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


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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, GDP-bound state and an inactive, GDP-bound state, regulating vesicular trafficking, nuclear-cyttoplasmic 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 β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 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 RPMI 1640 supplemented with 10% fetal bovine serum (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. STAT3−/− cells were from Dr. David Levy (New York University School of Medicine, New York, NY) and STAT3 reintegrated L1-HU (the NH2-terminal truncated STAT3, Δ1335STAT3F) and L1-WT (wild-type STAT3) cells were cultured in DMEM supplemented with 10% FBS.

Yeast two-hybrid analysis. Full-length human ARHI cDNA was cloned into plasmid pGBK7 containing the GAL4 DNA-binding domain as bait. Yeast AH109 was transformed with pGBK7-ARHI to screen a human ARHI-SKOv3 or ARHI-NTD-SKOv3 (the NH2-terminal deletion mutant ARHI)–inducible cell line5 was grown in McCoy’s medium supplemented with Trp, His, Leu, and Ade and incubated at 30°C for 14 days. A β-galactosidase colony-lift filter assay was done to eliminate the false-positive colonies. The plasmids were isolated from the positive colonies and transformed into Escherichia coli strain DH5α. Plasmid DNA was purified from E. coli and sequenced.
A biosensor assay was done to confirm putative positive clones according to the manufacturer’s instructions (BD Clontech). The AH109 yeast strain was transfected with test and control constructs and grown in 96-well plates with selective SD medium. In this medium, hybrid yeast clones that contained interacting proteins could grow and generate fluorescent light.

Wild-type and mutant a Ras homologues member 1 plasmids. Full-length ARHI, starting at the first in-frame ATG and extending to the stop codon (assigned as ARHI, nucleotides 3,355–4,144 [1]) and mutant ARHIs with truncation of either the first 34 residues of the NH2 terminus (assigned as NTD mutant, nucleotides 3,458–4,144 [1]) or the last 25 residues (assigned as CTD mutant, nucleotides 3,355–4,066 [1]) were generated by the PCR using a plasmid containing ARHI cDNA, including untranslated regions as template. The resulting 0.7-kb (full-length) or 0.6-kb (NTD or CTD mutants) DNA 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 as template. The following mutant plasmids that modified the effector domain of ARHI were generated: pcDNA-ARHI R193H-H225Q, pcDNA-ARHI R193H-R252H-E268Q, pcDNA-ARHI R63V-H64D, and pcDNA-ARHI R63V-H64D-N72D-T73R-C75R-Q76K (a construct that combined mutations in the effector domain). All constructs were confirmed by DNA sequencing.

Transient transfection 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 pcDNA-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 mM NaCl, 0.1% Tween 20) 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 33 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 three times with lysis buffer, boiled for 5 minutes, and loaded on polyacrylamide gels. Proteins were then electrotransferred 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 second 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

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adenovirus, transfected 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<sup>−/−</sup>), L1-IU (ΔI3STAT3F), and L1-WT (wild-type STAT3) cells were infected with adenovirus before harvested for cell lystate 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 adenovirus-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 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 peroxidase 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 adenovirus-Lacz, adenovirus-ARHI, or adenovirus-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-nitroblue-labeled oligonucleotide that contained the consensus binding site for STAT3. Competition analysis was done using oligonucleotides 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'-TCC-CTCCACCTGGAAGAAGCT-3' and 5'-GCATTTTCGGTTGCTGA-3'. The primers for Bcl-xl were 5'-CCCTGGACCCACCACACT-3' and 5'-GCCGATCCACACAGGATCT-3'. The primers for c-Fos were 5'-GGGGCCCTCCGT-GAGTCTTTGTA-3' and 5'-CACGGTGTTGTACCTCCTTCC-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control and the primers for GAPDH were 5'-GAAAGTGTAGAGGCTGGA-3' and 5'-GAAGATGTTGATGGGATTCT-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. Ps < 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 pGBK7-ARHI and pACT2-STAT3...
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 adenovirus expressing ARHI and stimulated with IL-6 (10 ng/mL) for 30 minutes before harvest. Proteins coimmunoprecipitated with anti-ARHI antibodies were analyzed by Western analysis with anti-STAT3 antibody (A1). 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 adenovirus expressing ARHI or adenovirus expressing LacZ. Cell extracts from infected cells were immunoprecipitated 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 adenolacZ, 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. 2A2).

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 endogenous 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.

![Image](https://www.aacrjournals.org/6705/cancerres.aacrjournals.org)
showed that starvation-induced growth arrest did not affect the ARHI expression and/or stimulation with IL-6. The results for 48 hours to induce growth arrest (30) before introducing the experiment has been set by culturing cells in serum-free medium. To examine the potential role of the effector domain of ARHI in the interaction with STAT3, ARHI plasmids with mutations on the effector domain of ARHI (pcDNA-ARHI^{66V-164D}, pcDNA-ARHI^NTD-T73S-C75R-Q76K, or pcDNA-ARHI^{66V-164D-NTD-T73S-C75R-Q76K} as well as the NH2- or COOH-terminal truncated ARHI mutant plasmids pcDNA-ARHINTD or pcDNA-ARHI^{CTD}) were transfected into SKBr3 cells and their interaction with STAT3 was examined by immunoprecipitation with anti-STAT3 antibody.

**A Ras homologue member I 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 in response to IL-6 stimulation. However, expression of the wild-type ARHI and the NH2-terminal deleted mutant reduced the levels of STAT3 phosphorylation by 30% to 40% (Fig. 4). This reduction in phospho-STAT3 levels is specific to ARHI because infection with the adeno-LacZ control virus had no effect on STAT3 phosphorylation when compared with uninfected control cells.

**A Ras homologue member I 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-A3, 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 STAT3 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 I 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 that had been infected with adeno-LacZ, adeno-ARHI, or adeno-NTD and then treated with or without IL-6 (10 ng/mL) for 30 minutes. Cell lysates were analyzed on Western blots using anti-ARHI, anti-STAT3, or anti-phospho-STAT3 antibodies (A). Average levels of STAT3 phosphorylation in the presence or absence ARHI were quantified by densitometry scanning of three independent experiments (B). * P < 0.05, compared with control.

![Figure 4. Expression of ARHI attenuates STAT3 phosphorylation. SKBr3 breast cancer cells were infected with adeno-LacZ, adeno-ARHI, or adeno-NTD and then treated with or without IL-6 (10 ng/mL) for 30 minutes. Cell lysates were analyzed on Western blots using anti-ARHI, anti-STAT3, or anti-phospho-STAT3 antibodies.](image-url)
the STAT3 consensus binding element from the \( \alpha2 \)-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 a 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-x\(_L\), 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-x\(_L\), and c-Fos mRNA levels \((P < 0.05; P < 0.01)\). The basal levels of Bcl-x\(_L\) and c-Fos transcripts were also reduced by ~40% and

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**Figure 5.** Subcellular colocalization of ARHI and STAT3 prevents nuclear translocation. ARHI-inducible ovarian cancer cell line ARHI-SKOv3 was cultured in chamber slides and ARHI expression was induced with doxycycline (Dox; 1 \( \mu \)g/mL) for 24 hours and immunofluorescently (A and C) or immunohistochemically (B) stained with anti-ARHI or anti-STAT3 antibody. The same cells were cultured in the same chamber slides with serum-free medium for 48 hours as a control to compare their STAT3 function in the “growth-arrested” status (41) with or without ARHI expression (A-A1-A-A3). To avoid overlapped staining from the cytosol content around the nucleus, Z-axis sections (0.2 \( \mu \)m/section) were cut by the confocal microscope and the center section that included the nucleus alone was selected (C). A small amount of ARHI and STAT3 could be detected in the nucleus. The ARHI or STAT3 localization in the subcellular fractions was confirmed by Western blot analysis (D). The cytoplasmic and the nuclear protein extracts were separated using NE-PER nuclear and a cytoplasmic extraction kit (Pierce Biotechnology, Rockford, IL).
transcripts (P < 0.05 for Bcl-xL and c-Fos).

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 STAT3Δ, 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 ARHI. Recently, Ma et al. found that R214/R215 in the coiled-coil domain of STAT3 could also interact with 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.

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ARHI Inhibits STAT3 in Breast and Ovarian Cancers

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 using ABI Prism 7000 Sequence Detector. Fold induction was normalized by GAPDH. Data include three independent experiments. *, P < 0.05; **, P < 0.01, compared with DOX- group. ▲, P < 0.05; ▲▲, P < 0.01, compared with cells without IL-6 stimulation.

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 using ABI Prism 7000 Sequence Detector. Fold induction was normalized by GAPDH. Data include three independent experiments. *, P < 0.05; **, P < 0.01, compared with DOX- group. ▲, P < 0.05; ▲▲, P < 0.01, compared with cells without IL-6 stimulation.

and GRIM-19 that interacted with the coiled-coil domain of STAT3 could block STAT3's nuclear translocation (40). ARHI binding to STAT3 in the nearby region, possibly by occupation of the NLS site of STAT3, may render importin protein incapable of binding to STAT3 and thus interfere with STAT3's nuclear translocation. Neither the NH₂ terminus nor the COOH terminus of ARHI was required for interaction with STAT3, because both truncated ARHI mutants could bind STAT3 with equal efficiency (Fig. 5A). Thus, the region of ARHI that associates with STAT3 must be in the conserved GTPase core structure that is shared among most Ras family members. Rac1, one of the Ras family members that interacts and activates STAT3, has been shown to interact with STAT3 and possibly through its effector domain (26). To examine the potential role of the ARHI effector domain in the interaction with STAT3, we constructed several ARHI mutants with various amino acid substitutions in this domain. Most of these changes are the charged residues that have been shown to be in Ras-Raf interaction (41). These mutations on the effector region of ARHI, however, failed to affect their interaction with STAT3 (Fig. 5A).

ARHI exhibited a modest inhibitory effect on STAT3 phosphorylation following IL-6 stimulation (Fig. 6). This inhibition might result from an indirect effect of ARHI, such as the inhibitory effect on down-regulation of the Ras/MAPK pathway (2, 22), because the tyrosine phosphorylation of STAT3 depends on stimulation through the Ras/MAPK signaling pathway (22). Nevertheless, a modest inhibition on STAT3 phosphorylation is unlikely to explain ARHI’s dramatic inhibitory effect on STAT3 activity. On the other hand, ARHI sequestered most of the activated STAT3 in the cytoplasm and prevented its translocation into the nucleus may contribute more in ARHI’s inhibitory function on STAT3. ARHI also inhibits STAT3 binding to its corresponding DNA elements and transcriptional activity in the nucleus. Thus, ARHI seems to have a dual effect on the inhibition of STAT3 activity in cancer. At the cytoplasmic level, ARHI sequesters STAT3 via direct binding. At the nuclear level, ARHI inhibits STAT3 DNA binding and transcriptional activity through a unique NH₂-terminal extension, which is absent in most of Ras family members. Deletion of this extension markedly decreased ARHI’s inhibitory effect on STAT3 activity. As the NH₂-terminal extension of ARHI is not required for binding to STAT3, the mechanism by which the NH₂-terminal extension of ARHI suppresses transcription still needs to be clarified. Other coregulators of STAT3 may be involved in this process.

Here, we have identified ARHI as a novel negative regulator of STAT3 signaling. Our results suggest that direct protein-protein interactions coordinate cross-talk between a Ras family member ARHI and the JAK/STAT pathways. In contrast to a Ras/Rho-dependent process that activates STAT signaling, ARHI, an imprinted tumor suppressor and a Ras family member, suppresses STAT3 activity in breast and ovarian cancers. Considering that STAT3 is constitutively activated in many breast and ovarian cancers, the loss of ARHI expression in the majority of breast and ovarian cancers may result in up-regulation of STAT3 activity, contributing to oncogenesis.

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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

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