2.6 times higher than that of mock-transfected A549 cells (Figure 4, A549, VNR/PIAS3 clones 1, 2, and 3). The relative sensitivity of PIAS3-transfected LCD cells to VNR was 6.8 and 6.7 times higher than the IC_{50} of mock-transfected LCD cells (Figure 4, LCD, VNR/PIAS3 clones 1 and 2). EBC1 cells transfected with PIAS3 also became about 2.0-fold more sensitive to CBDCA and VNR, respectively (Figure 4, EBC1, CBDCA/PIAS3 clone 1 and VNR/PIAS3 clone 2). The IC_{50} values of SOCS3-infected A549 and LCD cells to both CBDCA and VNR were, nevertheless, very similar to those of their respective mock-transfected cells (Figure 4, A549, CBDCA/SOCS3, and VNR/SOCS3; Figure 4, LCD, CBDCA/SOCS3, and VNR/SOCS3).

Overexpression of PIAS3 Suppressed Bcl-xL and Akt Phosphorylation in Lung Cancer Cells, Whereas Transduction of SOCS3 Did Not

It is known that the expression of Bcl-xL and Akt is upregulated by STAT3. These molecules protect cells from apoptosis and are associated with oncogenesis in cancer cells [27–29]. To identify molecules mediating the anticancer effects of PIAS3, we analyzed the activation of Bcl-xL expression, as well as the upregulation of the expression of extracellular signal–regulated kinase (Erk) through Ras, and of Akt through phospholipase Cγ and PI3-K.

In contrast to mock-transfected A549 cells, PIAS3-transfected cells showed that both Bcl-xL expression by CBDCA exposure (Figure 5A, PIAS3/Bcl-xL) and Akt phosphorylation by IL-6 stimulation were inhibited by PIAS3 overexpression (Figure 5B, A549/PIAS3/pAkt). Erk1/2 was similarly phosphorylated in response to IL-6 stimulation in both mock-transfected and PIAS3-transfected A549 cells (Figure 5B, A549/PIAS3 pErk1/2). Figure 5, A and B, presents the results obtained with A549/PIAS3 clone 1. Similar results were also confirmed in other A549 PIAS3 clones (data not shown). In PIAS3-overexpressing EBC1 cells, the expression of pAkt was weak before stimulation, and the enhanced expression of pAkt after IL-6 stimulation attenuated sooner than that in mock-transfected EBC1 cells (Figure 5B, EBC1/PIAS3/pAkt). Overexpression of PIAS3 in EBC1 cells

Figure 3. Overexpression of PIAS3, alone or combined with SOCS3, retarded the growth of human lung cancer cells. (A) Growth curves of the A549 transfectants of PIAS3 (A549/PIAS3 clone 1), alone or combined with SOCS3 infection, using an adenoviral vector. Cells were infected with either AdSOCS3 or AdCATK as control at an MOI of 20. (B) PIAS3 overexpression alone suppresses the cell growth of the other A549/PIAS3 clone (A549/PIAS3 clone 2). Effects of PIAS3 overexpression on the cell growth of LCD (C) and EBC1 (D) cells (LCD/PIAS3 clone 1 and EBC1/PIAS3 clone 1). Cells were grown in an RPMI medium containing 5% FBS. Viable cell numbers were counted using trypan blue dye exclusion at the indicated times after seeding. Each value is the average (points) of triplicate dishes ± SD (bars).
did not change the phosphorylation of Erk1/2 in response to IL-6 stimulation either (Figure 5B, EBC1/PIAS3/pErk1/2). Infection of A549 cells with AdSOCS3 caused the suppression of IL-6–stimulated STAT3 phosphorylation (Figure 5C, AdSOCS3/pSTAT3). However, CATK-transfected and SOCS3-transfected cells showed almost very similar levels of Bcl-xL expression by CBDCA exposure (Figure 5C, AdSOCS3/Bcl-xL) and phosphorylation levels of both Akt and Erk1/2 in response to IL-6 stimulation (Figure 5C, AdSOCS3/pAkt and AdSOCS3/pErk1/2).

Figure 4. Drug sensitivity of lung cancer cells overexpressing PIAS3 or SOCS3. The eight of bars on the left indicate the fold increase in drug sensitivity to CBDCA and VNR in A549 cells. The six of bars on the middle of the figure indicate the fold increase in drug sensitivity in LCD cells, and the two of bars on the right indicate the fold increase in drug sensitivity in EBC1 cells. The clones used in the PIAS3 study are indicated in each figure. Cells were transfected with AdSOCS3 or AdCATK (mock) at an MOI of 20. The fold increase in drug sensitivity was calculated as the IC50 values of mock-transfected cells divided by those of PIAS3-transfected or SOCS3-transfected cells. Values are expressed as mean ± SD from three individual experiments.

Figure 5. Overexpression of PIAS3 inhibits Akt phosphorylation and Bcl-xL expression in A549 cells. (A) Suppression of Bcl-xL by PIAS3 overexpression. Cells were pretreated for 24 hours with the indicated concentrations of CBDCA. Protein extracts were probed with the Bcl-xL antibody. (B) Overexpression of PIAS3 inhibited Akt phosphorylation but did not affect Erk1/2 phosphorylation in A549 and EBC1 cells. Exponentially growing cells in a medium containing 10% FBS were exposed to 100 ng/ml IL-6 for the indicated durations. The phosphorylation of Akt and Erk1/2 was determined using each corresponding phosphospecific antibody. (C) Effects of SOCS3 transduction on pSTAT3, pAkt, pErk1/2, and Bcl-xL in A549 cells. Cells were exposed to 100 ng/ml IL-6 for the indicated durations. Protein extracts were subjected to immunoblotting. The phosphorylation status of STAT3, Akt, and Erk1/2 was determined using each corresponding phosphospecific antibody. To detect Bcl-xL expression, cells were pretreated for 24 hours with indicated concentrations of CBDCA. Protein extracts were probed with the Bcl-xL antibody.
Blocking of PIAS3 with siRNA Accelerated Cell Proliferation and Deteriorated Sensitivity to Chemotherapeutic Agents in Lung Cancer Cells

To further confirm the effects of PIAS3 on the cell proliferation and chemosensitivity of lung cancer cells, we suppressed PIAS3 expression using siRNA. Although treatment with a PIAS3-specific siRNA significantly suppressed PIAS3 expression, it augmented the phosphorylation of Akt in A549, EBC1, and LCMS cells. However, the expression of total Akt was almost similar (Figure 6A). Downregulated PIAS3 expression was associated with an accelerated growth of both A549 and LCMS cells [Figure 6B, A549, control siRNA versus PIAS3 siRNA (P = .0475 on day 7); Figure 6C, LCMS, control siRNA versus PIAS3 siRNA (P = .0073 on day 7)]. The inhibition of PIAS3 expression also resulted in the reduced sensitivity of EBC1 and LCMS cells to CBDCA (Figure 6D).

PIAS3 Interacted with Akt In Vitro in Lung Cancer Cells

Although PIAS3 specifically binds to STAT3 [10], it is not known whether PIAS3 has interactions with Akt. We prepared protein extracts from PIAS3-overexpressing cells stimulated with IL-6. Proteins immunoprecipitated with Akt were subjected to protein immunoblotting with anti-PIAS3. PIAS3 was present in Akt immunoprecipitates from both A549 and EBC1 cells (Figure 7). Stimulation with IL-6 slightly enhanced the interaction of PIAS3 with Akt in both cell lines. PIAS3 was also present in Akt immunoprecipitates from wild-type A549 and EBC1 cells (data not shown).

Discussion

The JAK/STAT pathway is activated in the cancer cell lines used in this study (Figure 2C). Zhang et al. described that the downregulation of PIAS3 proteins is frequently seen in lymphoma cells and that loss of PIAS3 expression is partly responsible for maintaining high levels of activated STAT3.

Figure 6. Blocking of PIAS3 with siRNA accelerated cell proliferation, deteriorated chemosensitivity, and augmented Akt phosphorylation in lung cancer cells. (A) Although treatment with siRNA ablated PIAS3 expression, it augmented Akt phosphorylation. Cells were transfected with siRNA specific for PIAS3 or with control siRNA. After 48 hours of incubation, cells were harvested and protein extracts were probed with indicated antibodies. Growth acceleration of A549 (B) and LCMS (C) cells. Cells were transfected with siRNA specific for PIAS3 or with control siRNA. Viable cell numbers were counted using trypan blue dye exclusion at the indicated times after the transfection. Each value is the average (points) of triplicate dishes ± SD (bars). (D) Chemosensitivity of siRNA-treated EBC1 and LCMS cells for CBDCA. The fold increase of drug sensitivity was calculated as the IC50 values of control siRNA-transfected cells divided by those of PIAS3 siRNA-transfected cells. Values were expressed as mean ± SD from three individual experiments.

Figure 7. The in vitro interaction of PIAS3 with Akt. Protein extracts from PIAS3-overexpressing A549 and EBC1 cells, stimulated with (+) or without (−) IL-6, were immunoprecipitated (IP) with Akt. The blot was probed with anti-PIAS3 (top). The same blot was then reprobed with anti-Akt (bottom).
[20]. However, Wang and Banerjee [30] described that basal amounts of PIAS3 were present in various human cancers. In our study, immunoblotting analysis detected endogenous PIAS3 proteins in A549 and LCD cells (Figure 2B). Methylation-specific polymerase chain reaction showed that SOCS-3 was hypermethylated in A549 and LCD cells and, thus, the amount of transcripts of SOCS-3 was decreased (data not shown).

The phosphorylation of STAT3 was attenuated in PIAS3-transfected A549 and EBC1 cells, whereas it was not changed in PIAS3-transfected LCD cells (Figure 2C). Schmidt and Muller discussed the mechanisms mediating the downregulation of the transcriptional activity of STAT by PIAS. They presented a model in which PIAS interferes with the binding of STAT to target DNA by covering the DNA-binding domain of an activated STAT dimer. An alternative model is one wherein PIAS interacts transiently with STAT dimers to induce their dissociation and, consequently, the loss of DNA-binding activity [31].

We showed that treatment with the JAK inhibitor AG490 and the PI3-K inhibitor LY294002 contributed to the growth suppression of lung cancer cells (Figure 1). Both PI3-K/Akt and JAK/STAT are important regulators of cell survival and apoptosis. Akt suppresses apoptosis by interacting with and phosphorylating Bad, a cell survival factor [32] that inactivates the initiation of caspase-9 [33]. Overexpression of PIAS3 successfully downregulated the JAK/STAT pathways (Figure 2C) and retarded the growth of these cells (Figure 3). The magnitude of growth suppression by SOCS3 was, nevertheless, superior to that by PIAS3 (Figure 3A). Because the methods of transfection were different between the PIAS and SOCS genes, we speculate that the transient but strong gene expression by adeno-viruses caused more significant inhibitory effects on cell growth.

In a previous report, overexpression of KchAP, a member of the PIAS family in prostate cancer cells, induced apoptosis associated with increased levels of p53 and p21 [21]. The authors, however, did not refer to changes in drug sensitivity or in the expression of antiapoptotic molecules such as Akt, Bcl-xL, and Erk1/2. We demonstrated that the overexpression of PIAS3 in lung cancer cells increased drug sensitivity (Figure 4), suppression of cell growth (Figure 3), and inhibition of both Akt phosphorylation in response to IL-6 and Bcl-xL expression in exposure to CBDCA (Figure 5, A and B). However, transduction of SOCS-3 only retarded growth (Figure 3A) without affecting drug sensitivity or Akt phosphorylation (Figures 4 and 5C).

Treatment of A549 cells with LY294002 increased drug sensitivity as well as growth suppression, but AG490 treatment did not alter the drug sensitivity of A549 cells (Figure 1D). Roberts [34], using cDNA microarray analysis, demonstrated a significant association of STAT1 expression with decreased sensitivity to cisplatin in human ovarian cancer cell lines. However, Brognard et al. [35] reported that constitutive Akt/protein kinase B (PKB) activity was detected in 16 of 17 NSCLC cell lines, including A549. The PI3-K inhibitors LY294002 and wortmannin inhibited Akt/PKB phosphorylation and potentiated chemotherapy-induced apoptosis in cancer cells. Knuefermann et al. also reported that HER2/PI3-K—dependent activation of Akt plays an important role in conferring broad-spectrum drug resistance on breast cancer cells. They speculate that Akt-mediated resistance to chemotherapeutic agents with different mechanisms of action is likely because of the overall antiapoptotic activity of Akt [36]. As shown in Figures 4, Figures 5, Figures 6, we speculate that the levels of phosphorylated Akt are regulated by PIAS3 and are inversely correlated with the drug sensitivity of lung cancer cells. Drew et al. [37] showed that stimulation with IL-6 enhanced the expression of multidrug resistance—associated proteins (MRPs) and STAT proteins. Expression of MRPs was suppressed by the inhibition of Akt with the PI3-K inhibitor. However, the inhibition of mitogen-activated protein kinase/Erk1/2 did not have an inhibitory effect on the enhanced transcription of MRP. The suppression of MRP expression caused by Akt inactivation might account for the increase of drug sensitivity by PIAS3. PIAS3 was present in Akt immunoprecipitates from A549 and EBC1 cells (Figure 7). The intensity of the bands indicating the PIAS3 proteins associated with Akt was enhanced by stimulation with IL-6. There has been no previous report on the involvement of PIAS3 in Akt activation. This result implies that PIAS3 affects the phosphorylation of Akt, although it is necessary to elucidate the mechanism.

In conclusion, using PIAS3 in targeting the JAK/STAT signaling pathways against cancer has the advantages of restoring sensitivity to chemotherapeutic drugs and inhibiting cell growth.

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