A Ras Homologue Member I Directly Inhibits Signal Transducers and Activators of Transcription 3 Translocation and Activity in Human Breast and Ovarian Cancer Cells

Arata Nishimoto,1 Yinhua Yu,1 Zhen Lu,1 Xiang Mao,2 Zhiyong Ren,2 Stephanie S. Watowich,3 Gordon B. Mills,4 Warren S-L. Liao,2 Xiaomin Chen,2 Robert C. Bast Jr.,1 and Robert Z. Luo1

Departments of Experimental Therapeutics,1 Biochemistry and Molecular Biology,1 Immunology, and1 Molecular Therapeutics, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

Abstract
A Ras homologue member I (ARHI) is a novel imprinted tumor suppressor gene whose expression is frequently lost in breast and ovarian cancers. This small GTP-binding protein is a member of the Ras superfamily with significant homology to both Ras and Rap. Unlike the Ras oncogene, however, ARHI inhibits tumor cell growth. To elucidate the mechanisms by which ARHI inhibits cancer growth, we screened a human breast epithelial cell cDNA library using a yeast two-hybrid system for ARHI-interacting proteins. ARHI was found to interact with signal transducers and activators of transcription (STAT) 3, a latent transcription factor that transduces signals from the cell surface to the nucleus and activates gene transcription. STAT3 is frequently phosphorylated and activated in breast and ovarian cancers, where cytokines and growth factors up-regulate STAT3 and stimulate proliferation. The ARHI-STAT3 interaction was confirmed by coimmunoprecipitation in mammalian cells and shown to be specific for STAT3 but not STAT1 or STAT5a. When ARHI and STAT3 were coexpressed in SKOV3 cells, ARHI formed a complex with STAT3 in the cytoplasm and prevented interleukin-6–induced STAT3 accumulation in the nucleus. ARHI markedly reduced STAT3 binding to DNA and STAT3-dependent promoter activity while only moderately affecting STAT3 phosphorylation. Deletion of the NH2 terminus of ARHI significantly compromised its inhibitory activity, suggesting that this unique NH2-terminal extension contributes to ARHI's inhibition of STAT3-mediated transcriptional activity. Thus, the physical association between STAT3 and ARHI as well as the functional inhibition of STAT3 transcriptional activity by ARHI suggests a novel mechanism through which a putative tumor suppressor gene can inhibit STAT3 activity in breast and ovarian cancers. (Cancer Res 2005; 65(15): 6701-10)

Introduction
Recently, our group has identified a Ras homologue member I (ARHI), a novel maternally imprinted tumor suppressor gene that encodes a 26-kDa GTP-binding protein with 60% homology to Ras and Rap (1). ARHI shares several conserved sequences with the GTP-binding domains of the Ras protein but differs from Ras in residues critical for GTPase activity and in the effector domain. Due to these sequence differences, ARHI is likely to exist in the constitutively GTP-bound form and is likely to have different binding partners. Furthermore, ARHI contains a unique extension of 34 amino acids at the NH2 terminus, which is rarely encountered in other Ras family members (2). ARHI is expressed in normal breast and ovarian epithelial cells, but its expression is lost or markedly down-regulated in the majority of breast and ovarian cancers. The ARHI gene maps to a locus on human chromosome 1p31 that has been associated with a high frequency of loss of heterozygosity (40%) in breast and ovarian cancers (1, 3). Introduction of this gene into cancer cells that lack ARHI expression inhibits proliferation and motility of cancer cells. ARHI truncates signaling through Ras/mitogen-activated protein kinase (MAPK), activates c-Jun NH2-terminal kinase, induces p21WAF1/CIP1, down-regulates cyclin D1, and triggers apoptosis (1, 2, 4). Although most members of the Ras superfamily are proto-oncogenes or positive growth regulators, ARHI is one of the rare tumor suppressor genes in this superfamily. Interestingly, the NH2 terminus that differentiates ARHI from Ras seems essential for its suppressive activity (2). Molecular mechanisms by which ARHI inhibits proliferation and motility remain to be fully elucidated.

The signal transducers and activators of transcription (STAT) proteins play important roles in regulating fundamental cellular processes, including cell growth, differentiation, and survival (5, 6). These proteins exhibit dual functions as STAT. STATs are latent transcription factors that are activated by phosphorylation of a conserved tyrosine residue in response to extracellular signaling molecules, such as cytokines and growth factors. STATs can also be activated by intracellular molecules, including some oncogenes and nonreceptor tyrosine kinases. Activated STATs form homodimers or heterodimers through reciprocal interactions between their SH2 domains and phosphorylated tyrosine residues. STAT dimers then translocate to the nucleus where they bind to consensus response elements through their DNA-binding domains and activate transcription of target genes (7, 8). Within the structures of STAT proteins, the NH2-terminal and the coiled-coil domains are likely to be the major regions involved in protein-protein interaction and in regulating their function and activity (9–11).

Accumulating evidence shows that, among the STATs, abnormal activation of STAT3 signaling plays a critical role in oncogenesis (12). STAT3 is overexpressed and constitutively activated in a wide variety of human malignancies, including breast and ovarian carcinomas (13–15). Accordingly, STAT3 expression is up-regulated through a self-activation loop and STAT3 is a survival and mitogenic transducer that up-regulates the apoptosis suppressor Bcl-xL and the cell cycle activators c-Myc and cyclin D1 (15–18). STAT3 has been classified as a potential oncogene, because a gain-of-function...
mutation renders cells transformed and tumorigenic (14, 16). Constitutive activation of STAT3 has been observed in most invasive breast cancer samples and many breast and ovarian cell lines but not in normal breast and ovarian surface epithelial cells (14, 18–21). Hence, identifying mediators of STAT3 activation may enhance our understanding of malignant transformation events and provide novel molecular targets for therapeutic intervention in these cancers.

Recent studies have suggested a link between STAT3 activity and some small GTPases, including Rac1 or RhoA (22–26). Small GTPases comprise a family of >100 monomeric G proteins that function as molecular switches in cellular signaling. Small G proteins cycle between an active, GTP-bound state and an inactive, GDP-bound state, regulating vesicular trafficking, nuclear-cytoplasmic trafficking, spindle microtubule assembly events, and cytoskeletal rearrangement (27). Constitutive activation of many Ras family members has led to the induction of cellular transformation and oncogenesis (28). Although both Rac1 and RhoA bind and up-regulate STAT3 activity, our data indicate that ARHI, also a Ras superfamily member, directly binds STAT3 and inhibits its activity. Studies with ARHI mutants suggest that the unique NH2-terminal extension of ARHI plays an important role in down-regulating STAT3 activity. Taken together, these observations document a novel negative regulatory mechanism in STAT3 signaling, where a Ras family member negatively modulates STAT transcriptional activities.

Materials and Methods

Reagents. A polyclonal antibody against phospho-STAT3-Tyr705 was obtained from Cell Signaling Technology (Beverly, MA). Antibodies against STAT3, STAT1, STAT5a, and STAT5b were obtained from Cell Signaling Technology (Beverly, MA). Monoclonal and polyclonal antibodies against ARHI were generated in our laboratory.

Cells. Normal breast epithelial cells NBE234 and NBE231 and normal ovarian epithelial cells OSE113, OSE115, OSE106, and OSE120 were cultured as described previously (29). SKBr3, BT20, MDA-MB-468, and MDA-MB-435 breast cancer cells were maintained in RPMI1640 supplemented with 10% FBS. OVCA420, SKOv3, DOV13, and OVCA429 ovarian cancer cells were grown in McCoy’s medium containing 10% FBS. A Tet-On ARHI-SKOv3 or ARHI-NTD-SKOv3 (the NH2-terminal deletion mutant ARHI)–inducible cell line was grown in McCoy’s medium supplemented with 10% FBS, 200 μg/mL G418, and 0.12 μg/mL puromycin. ARHI expression was induced by adding 1 μg/mL doxycycline to the culture medium. The resulting 0.7-kb (full-length) or 0.6-kb (NTD or CTD mutants) ARHI-cDNA was subcloned into pcDNA3 expression vector (Invitrogen, Carlsbad, CA). Other mutant ARHI plasmids were constructed by site-directed mutagenesis (QuickChange site-directed mutagenesis kit, Stratagene, La Jolla, CA). Briefly, synthetic oligonucleotide primers containing the mutated sequences were synthesized and mixed with components of the thermal cycling reaction that contained a supercoiled double-stranded full-length pcDNA3-ARHI plasmid template. The resulting mutant plasmids that modified the effector domain of ARHI were generated: pcDNA-ARHH64D-N72D-C75R-Q76K, pcDNA-ARHIH64D-N72D-T73S-C75R-Q76K, and pcDNA-ARHIH64D-N72D-T73S-C75R-Q76K (a construct that combined mutations in the effector domain). All constructs were confirmed by DNA sequencing.

Transient transfections and luciferase assays. For luciferase reporter assays, SKBr3 cells were seeded (2 × 104 per well) in six-well plates for 24 hours before transfection. All transfection experiments were done in triplicate using FuGene6 (Roche Diagnostics, Indianapolis, IN). Cells were cotransfected with 0.4 μg luciferase reporter construct that contained three copies of the STAT3 DNA-binding sequence from the α2-macroglobulin promoter and 1.6 μg pcDNA-ARHI or pcDNA3-ARHI-NTD or an empty pcDNA3 vector. Twenty-four hours after transfection, cultures were treated with diluent or with interleukin-6 (IL-6; 10 ng/mL) for 6 hours, and cell extracts were prepared for luciferase assays. Transfected cells were washed twice with PBS, and lysis buffer (500 μL) was added to each well. Extracts were then used to measure luciferase activity by integrating total light emission over a 10-second period. Luciferase activity was normalized based on protein concentration and luciferase activities were assayed as described in the manufacturer’s protocol (Promega Corp., Madison, WI).

Adenovirus infection. A binary adenoviral vector system was constructed to express ARHI or ARHI-NTD mutant proteins in cancer cells (4). Adeno-LacZ was used as a control virus. Ovarian and breast cancer cells were grown as monolayers in 60 mm culture dishes with 4 mL culture medium for 24 hours before infection with adenoviral vectors. Supernatant medium was aspirated and replaced with fresh medium containing different combinations of adenoviral vectors. A ratio of 30 to 50 plaque-forming units per cell was used to infect cells, and cells were harvested 72 hours after infection.

Immunoprecipitation and Western blot analysis. Immunoprecipitation reactions were done with cell lysates that had been precleared with 50 μL protein G-Sepharose (Amersham Biosciences, Piscataway, NJ) for 30 minutes at 4°C. Cleared extracts were immunoprecipitated with 4 μg of the indicated antibodies overnight at 4°C followed by the addition of 40 μL protein G-Sepharose for 4 hours at 4°C. Immunoprecipitates were washed thrice with lysis buffer, boiled for 5 minutes, and loaded on polyacrylamide gel. Proteins were then electrophoresed overnight to polyvinylidene difluoride (PVDF) membranes. Blots were washed five times and incubated for 1 hour at room temperature in TBST [20 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 0.1% Tween 20] buffer that contained 5% milk and 2% bovine serum albumin (BSA). Blots were then incubated with the indicated antibodies in TBST for 2 hours, washed five times, and incubated with secondary antibodies conjugated to horseradish peroxidase (HRP). Blots were developed by using an enhanced chemiluminescence system (Amersham Biosciences).

Because ARHI was lost or markedly down-regulated in all ovarian or breast cancer cells used in our studies, we infected these cells with...
ARHI Inhibits STAT3 in Breast and Ovarian Cancers

adeno-ARHI virus, transduced them with ARHI plasmid constructs, or used the doxycycline-inducible ARHI-SKOV3 cells to reintroduce ARHI into cancer cells to study the ARHI-STAT3 interaction.

To examine the interaction site on STAT3, L1 (STAT3-ΔC), L1-U (Δ1ISSTAT3F), and L1-WT (wild-type STAT3) cells were infected with adeno-ARHI before harvest for cell lysate preparation. Anti-ARHI antibody was used to immunoprecipitate ARHI and its interacting proteins. Proteins in the immune complexes were then subjected to Western blotting with anti-ARHI or anti-STAT3 antibodies. Additionally, soluble full-length or NH2-terminal truncated STAT3 protein (2 μg) purified from an E. coli expressing system was added to the cell lysate prepared from adeno-ARHI-infected L1 cells and incubated with anti-ARHI antibody overnight at 4°C. Immunoprecipitated proteins were then identified by Western blotting.

**Immunostaining.** For immunofluorescent staining, Tet-On-inducible ARHI-SKOV3 cells were grown on collagen-coated chamber slides (BD Bioscience, Bedford, MA) in McCoy's medium supplemented with 10% FBS, 100 μg/ml G418, and 0.12 μg/ml puromycin until cells reached 30% confluence. To induce ARHI expression, ARHI-SKOV3 cells were treated with doxycycline (1 μg/ml) for 24 hours before they were fixed with 4% paraformaldehyde and 3% BSA in PBS and washed with PBS. After permeabilization with 0.5% Triton X-100 for 30 minutes, cells were washed and blocked with 3% normal goat serum and 0.1% BSA in PBS followed by incubation with primary antibodies in 0.1% BSA in PBS. After washing with 0.1% BSA in PBS, cells were incubated with the appropriate secondary antibodies conjugated with Alexa Fluor 488 or 594 (Molecular Probes, Eugene, OR). Cells were then washed thrice with PBS and mounted with Antifade mounting medium (Vector Laboratories, Burlingame, CA) and examined using a confocal microscope (Olympus Fluoview 500, Olympus, Inc., Melville, NY).

For nuclear section imaging, a Z-axis image section was cut in 0.2 μm sections by the confocal microscope and the center section of the nucleus was selected.

Immunohistochemical staining was done on the chamber slides with the same fixation and permeabilization procedures used for fluorescent staining. Endogenous peroxidase activity was quenched by incubating sections in 3% hydrogen peroxide. Slides were incubated in Ultra V Block for 5 minutes at room temperature to block nonspecific staining. Anti-ARHI or anti-STAT3 antibody was applied at 1 μg/ml in 1% BSA in PBS at 4°C overnight. Biotinylated secondary antibodies were detected with streptavidin peroxi-
dase by using the UltraVision Large Detection System Anti-Polyvalent HRP (Lab Vision, Fremont, CA). A diamobenzidine tetrachloride supersensitive substrate kit (Biogenex, San Ramon, CA) was used to visualize the antibody-antigen complexes.

**Electrophoretic mobility shift assay.** Nuclear extracts were prepared from SKBr3 cells infected with adeno-LacZ, adeno-ARHI, or adeno-NTD after stimulation with IL-6 (10 ng/ml) for 30 minutes before harvest. Electrophoretic mobility shift assay (EMSA) was done with a double-stranded, 32P-niadiolabeled oligonucleotide that contained the consensus binding site for STAT3. Competition analysis was done using oligonucleo-
tides for the consensus binding site for STAT3 or mutant oligonucleotides of the STAT3-binding motif or oligonucleotides of the nuclear factor-κB-binding element. Supershift analysis was done using antibodies against normal IgG, STAT1, or STAT3. The protein-DNA complexes were resolved on a nondenaturing polyacrylamide gel and were autoradiographed.

**Real-time quantitative reverse transcription-PCR.** Real-time quantitative reverse transcription-PCR (RQ RT-PCR) was used to measure the expression levels of the STAT3-responsive genes in cells treated with or without IL-6 and/or doxycycline. Total RNA from cells was purified and the cDNA was synthesized using 2 μg total RNA. The reverse transcriptase reaction was done according to the manufacturer's instructions using oligo(dT)16 and SuperScript II reverse transcriptase (Invitrogen) followed by SYBR Green real-time PCR (ABI Prism 7000 Sequence Detection System, Applied Biosystems, Foster City, CA). The primers for c-Myc were 5'-GCCATCCACTGCGAGAGCT-3' and 5'-GAAATTTGAAGGGCAGGAGTC-3'. The thermal cycling conditions for each primer set consisted of 2 minutes at 50°C and 10 minutes at 95°C followed by 40 cycles for 15 seconds at 95°C and 1 minute at 60°C. All reactions were done using ABI Prism 7000 Sequence Detector. The expression levels were normalized by the GAPDH level.

**Statistical analysis.** The data are expressed as means ± SE. Statistical comparisons between groups were done using the Student's t test. P < 0.05 were considered statistically significant.

**Results**

A Ras homologue member I interacts with signal transducers and activators of transcription 3 in a yeast two-hybrid system. To identify the proteins that interact with ARHI, a yeast two-hybrid screen of a human breast epithelial cDNA library was done using full-length ARHI as bait. A cDNA fragment obtained in the yeast two-hybrid analysis matched with the sequence of human STAT3 mRNA encoding a protein fragment that contained the first 400 amino acid residues. This fragment encompassed the STAT3 NH2-terminal domain, the coiled-coil domain, and part of the DNA-binding domain. Two independent clones containing different lengths of STAT3 cDNA encoding the NH2-terminal regions were identified among several other ARHI-interacting clones. The ARHI-STAT3 interaction was subsequently confirmed in a yeast two-hybrid interaction assay (Fig. 1A). Yeast expression constructs that contained ARHI, STAT3 fragments, or control cDNA fragments were cotransformed into yeast strain AH109 on SD medium that lacked histidine and leucine. The transformed cells were examined on plates that contained SD/glucose/-Trp/-Leu/-His selection medium. The selected colonies were then tested for β-galactosidase activity. Only yeast cells containing both pGBKTK7-ARHI and pACT2-STAT3

![Figure 1](https://cancerres.aacrjournals.org/attachment/0052694a.png)

Figure 1. ARHI interacts with STAT3 in yeast two-hybrid analysis. To examine the interaction of ARHI and STAT3 in yeast, ARHI (bait), STAT3 (target), and control yeast constructs were transformed into yeast strain AH109, cultured in nutrition-depleted medium, and analyzed for β-galactosidase activity. Only colonies that contained ARHI + STAT3 or the positive control [p53 + T (+C)] displayed β-galactosidase activity indicated by a blue color. Noninteracting protein lamin (Lam) was used as the negative control (-C, A). A biosensor assay was also done and further confirmed the specific interaction between ARHI and STAT3 (B). *, P < 0.001, compared with control.
grew and exhibited a blue color staining for β-galactosidase activity comparable with the positive control. This transformant was further analyzed using a biosensor assay following the manufacturer's protocol (Invitrogen). Only the positive clones were able to grow in the selective medium and display fluorescence (Fig. 1B). Thus, yeast two-hybrid analysis resulted in the identification of STAT3 as an ARHI-interacting protein.

A Ras homologue member I associates with endogenous signal transducers and activators of transcription 3 in breast and ovarian cancer cells. To show the relevance of this interaction in vivo, we investigated the association of ARHI with endogenous STAT3 in human cells. Immunoprecipitation of cell lysates prepared from OSE115 normal ovarian epithelial cells with an anti-ARHI antibody showed that ARHI associated with STAT3. To overcome the down-regulated ARHI expression in cancer cells, SKBr3 breast cancer cells were infected with adeno-LacZ, adeno-ARHI, or adeno-NTD and stimulated with IL-6 (10 ng/mL) for 30 minutes before harvest. Proteins communoprecipitated with anti-ARHI antibodies were analyzed by Western analysis with anti-STAT3 or anti-phospho-STAT3 antibody. Conversely, proteins immunoprecipitated with anti-STAT3 antibody were analyzed by Western analysis with anti-ARHI antibody (A2). A similar association was observed in SKBr3 breast cancer cells and DOV13 ovarian cancer cells without IL-6 stimulation (A3). To examine the specificity of ARHI-STAT3 interaction, SKBr3 breast cancer cells that express endogenous STAT5a and MDA-MB-468 breast cancer cells that express STAT1 were infected with adeno-LacZ or adeno-ARHI. Cell extracts from infected cells were communoprecipitated with anti-ARHI antibody and Western blotted with anti-STAT1 or anti-STAT5a antibodies (B1 and B2).

Figure 2. ARHI interacts with endogenous STAT3. Cell lysates from OSE115 normal ovarian epithelial cells were incubated with anti-ARHI antibody overnight at 4°C and the immunoprecipitates were analyzed by Western blots with anti-STAT3, anti-ARHI, or preimmunized IgG. Note that due to relatively low ARHI levels in normal epithelial cell lysates only immunoprecipitated samples showed ARHI protein signal (A1). To overcome the down-regulated ARHI expression in cancer cells, SKBr3 breast cancer cells were infected with adeno-LacZ, adeno-ARHI, or adeno-NTD and stimulated with IL-6 (10 ng/mL) for 30 minutes before harvest. Proteins communoprecipitated with anti-ARHI antibodies were analyzed by Western analysis with anti-STAT3 or anti-phospho-STAT3 antibody. Conversely, proteins immunoprecipitated with anti-STAT3 antibody were analyzed by Western analysis with anti-ARHI antibody (A2). A similar association was observed in SKBr3 breast cancer cells and DOV13 ovarian cancer cells without IL-6 stimulation (A3). To examine the specificity of ARHI-STAT3 interaction, SKBr3 breast cancer cells that express endogenous STAT5a and MDA-MB-468 breast cancer cells that express STAT1 were infected with adeno-LacZ or adeno-ARHI. Cell extracts from infected cells were communoprecipitated with anti-ARHI antibody and Western blotted with anti-STAT1 or anti-STAT5a antibodies (B1 and B2).
Because ARHI expression was either absent or dramatically down-regulated in most ovarian or breast cancer cells, we induced ARHI expression in these cells using a dual adenoviral vector system (4). Human breast cancer cell line SKBr3 or the ovarian cancer cell line DOV13 were infected with adenov-LacZ, adeno-ARHI, or adeno-NTD (containing the NH₂-terminal deletion mutant of ARHI). Seventy-two hours after infection, cells were treated with IL-6 (10 ng/mL) or with diluent for 30 minutes before harvested for protein extract preparation. Proteins immunoprecipitated with anti-ARHI or anti-STAT3 antibody were probed by Western analysis with anti-STAT3, anti-phosphorylated STAT3, or anti-ARHI antibody. In SKBr3 cell extracts from adeno-NTD or adeno-ARHI-infected cells but not from adeno-LacZ-infected cells, both the nonphosphorylated and the phosphorylated STAT3 were coimmunoprecipitated by anti-ARHI antibodies (Fig. 2A2). This result suggests that ARHI can interact with both phosphorylated and nonphosphorylated STAT3. Thus, the association of ARHI and STAT3 seems to be independent of STAT3 protein phosphorylation. Furthermore, both the full-length ARHI and the NH₂-terminal truncated ARHI mutant (NTD) were coimmunoprecipitated with STAT3 (Fig. 2A2), indicating that the NH₂-terminal domain of ARHI is not required for interaction with STAT3. Adeno-LacZ-infected cell extracts and anti-IgG antibody were included as negative controls for specificity in these immunoprecipitation assays. A similar profile of interaction between ARHI and endogenous STAT3 was observed in the adeno-ARHI-infected human ovarian cancer cell line, DOV13 (Fig. 2A3).

A Ras homologue member I does not associate with signal transducers and activators of transcription 1 or 5a. To show the specificity of ARHI-STAT3 interaction, we examined whether ARHI could interact with other STAT proteins. SKBr3 and MDA-MB-468 breast cancer cells that express endogenous STAT5a and STAT1, respectively, were infected with adeno-ARHI or adeno-LacZ. The cell lysates from these infected cells were immunoprecipitated with anti-ARHI and Western blotted for STAT5a and STAT1. In contrast to STAT3, neither STAT5a nor STAT1 was detected in the anti-ARHI immunoprecipitates despite their abundant expression in these cells (Fig. 2B). Taken together, our results strongly suggest that the interaction between ARHI and STAT3 is highly specific.

A Ras homologue member I interacts with the NH₂ terminus of signal transducers and activators of transcription 3. To examine the potential binding site of ARHI in the association with STAT3, we conducted immunoprecipitation experiments by transfecting ARHI mutant constructs into SKBr3 breast cancer cells in which endogenous ARHI expression was not detectable. Surprisingly, mutations of the ARHI effector domain as well as the NH₂- and COOH-terminal truncation mutants did not affect their binding to STAT3 (Fig. 3A).

We also mapped the ARHI-binding site on STAT3 using immunoprecipitation (Fig. 3B) and pull-down (Fig. 3C) experiments. In contrast to the ARHI deletion studies, truncation of the NH₂ terminus of STAT3 abolished its interaction with ARHI.
showed that starvation-induced growth arrest did not affect the
for 48 hours to induce growth arrest (30) before introducing
experiment has been set by culturing cells in serum-free medium
regulate its target genes. To exclude that the potential growth
potentially gain access to STAT3-dependent DNA elements and
Consequently, a certain amount of activated STAT could
A Ras homologue member 1 inhibits phosphorylation of
signal transducers and activators of transcription 3. We next
examined the effect of ARHI on tyrosine phosphorylation of
STAT3 in response to IL-6 stimulation. SKBr3 cells were infected
with adeno-LacZ, adeno-ARHI, or adeno-NTD. Before harvest,
cells were stimulated with IL-6 (10 ng/mL) for 30 minutes and
Western analysis was done with anti-phosphorylated STAT3 antibody. Phosphorylated STAT3 increased markedly in SKBr3
cells were stimulated with IL-6 (10 ng/mL) for 30 minutes and
with adeno-LacZ, adeno-ARHI, or adeno-NTD. Before harvest,
cells were stimulated with IL-6 (10 ng/mL) for 30 minutes and
interactions with ARHI.

A Ras homologue member 1 colocalizes with signal
transducers and activators of transcription 3 and prevents
nuclear translocation. To elucidate the mechanism(s) by which
ARHI inhibits STAT3 activity, we examined the cellular localization
of STAT3 and its interaction with ARHI in intact cells. Because ARHI expression was either lost or markedly down-
regulated in most breast and ovarian cancer cells, we established
a Tet-On-inducible ovarian cancer cell line ARHI-SKOV3 that
expresses modest levels of ARHI (i.e., similar to those in normal
epithelial cells) in the presence of doxycycline. As expected, in
the absence of ARHI, IL-6-activated endogenous STAT3 and
induced STAT3 translocation into the nucleus (Fig. 5A, A2, A-C2,
and B1). However, induction of ARHI expression with doxycycline
prevented IL-6-mediated STAT3 translocation to the nucleus
(Fig. 5A, A3, A-E2, B2, and C2), although the level of STAT3
phosphorylation was not drastically reduced in the presence of
ARHI (Fig. 5D). In these ARHI-expressing cells, STAT3 colocalized
with ARHI and restricted largely to the cytoplasm (Fig. 5A-D3,
A-E3, B2-B3, and C3). Although most STAT3 was retained in the
cytoplasm, a small amount of ARHI and STAT3 could,
however, be detected in the nucleus by confocal microscopy and
Western blot analysis of subcellular fractions (Fig. 5C and D).
Consequently, a certain amount of activated STAT could
potentially gain access to STAT3-dependent DNA elements and
regulate its target genes. To exclude that the potential growth
arrest triggered down-regulation in signal pathways, a control
experiment has been set by culturing cells in serum-free medium
for 48 hours to induce growth arrest (30) before introducing
ARHI expression and/or stimulation with IL-6. The results
showed that starvation-induced growth arrest did not affect the
IL-6-stimulated STAT3 translocation and ARHI’s inhibitory
activity (Fig. 5A-A1-A3). A Ras homologue member 1 inhibits signal transducers and activators of transcription 3–dependent transcriptional activity. The colocalization of STAT3 and ARHI and the impaired nuclear translocation of STAT3 might prevent its interaction with specific DNA elements. To examine this possibility, nuclear extracts were prepared from SKBr3 cells that had been infected with adeno-LacZ, adeno-ARHI, or adeno-NTD. Nuclear extracts were prepared 24 hours after infection and analyzed in EMSA with a double-stranded 32P-oligonucleotide from the consensus STAT3-binding site. Oligonucleotide competition and antibody supershift experiments were used to confirm the specificity of our EMSA results (Fig. 6A-4). As expected, infection of SKOV3 and DOV13 cells with adeno-ARHI almost completely abolished STAT3 DNA-binding activity (Fig. 6A-4). In contrast, control cells infected with adeno-LacZ had no inhibitory effects on STAT3 DNA binding. Consistent with the marked decrease in inhibiting the clonogenic growth of cancer cells found in our earlier studies (2), adeno-NTD exhibited less inhibition of STAT3 DNA binding.

To determine whether ARHI ultimately affects STAT3 transcriptional activity, luciferase reporter assays were carried out in SKBr3 cells. A luciferase reporter construct that contained three copies of

Cancer Res 2005; 65: (15). August 1, 2005 6706 www.aacrjournals.org

Downloaded from cancerres.aacrjournals.org on November 10, 2017. © 2005 American Association for Cancer Research.
the STAT3 consensus binding element from the $\alpha_2$-macrogloblin promoter was cotransfected with expression plasmids containing either full-length ARHI cDNA or the NH$_2$-terminal truncation of ARHI. Addition of IL-6 resulted in an 6-fold increase in luciferase activity (Fig. 6B). However, cotransfection of ARHI significantly inhibited luciferase activity with or without IL-6 stimulation. Consistent with earlier results, NTD-ARHI mutant also exerted inhibitory effects on reporter gene activity albeit at much lower levels than the wild-type ARHI. These results suggested that the NH$_2$ terminus of ARHI might play an important role in inhibiting STAT3’s transcriptional activity in the nucleus. Expression levels of ARHI and phosphorylated STAT3 were monitored in all extracts and shown to be equivalent in the different transfectants (data not shown). Thus, ARHI seems to be a negative coregulator of STAT3-mediated transcription.

**Signal transducers and activators of transcription 3 is activated in breast and ovarian cancer cells.** We examined STAT3 tyrosine phosphorylation status in normal breast epithelial cells, breast cancer cells, normal ovarian epithelial cells, and ovarian cancer cells (Fig. 7A and B). Total STAT3 expression varied between cell lines, but STAT3 protein levels were not significantly different between normal and cancer cells. STAT3, however, was constitutively activated in several breast and ovarian cancer cell lines even without IL-6 stimulation. STAT3 activation was increased in both breast (MDA-MB-435 and SKBr3) and ovarian (DOV13 and OVCA429) cancer cells in response to IL-6 stimulation. In contrast, no significant STAT phosphorylation was observed in normal epithelial cells with or without IL-6 stimulation.

**The expression of signal transducers and activators of transcription 3–responsive genes is attenuated in SKOv3 cells expressing a Ras homologue member I.** To further evaluate the effects of ARHI on STAT3-induced genes, we used the inducible ARHI-SKOv3 or ARHI-NTD-SKOv3 ovarian cancer cell line that express modest levels of ARHI after induction by doxycycline. Cells were cultured with or without 1 $\mu$g/mL doxycycline for the indicated times, and total RNA was extracted for RQ RT-PCR analysis. As shown in Fig. 7C, the levels of STAT3-responsive transcripts of c-Myc, Bcl-xL, and c-Fos were markedly increased after IL-6 stimulation ($P < 0.05; P < 0.01$). Induction of ARHI by doxycycline, however, significantly reduced IL-6-stimulated c-Myc, Bcl-xL, and c-Fos mRNA levels ($P < 0.05; P < 0.01$). The basal levels of Bcl-xL and c-Fos transcripts were also reduced by ~40% and
80%, respectively ($P < 0.05$). On the other hand, no significant reduction was observed for all three transcripts after induction of NTD-ARHI mutant, although IL-6 stimulation increased levels of all transcripts ($P < 0.05$ for Bcl-xL and c-Fos).

**Discussion**

STATs are transcription factors that mediate normal biological responses to cytokines and growth factors. In normal cells, the ligand-dependent activation of the STATs is a tightly regulated and transient process. Persistent activation of STATs, including STAT3, has been associated with oncogenesis. STAT3 is constitutively activated in many breast and ovarian cancer cell lines and tumors but not in normal breast and ovarian epithelial cells (14, 19–21, 31). STAT3 has been classified as an oncogene in that constitutively active STAT3 is sufficient to mediate cellular transformation in cultured cells and to induce tumor formation in immunosuppressed mice (16, 32). Activated STATs may contribute to oncogenesis through up-regulation of genes encoding apoptosis inhibitors and cell cycle regulators, such as Bcl-xL, c-Fos, and c-Myc. Further, the status of STAT3 activation serves as an independent prognostic factor in breast cancer (33). With these properties and characteristics, STAT3 has emerged as an excellent target for therapeutic intervention in several types of cancers (34).

STAT activities can be decreased by several negative regulatory mechanisms (reviewed in refs. 11, 35). One factor is the suppressors of cytokine signaling, which is found in the cytoplasm and inhibits the Janus-activated kinase (JAK)-STAT pathway by negative feedback mechanism. The other negative regulatory protein is the protein inhibitor of activated STAT (PIAS) that is found in the nucleus and blocks binding of activated STAT to DNA. Finally, a naturally occurring COOH-terminal truncated STATs, such as STAT3Δβ, function as dominant negatives when overexpressed in cells.

In this study, we report a novel STAT3-interacting protein, ARHI, an imprinted Ras family tumor suppressor gene. ARHI physically interacts with STAT3 and prevents STAT3 from being translocated to and/or accumulated in the nucleus and hence unable to activate its target genes. Consistent with the negative effects of ARHI on STAT3 activity, the expression of ARHI in cells is inversely connected with STAT3 activity. STAT3 constitutively activated in many breast and ovarian cancers but not in normal epithelial cells, whereas ARHI expression is readily detectable in normal breast and ovarian epithelial cells but is frequently lost or markedly down-regulated in breast and ovarian cancer cells (1, 36). Thus, the loss of ARHI expression in ovarian and breast cancer cells provide one mechanism by which contributes to their high constitutive STAT3 activity.

Recent studies have suggested a link between STAT3 activity and small GTPases (22, 25, 26). Constitutively activated Ras and Rho small G proteins have been reported to activate STAT proteins. Expression of dominant-negative Ras, Rac1, and MKK1 can completely abrogate STAT3 function (22). Recently, Rac1 and RhoA have been shown to interact directly with STAT3 and constitutively active Rac1 and RhoA are able to induce STAT3 tyrosine phosphorylation, STAT3 DNA binding, and STAT3-dependent gene transcription (25, 26). In this study, we report, for the first time, that ARHI, a Ras family member, can also interact directly with STAT3. However, in contrast to Rac1 and RhoA, ARHI suppresses STAT3 transcriptional activity.

Yeast two-hybrid analysis indicated that ARHI interacts with STAT3's NH2-terminal 400–amino acid residues that include the NH2-terminal and coiled-coil domains. Interestingly, these two domains have been implicated in protein-protein interaction with other proteins, including PIAS and c-Jun (10, 37, 38). In this study, we provided evidence that the NH2 terminus of STAT3 is involved in the interaction with a regulatory protein ARH. Recently, Ma et al. found that R214/R215 in the coiled-coil domain of STAT3 could also function as a nuclear localization sequence (NLS) for STAT3 (39).
ARHI Inhibits STAT3 in Breast and Ovarian Cancers

Figure 7. STAT3 phosphorylation is increased in breast and ovarian cancer cells and ARHI inhibits expression of STAT3-responsive genes. To compare the phosphorylation status of STAT3 between breast (B1) and ovarian (B2) cancers and in normal epithelial cells, Western blot analysis has been used to detect phosphorylated STAT3 in these cells with or without IL-6 stimulation. β-actin was used as an internal control for protein levels. To examine the effect of ARHI expression on STAT3 target genes, the mRNA levels of STAT3-responsive genes c-Myc, Bcl-xL, and c-Fos were measured by RQ RT-PCR. Inducible ARHI-SKOv3 or ARHI-NTD-SKOv3 cells were treated with or without 1 μg/mL doxycycline for the indicated times to induce ARHI expression. Cells were incubated with or without 10 ng/mL IL-6 for 30 minutes as indicated. Total RNA was extracted and subjected to RT-PCR analysis. All reactions were done using ABI Prism 7000 Sequence Detector. Fold induction was normalized by h-actin. Data include three independent experiments. *, P < 0.05; **, P < 0.01, compared with DOX-treated groups. ▲, P < 0.05; ▲▲, P < 0.01, compared with cells without IL-6 stimulation.

acknowledgments

Received 1/14/2005; revised 5/12/2005; accepted 5/20/2005.

Grant support: NIH grants CA64602 (R.C. Bast) and CA80957 (Y. Yu), NCI grant R24CA124743-05 (R.C. Bast), and M.D. Anderson Cancer Center support grant (R.Z. Luo).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
References


A Ras Homologue Member I Directly Inhibits Signal Transducers and Activators of Transcription 3 Translocation and Activity in Human Breast and Ovarian Cancer Cells

Arata Nishimoto, Yinhua Yu, Zhen Lu, et al.