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The Association and Nuclear Translocation of the PIAS3-STAT3 Complex Is Ligand and Time Dependent

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Abstract

The epidermal growth factor (EGF) receptor activation of downstream signal transducers and activators of transcription 3 (STAT3) plays a crucial role in the pathogenesis of lung cancer. STAT3 transcriptional activity can be negatively regulated by protein inhibitor of activated STAT3 (PIAS3). We investigated the time-dependent PIAS3 shuffling and binding to STAT3 in an EGF-dependent model in lung cancer by using confocal microscopy, immunoprecipitation, luciferase reporter assay, and protein analysis of segregated cellular components. We also explored the role of phosphorylation at Tyr705 of STAT3 in the formation and intracellular shuffling of the PIAS3-STAT3 complex. In a growth factor–free state, PIAS3 was localized to the cytoplasm and unbound to STAT3 in both H520 and A549 cells. On exposure to EGF, we observed STAT3 phosphorylation and rapid formation of the PIAS3-STAT3 complex. Within 5 minutes, there was a progressive translocation of the complex to the nucleus, and by 10 minutes, PIAS3 was uniquely localized to the nuclear compartment. After 30 minutes, PIAS3 returned to the cytoplasm. Using site-directed mutagenesis, we substituted Tyr705 of STAT3 with a phenylalanine. Despite EGF stimulation, we observed a significant decrease in PIAS3-STAT3 binding and a significant reduction in nuclear translocation of PIAS3. Furthermore, there was a significant reduction in the capacity of PIAS3 to reduce STAT3-mediated gene transcription. In wild-type STAT3 cells, increasing concentrations of PIAS3 resulted in a proportional decrease in STAT3 phosphorylation. These data suggest an important role for the negative regulatory effect of PIAS3 on STAT3 in EGF-driven tumors. (Mol Cancer Res 2009;7(11):1854–60)

Introduction

Signal transducers and activators of transcription (STAT) are latent cytoplasmic transcription factors that are activated (phosphorylated) by upstream receptor and nonreceptor tyrosine kinases. On activation, dimer formation occurs with subsequent nuclear translocation and modulation of gene transcription of its target genes. This pathway is of importance because of its functions in hematopoiesis, immune response, and oncogenesis (1). Of these seven STATs (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6), STAT3 is of particular importance due to its involvement in a wide spectrum of biological functions. It is activated (phosphorylated) by a variety of cytokines and growth factors, such as platelet-derived growth factor and epidermal growth factor (EGF), in a large number of human malignancies (2). STAT proteins have three families of natural inhibitors: the protein inhibitors of activated STAT (PIAS; ref. 3), the suppressors of cytokine signaling (4-6), and the Src homology 2–containing phosphatase (7). All three are known to participate in the negative regulation of the STAT signal transduction pathway (8).

PIAS3 belongs to a multigene family, which was first identified as a transcriptional repressor of activated STAT3 that inhibits the transactivation of a STAT3-responsive reporter gene and the DNA-binding activity of STAT3 (3, 9). Limited information exists on its relevance and function in solid tumors. Foremost, the intracellular kinetics and trafficking of PIAS3 in the setting receptor tyrosine kinase activation of solid tumors is unknown. PIAS3 is expressed in prostate cancer cells and functions as a transcriptional cofactor for the androgen receptor, and its overexpression can induce apoptosis in prostate cancer cells (10). Most recently, a loss of PIAS3 protein expression (but not mRNA) has been shown in glioblastoma multiforme with an increase in STAT3 phosphorylation and activity (11). We have previously shown in lung cancer that EGF receptor activation leads to PIAS3-STAT3 binding (12). We have also shown the absence of PIAS3 expression in 50% of lung cancer specimens with epigenetic changes as a potential mechanism for this loss (13).

This work aims to determine the kinetics of EGF receptor–induced PIAS3-STAT3 interaction in a lung cancer model as well as the importance of STAT3 tyrosine phosphorylation site for this association. We have shown the time-dependent cellular localization of PIAS3 under the influence of EGF and the importance of the phosphorylation on the tyrosine residue at position 705 of STAT3 in the formation of a complex with PIAS3 to inhibit STAT3-mediated transcription. We also show that PIAS3 intracellular concentration affects STAT3 Tyr705 phosphorylation and transcriptional activity.

Results

Time- and Ligand-Dependent PIAS3 Localization

We investigated the time- and ligand-dependent subcellular localization of PIAS3 protein in two different non–small cell lung carcinoma (NSCLC) cell lines using confocal laser-scanning microscopy. For this purpose, we initially analyzed

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our cells in the serum-free state without the presence of ligand. Subsequently, cells were stimulated with EGF at a concentration of 20 ng/mL, which activates the EGF receptor-STAT3 signaling pathway in lung cancer cells. This leads to the formation of a STAT3-PIAS3 complex. We found that, in the unstimulated cells, in both cell lines (Fig. 1A and B), PIAS3 resides mainly in the cytoplasm. Within 5 minutes of stimulation with EGF, there is evidence of PIAS3 migration toward the nucleus. This translocation remains evident for the 10- and 20-minute time points, but by 30 minutes, PIAS3 returns to the cytoplasmic compartment despite the presence of EGF.

Tyr705 of STAT3 Is Critical for Ligand-Dependent PIAS3-STAT3 Association

Given the fact that Tyr705 phosphorylation of STAT3 is an important process for STAT3 homodimer formation and nuclear translocation, we further investigated the importance Tyr705 of STAT3 in the PIAS3-STAT3 complex formation and PIAS3 nuclear translocation. We transiently cotransfected A549 and H520 cell lines with either the wild-type (WT) or the mutant Y705-Y705F STAT3 gene construct. After exposure to the EGF ligand, the cells were lysed and subjected to immunoprecipitation with an anti-PIAS3 antibody. The recovered immune complexes were subjected to immunoblotting analysis with an anti-STAT3 antibody. This showed that PIAS3 interacted significantly more with WT-STAT3–transfected as compared with mutant STAT3–transfected cells (Fig. 2). This result indicates that the presence of Y705 residue is important for the complete association of STAT3 with PIAS3. However, as seen in Fig. 2, a small band still appears for the mutant Y705–transfected cells. This most likely reflects the endogenous WT-STAT3 in these cells.

Tyr705 of STAT3 Also Affects PIAS3 Nuclear Translocation

To further show the importance of Y705 of STAT3 in EGF-induced PIAS3 nuclear translocation, we obtained nuclear and cytoplasmic extracts of A549 cells that were stimulated with EGF for 10 minutes. As had been seen in Fig. 1, we would expect maximum PIAS3 nuclear translocation to occur at the 10-minute time point. Figure 3A shows that at the 10-minute time point in mutant STAT–transfected cells, very little PIAS3

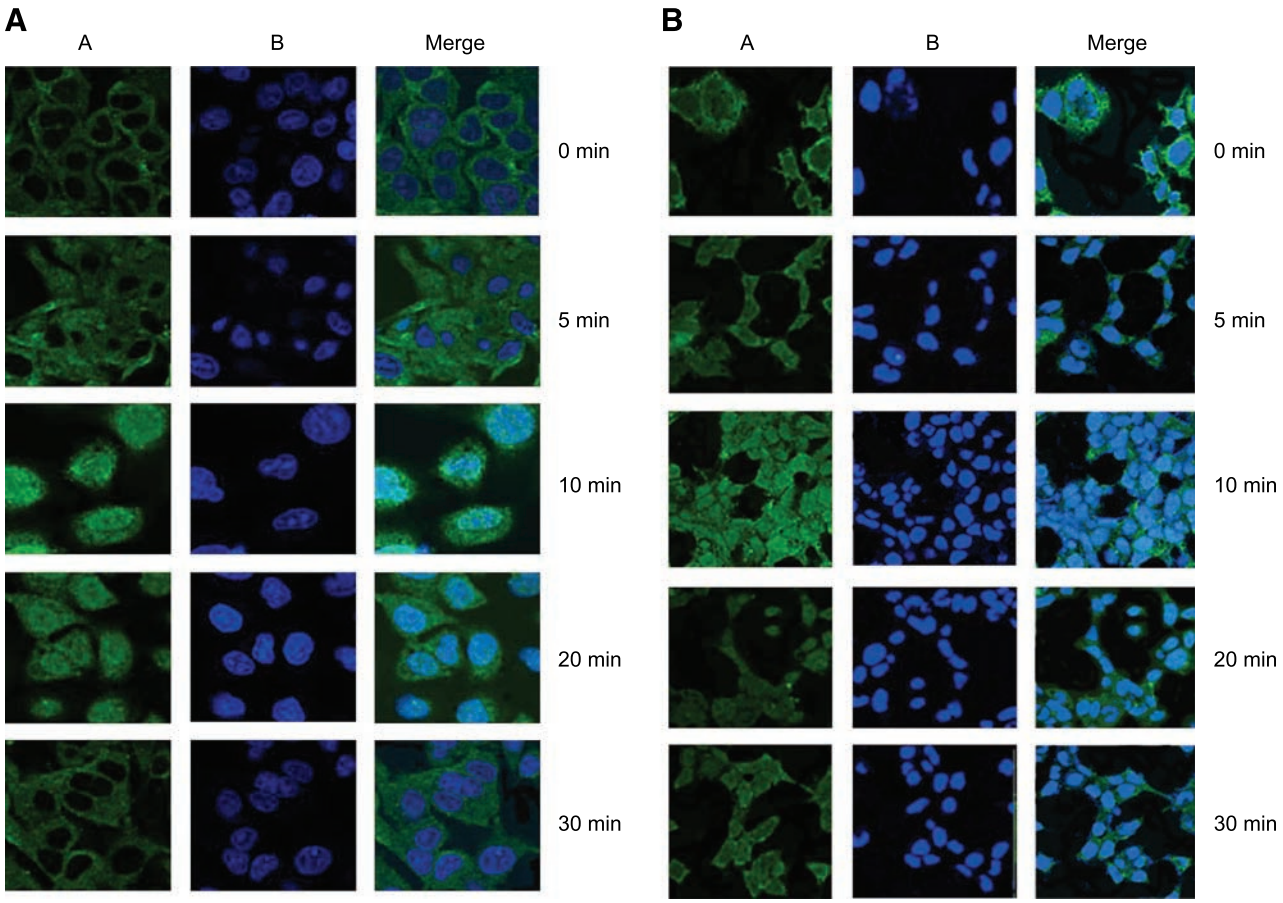

FIGURE 1. Time-dependent subcellular localization of PIAS3 on EGF stimulation. **A.** A549 cells were seeded on a poly-L-lysine–treated glass-bottomed culture dish, starved for at least 12 h in serum-free medium, and unstimulated or stimulated with 20 μg/mL EGF for 0 to 30 min. At every 5-min interval, cells were fixed, permeabilized, and stained as described in Materials and Methods. **B.** H520 cells were seeded on a poly-L-lysine–treated glass-bottomed culture dish, starved for at least 24 h in serum-free medium, and unstimulated or stimulated with 20 ng/mL EGF for 0 to 30 min. At every 5-min interval, cells were fixed, permeabilized, and stained as described above. **A.** DRAQ5 counterstain (nuclei, *blue*). **B.** Antirabbit IgG conjugated with Alexa Fluor 488 counterstain (PIAS3, *green*).

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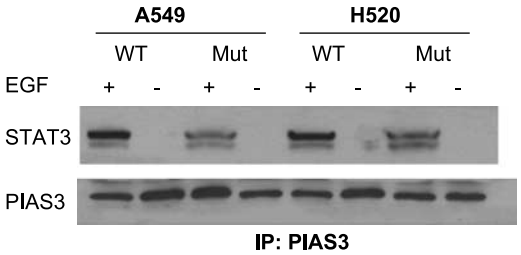

FIGURE 2. Immunoprecipitation (*IP*) of PIAS3 with whole protein extracts of NSCLC A549 and H520 cell lines transfected with WT and Tyr705 mutant STAT3 expression constructs with FLAG tagged pcDNA, EGF stimulated or unstimulated after 48 h transfection. Immunoprecipitates were resolved by Western blotting (*WB*) and membranes were probed with an anti-STAT3 and anti-PIAS3 antibodies. High expression of pSTAT3 is observed in WT-transfected cells (*lanes 1* and *3*) whereas lower pSTAT3 levels are observed in mutant-transfected cells (*lanes 2* and *4*). In both situations, STAT3 coprecipitated with PIAS3, suggesting increased PIAS3-STAT3 binding.

exists in the nucleus as compared with the cytoplasmic compartment (nuclear/cytoplasmic ratio of 0.62 based on densitometric analysis of bands). This shows the absence of significant STAT3 translocation to the nucleus when Y705 of STAT3 is mutated. On the other hand, in the WT-transfected cells, the inverse can be seen, with significantly more PIAS3 in the nucleus (nuclear/cytoplasmic ratio of 2.74). To further corroborate these findings, we used confocal laser-scanning microscopy in our WT- or mutant-transfected cells. Once again, cells were visualized after 10 minutes of exposure to EGF. The WT cells showed significant nuclear translocation of PIAS3, whereas much less was observed in the mutant cells (Fig. 3B).

Negative Functional Role of PIAS3 in STAT3 Transcriptional Activity: Crucial Role for Y705 of STAT3

To analyze the functional aspects of Y705 in the negative regulatory effects of PIAS3 on STAT3 transcriptional activity, we cotransfected the A549 cell line with WT Y705 or mutant Y705F STAT3 along with a pCMV5 vector containing PIAS3 gene. To determine the transcriptional capacity of STAT3, a pTA-Luc vector containing the luciferase reporter gene under the transcriptional control of STAT3 was used. In the absence of EGF, the luciferase activity is minimal. However, with EGF stimulation and WT-STAT3, a significant increase in luciferase activity is seen (Fig. 4). Cotransfection with PIAS3 expression construct results in a substantial decrease in luciferase expression when WT-STAT3 is present ($P < 0.0001$) and no significant effect when PIAS3 is cotransfected with mutant STAT3. These data indicate that Y705 is critical for the negative regulatory effects of PIAS3 on STAT3 transcriptional activity.

The Negative Regulation of STAT3 Transcriptional Activity Is PIAS3 Dose Dependent

We then hypothesized that the intracellular amount of PIAS3 would determine the negative regulatory effect on the ability of STAT3 to modulate the transcription of its target genes. Using our luciferase expression vector containing the STAT3 binding sequence, we progressively increased the amount of intracellular PIAS3 by increasing the amount of transfected PIAS3-containing pCMV vector. EGF results in a significant increase in luciferase activity. In both A549 and H520 cell lines, with increasing concentrations of PIAS3, a clear dose-dependent decrease in STAT3 transcriptional activity can be seen (Fig. 5A and B).

The Dose-Dependent Effect of PIAS3 on STAT3 Transcriptional Activity May Result from the Effect of STAT3 Y705 Phosphorylation Status

To elucidate the potential mechanism by which a dose-dependent effect of PIAS3 would result in decreased STAT3 transcriptional activity, we looked at the relation between STAT3 phosphorylation status of the Y705 site in the presence of EGF and increasing doses of transfected PIAS3. Transfection with the same vector not containing the PIAS3 gene was used as control (Fig. 6A and B). In this experiment, both A549 and H520 cell lines were transfected with increasing amounts of PIAS3 or empty vectors. After stimulation with 20 ng/mL EGF, cells were harvested and nuclear cell lysates were prepared. By immunoblotting with anti–phosphospecific STAT3

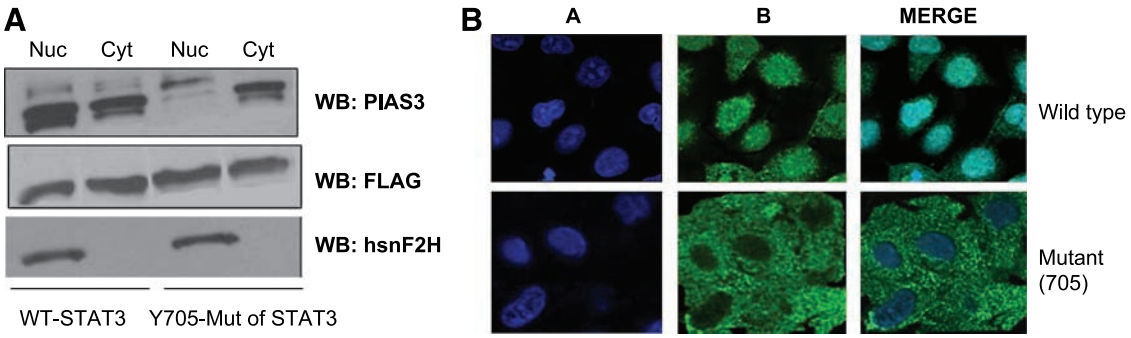

FIGURE 3. **A.** Western blot analysis. A549 cells were transiently transfected with a plasmid containing WT Y705 and mutant (Y705F) STAT3 expression vector. After 48 h of transfection, cells were starved for 12 h in serum-free medium and cells were stimulated with 20 μg/mL EGF for 10 min; nuclear cell lysates (*Nuc*) and cytoplasmic cell lysates (*Cyt*) of cell lines transfected with WT and Tyr705 mutant plasmids were subjected to SDS-PAGE and blotted with anti-PIAS3. **B.** A549 cells were incubated on a poly-L-lysine–treated glass-bottomed culture dish and were transiently transfected with a plasmid containing WT Y705 and mutant (Y705F) STAT3 expression vector. Both transfected cell lines were starved for at least for 12 h in serum-free medium and were stimulated with 20 μg/mL EGF for 10 min. Cells were fixed, permeabilized, and immunostained. WT cells show complete shuttling of PIAS3 into the nucleus, whereas mutant (Y705F) cells could not accumulate completely into the nucleus, suggesting that the presence of Tyr705 is essential for efficient translocation. **A.** DRAQ5 counterstain (nuclei, *blue*). **B.** Antirabbit IgG conjugated with Alexa Fluor 488 counterstain (PIAS3, *green*).

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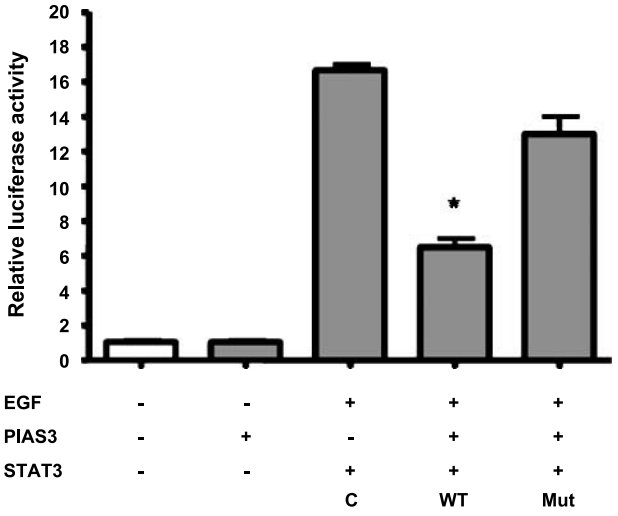

FIGURE 4. Effect of mutation of STAT3 at Y705 on PIAS3-STAT3 association. PIAS3 significantly inhibits STAT3-regulated gene expression in WT-STAT3 but fails to do so in cell lines transfected with Y705F mutant of STAT3. After 48 h of cotransfection with WT or Y705F mutant with luciferase reporter pTA-Luc vector or empty pTA-Luc vector, with or without PIAS3, cells were then stimulated with EGF for 10 min. The luciferase activity of lysed cells was measured and normalized against the protein concentration. Columns, mean of four experiments; bars, SEM. The luciferase activities of WT and mutant were compared with the control (*C*), which is without PIAS3 transfection.

antibody (against Y705 site), we found that the phospho-STAT3 protein levels were significantly reduced in the nucleus, and this reduction was PIAS3 dose dependent. Similar results were seen in both cell lines. Anti-hsnf2H antibody was used as a loading control for nuclear extracts. This result suggests that PIAS3 inhibits STAT3 by potentially accelerating dephosphorylation process, as we had previously shown that PIAS3 does not effect the initial phosphorylation of STAT3.

Discussion

STAT3 activation has been observed to occur in many solid tumors and hematologic malignancies and is correlated with proliferative and antiapoptotic effects in malignancies (14-16). There are several physiologic STAT inhibitors, and one of which is the PIAS. PIAS proteins represent a group of five proteins, PIAS1, PIAS3, PIASxα, PIASxβ, and PIASy, which are known to block the STAT-DNA binding activity (17). These proteins contain several conserved domains including the NH_2-terminal SAP (Saf-A/B, Acinus, and PIAS) box with the LXXLL signature; the Zn finger/RING domain, which is essential for small ubiquitin–related modifier ligase activity; and a COOH-terminal acidic domain, which is involved in binding to other nuclear coactivators (18) and (19). Subsequent studies in many cell systems determined that PIAS proteins interact with and modulate a broad array of nuclear proteins. The suggested mechanism of action of PIAS3 is substrate modification through the ability of PIAS proteins to function as E3 ligases and mediate small ubiquitin–related modifier-1 modification of transcription factors (2, 20-23). PIAS3 is known to inhibit gene induction through activated STAT3 by blocking the DNA binding activity of the transcription factor (3).

In this report, we have shown that EGF stimulation in NSCLC cells leads to the activation of STAT3 and its complex formation with PIAS3. This occurs within minutes of exposure to EGF, suggesting a negative feedback loop to control STAT3 signaling. PIAS3 subsequently returns to the cytoplasm by 30 minutes of EGF stimulation. This corresponds to the same time frame in which STAT3 homodimers form with EGF stimulation, as described by Wang and Banerjee (24). Previous data in human and murine cell lines have determined that PIAS3 localizes to nuclear and cytoplasmic compartments; however, the kinetics of trafficking among the two compartments was unknown (3). The function of PIAS3 as a transcription factor modulator suggests that the nucleus is its site of activity. Here, we determined the presence of subcellular localization of PIAS3 protein by *in situ* immunofluorescence analysis and immunoblot analysis of nuclear and cytoplasmic extracts. Our data suggest that its localization is time and ligand dependent. Both PIAS3 binding to STAT3 and its nuclear translocation are also strongly dependent on the presence of Tyr705 of STAT3. The importance of Tyr705 of STAT3 for PIAS3-STAT3 interaction had not been previously described.

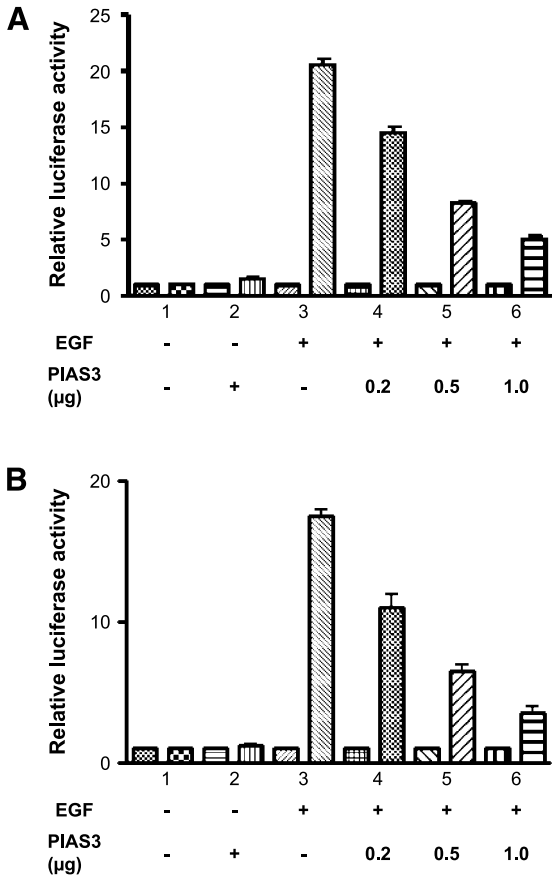

FIGURE 5. PIAS3 regulates EGF-dependent transcriptional activation of STAT3 in A549 (**A**) and H520 (**B**) cell lines. Cells were transfected with luciferase reporter pSTAT3-LUC vector (1 μg) together with either PIAS3 expression constructs or the empty pCMV vector in increasing concentration and then stimulated or unstimulated with EGF; after 48 h of transfection, cells were harvested and relative luciferase activity was measured. Columns, relative luciferase activity from triplicate experiments.

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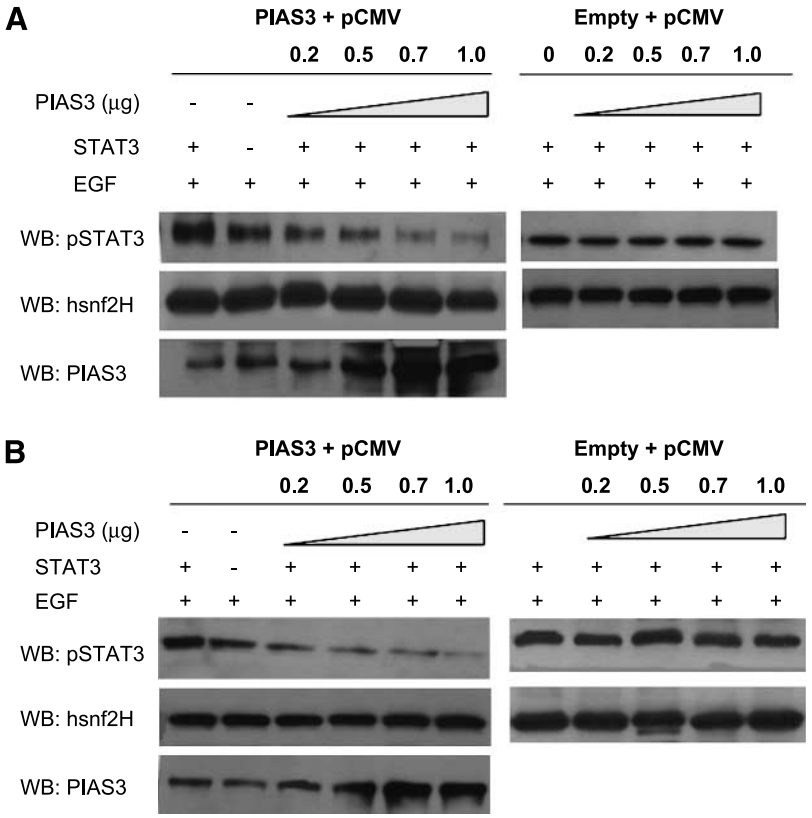

FIGURE 6. Transfection of PIAS3 expression constructs or the empty pCMV5 vector in increasing concentration results in downregulation of pSTAT3. Cells were transfected with increasing concentration of PIAS3 and stimulated or unstimulated with EGF; after 48 h of transfection, cells were harvested and nuclear extracts were prepared and immunoblotted for pSTAT3 and hsnf2H as a nuclear extract loading control. **A.** A549 cell line. **B.** H520 cell line.

Phosphorylation is known as an important process in the formation of protein complexes. To evaluate the importance of phosphorylation at Tyr705 of STAT3 in the formation of the PIAS3-STAT3 complex, we constructed Tyr705 mutants in which tyrosine residue is substituted with phenylalanine, which mimics the charge of hydroxyl group of tyrosine. The WT human STAT3 and the Y705F mutant of STAT3 cloned in to pcDNA3 vector were transfected into A549 and H520 cell lines and stimulated or unstimulated with EGF, and the cell lysates were immunoprecipitated with anti-PIAS3 antibody. Substitution of tyrosine with phenylalanine resulted in decreased binding with PIAS3. These results show that phosphorylation at Tyr705 of STAT3 is critical in the PIAS3-STAT3 association. In addition, PIAS3 trafficking to the nucleus is hampered in Y705F mutant STAT3 cells.

To understand the functional aspects of Y705 in the negative regulatory effects of PIAS3 on STAT3 transcriptional activity, we cotransfected the A549 cell line with WT Y705 or mutant Y705F STAT3 cloned into luciferase reporter vector under the control of a TA promoter, along with the pCMV5 vector containing PIAS3 expression construct. We found that PIAS3 inhibited WT STAT3 transcriptional activity but failed to inhibit mutant STAT3 (Fig. 4). In the PIAS3 region of interaction, Levy et al. (25) showed that a short stretch of 50 amino acids in the PINIT domain of PIAS3 is directly responsible for the binding and downregulation of STAT3. We now show that Tyr705 of STAT3 is critical in the STAT3 region of interaction.

We also show that the negative inhibitory effect of PIAS3 is concentration dependent. Increasing intracellular levels of PIAS3 clearly proportionally increase the negative inhibitory effect on STAT3 signal transduction. A549 and H520 cells were cotransfected with the luciferase reporter construct containing the STAT3-encoding gene under the TA promoter in the presence of increasing amount of PIAS3 expression vector. EGF-induced STAT3 activation was inhibited by the expression of PIAS3 in a dose-dependent manner. Recently, we showed that PIAS3 protein is not expressed in roughly 50% of human NSCLC specimens (13), with a similar downregulation shown in glioblastoma multiforme specimens (11). This thus sets the stage to consider upregulation of PIAS3 as a potential antitumor strategy in lung cancer. Our data suggest that this concentration-dependent effect occurs by two mechanisms that may be related. First, we show that there is PIAS3 concentration–dependent decrease in STAT3 transcriptional activity. This is consistent with the previously described effect of PIAS3 on the DNA binding activity of STAT3 (3). However, we also show a potential novel mechanism by which increasing PIAS3 concentration may determine its anti-STAT3 activity. We thus show that increasing PIAS3 concentration decreases phosphorylation of Tyr705 of STAT3. This suggests that PIAS3 may have a role in the dephosphorylation process of STAT3. As PIAS3 does not

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alter the capacity of EGF stimulation to initially induce STAT3 phosphorylation, our data suggest that PIAS3 may accelerate the dephosphorylation of phospho-STAT3.

In this study, we have presented experimental evidence that supports the hypothesis of the functional and biochemical interactions between STAT3 and PIAS3 in NSCLC. Understanding the mechanism of interactions between the STAT3 and PIAS3 in more detail would be important in providing the basis for drug development in NSCLC.

Materials and Methods

Cell Lines

Human pulmonary epithelial cell lines A549 and H520 were purchased from the American Type Culture Collection. A549 and H520 were maintained in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hyclone), 50 unit/mL penicillin, and 50 μg/mL streptomycin in a 5% CO_2 humidified incubator at 37°C. Cell number and viability were assessed by trypan blue (Sigma) dye exclusion using a hemocytometer.

Confocal Microscopy

A549 and H520 cells were incubated on a glass-bottomed culture dish (MatTek Corp.) and were starved for at least 12 h in serum-free medium. Cells were unstimulated or stimulated with 20 ng/mL EGF for 5 to 30 min and then fixed with 4% formaldehyde diluted in PBS. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min. After 1-h blocking with 1% bovine serum albumin in PBS, cells were incubated with the rabbit polyclonal anti-PIAS3 (Santa Cruz Biotechnology, Inc.; 1:100) antibody at room temperature for 2 h and washed three to four times with PBS. Incubation with Alexa Fluor 488–conjugated goat anti-rabbit IgG (Molecular Probes; 1:500) was done for 1 h at room temperature, followed by repeated washes using PBS. After the subsequent staining with 1 μmol DRAQ5 in PBS for 30 min, confocal images were obtained on Zeiss LSM510 NLO laser-scanning microscope using single-line (488 nm) or multitrack sequential excitation (488 and 633 nm). Images were acquired and processed with Zeiss LSM Image Browser software.

Plasmid Construction

The STAT3 WT and mutant (Y705F) constructs were generated as follows. The cDNAs encoding the open reading frame of human STAT3 and STAT3 mutant, in which tyrosine is replaced with phenylalanine at position 705, were subcloned into the *Nhe*I and *Xho*I sites at the multiple cloning site of the luciferase reporter pTA-Luc vector (Clontech). The FLAG-tagged pcDNA3 vector containing the coding region for WT-STAT3 and Tyr705 mutant STAT3 was generously provided by Dr. J. Stark at Lerner Research Institute (Cleveland, OH). The cDNA encoding PIAS3 in a pCMV5 expression vector was generously provided by Dr. Ken Shuai from the David Geffen School of Medicine, University of California at Los Angeles (Los Angeles, CA). The fidelity of all the constructs was verified by direct sequencing.

Transient Transfection and Luciferase Assay

A549 cells were seeded at 1×10^5 per well in six-well plates. The HD FuGENE (Roche) was used as a transfection reagent to cotransfect the cells with luciferase reporter construct pSTAT3 TA-Luc [WT or mutant (Y705F)] or pTA-Luc alone as a nonspecific control and with pcMV5 PIAS3 expression construct or pcMV5 alone as a control. The cells were incubated in DMEM/HF12 medium for 48 h, treated or not with 20 ng/mL EGF for 15 min; cells were washed with cold PBS, lysed with passive lyses buffer (Promega), and then centrifuged at 12,000 × *g* for 4 min. The supernatant was collected and stored at −80°C until assessment of luciferase activity. Luminescence was read in a Berthold luminometer (Lumat LB9501) after briefly mixing the supernatant (20 μL) with 100 μL of firefly luciferase assay substrate solution. The activity of luciferase was normalized to protein concentrations in lysate. Transfections were repeated at least three times, and the relative changes are presented as mean ± SE.

Coimmunoprecipitation

A549 cells were transfected with the WT or mutant Y705F STAT3 with a luciferase reporter vector. Transfection was carried out as described above with the transfection reagent HD FuGENE (Roche). After 24 h of transfection, medium was replaced with serum-free medium and incubated overnight at 37°C. The next day, cells were stimulated for 10 min with 20 ng/mL EGF. Nuclear and cytoplasmic cell lysates were prepared separately using Panomics kit following the manufacturer's instructions. One hundred micrograms of protein lysate were complexed with 2 μg of anti-PIAS3 antibody (Santa Cruz Biotechnology) for 2 h at 4°C on a rocking platform. Protein A-Sepharose beads (50 μL; Zymed Laboratories, Inc.) were added; the solution was incubated for 1 h, washed thrice with lysis buffer, and centrifuged; and the supernatant removed. The bead, protein-antibody complex was then resuspended in 1× Laemmli buffer and boiled at 95°C for 5 min; 10 μL of the supernatant containing the protein-antibody complex were resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, and detected by immunoblotting with the appropriate antibodies using the enhanced chemiluminescence substrate from Amersham Pharmacia Corp.

Western Blotting

To obtain protein lysate, cells that were in log-phase growth (50-70% confluence) were lysed in buffer containing 1% Triton X-100, 0.15 mol/L sodium chloride, 50 mmol/L Tris (pH 7.4), and protease inhibitors (50 μg/mL aprotinin, 50 μg/mL pepstatin, 10 μg/mL leupeptin, 0.4 mmol/L EDTA, 0.4 mmol/L sodium orthovanadate, 10 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, 5 mmol/L phenylmethylsulfonyl fluoride). Protein concentrations were determined by the Coomassie method (Bio-Rad Protein Assay, Invitrogen). Fifteen micrograms of protein from each sample were separated on 10% SDS-PAGE, transferred onto a polyvinylidene difluoride membrane (Immobilon, Millipore), and blotted with specific antibodies (1:200 dilution for all primary antibodies and 1:5,000 for all secondary antibodies; Santa Cruz Biotechnology).

Statistical Analysis

All the described experiments were done more than three times, and the data are presented as mean values ± SEM. *P* values were determined by *t* test using Prism software. $P < 0.05$ was considered statistically significant.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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