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<th>STAT1 Represses Skp2 Gene Transcription to Promote p27(^{kip1}) Stabilization in Ras-Transformed Cells</th>
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<td><strong>Shuo Wang(^1), Jennifer F. Raven(^1,2), and Antonis E. Koromilas(^1,3)</strong></td>
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**Abstract**

The S-phase kinase-associated protein 2 (Skp2) is an F-box protein that serves as a subunit of the Skp1-Cullin-F-box ubiquitin protein ligase complex. Skp2 is overexpressed in many tumors and promotes tumor formation through its ability to induce the degradation of proteins with antiproliferative and tumor-suppressor functions, such as p27\(^{kip1}\). The signal transducer and activator of transcription 1 (STAT1) is a key regulator of the immune system through its capacity to act downstream of interferons. STAT1 exhibits tumor-suppressor properties by inhibiting oncogenic pathways and promoting tumor immunosurveillance. Previous work established the antitumor function of STAT1 in Ras-transformed cells through the induction of p27\(^{kip1}\) at the transcriptional level. Herein, we unveil a novel pathway used by STAT1 to upregulate p27\(^{kip1}\). Specifically, we show that STAT1 impedes Skp2 gene transcription by binding to Skp2 promoter DNA *in vitro* and *in vivo*. Decreased Skp2 expression by STAT1 is accompanied by the increased stability of p27\(^{kip1}\) in Ras-transformed cells. We further show that impaired expression of STAT1 in human colon cancer cells containing an activated form of K-Ras is associated with the upregulation of Skp2 and downregulation of p27\(^{kip1}\). Our study identifies Skp2 as a new target gene of STAT1 in Ras-transformed cells with profound implications in cell transformation and tumorigenesis. *Mol Cancer Res; 8(5); OF1–8. ©2010 AACR.*

**Introduction**

The S-phase kinase-associated protein 2 (Skp2) is a member of the F-box family of substrate-recognition subunits of the Skp1-Cullin-F-box protein (SCF) complex, which functions as a ubiquitin ligase (1). Skp2 mediates the ubiquitination and the proteasomal degradation of several proteins with antiproliferative and tumor-suppressor properties, including the cyclin-dependent kinase inhibitor p27\(^{kip1}\). Increased Skp2 expression is associated with downregulation of p27\(^{kip1}\) in a wide range of human tumors, and this correlates with poor prognosis (1). Skp2 upregulation in tumors is often seen in conjunction with the activation of oncogenic pathways, such as the Ras–mitogen-activated protein kinase pathway, as shown in mouse cancer models and clinical specimen of human cancers (4, 5). However, the molecular mechanisms that contribute to Skp2 upregulation in tumors are not fully understood, although several possible explanations have been provided. At the genomic level, amplification of a specific region of chromosome 5p13, which is the locus of the Skp2 gene, was observed in various human tumors (6-8). Also, transcriptional factors that are activated in oncogenic pathways, including E2F1, Sp1, or Elk-1, were reported to participate in the transcriptional induction of Skp2 gene (9-11). Furthermore, formation of the SCF complex as well as the ubiquitin ligase activity, localization, and stability of Skp2 were shown to be affected by Akt/PKB–mediated phosphorylation at serine 72 (S72; refs. 12-14).

The signal transducers and activators of transcription (STAT) is a family of seven cytoplasmic proteins with roles as signal messengers and transcription factors that participate in cellular responses to cytokines and growth factors (15, 16). STAT1, the prototypical family member, plays an essential role in innate immunity by protecting the host from infections with viruses and other pathogens. STAT1 functions downstream of interferon (IFN) receptors and mediates the transcriptional induction of genes with antiviral and immune-regulatory properties (15, 16). Phosphorylation of STAT1 at tyrosine (Y) 701 is essential for DNA binding and gene transcription, whereas phosphorylation at serine (S) 727 enhances the transactivation capacity of STAT1 (15, 16). STAT1 exhibits tumor-suppressor functions through its ability to promote the immunosurveillance of tumors in mouse models (17). At the cellular level, STAT1 inhibits the
proliferation of tumor cells in response to IFN-γ through the upregulation of the cyclin-dependent kinase inhibitor p27^Kip1 (18). Also, STAT1 promotes apoptosis by increasing the expression of caspase-2, caspase-3, and caspase-7; the expression of inducible nitric oxide synthase; or by decreasing the expression of antiapoptotic genes (i.e., Bcl-xL, Bcl-2; ref. 18). The antitumor activity of STAT1 is further supported by its ability to inhibit angiogenesis and tumor metastasis (18). Although the majority of data favor an antitumor function of STAT1, some studies provide compelling evidence for a tumor-promoting activity of STAT1 in mouse leukemogenesis (19). The ability of STAT1 to regulate tumor formation is likely to be affected by its site-specific phosphorylation. Specifically, STAT1 Y701 phosphorylation has been documented in many blood tumors, including multiple myeloma, erythroleukemia, and acute myelogenous leukemia as well as in breast and head and neck cancers (20, 21). Also, STAT1 Y701 phosphorylation was shown to be an indicator of better survival of breast cancer patients independent of other known prognostic factors (22). Furthermore, STAT1 phosphorylation at S727 is enhanced in Wilms’ tumor, which may represent a pro-survival event (23). Despite the numerous studies that implicate STAT1 phosphorylation in cancer, the precise role of phosphorylated STAT1 in tumor development remains elusive.

Recent work by our group showed an important role of STAT1 in Ras-mediated transformation (24, 25). We previously showed that STAT1 exhibits a strong antitumor function in Ras-transformed cells and tissues by upregulating p27^Kip1 (24). Although STAT1 induces p27^Kip1 at the transcriptional level (24), herein we provide strong evidence for a novel mechanism of p27^Kip1 induction by STAT1 in Ras-transformed cells. That is, we show that STAT1 suppresses Skp2 expression and stabilizes p27^Kip1 in Ras-transformed cells. For example, reconstitution of Ha-RasG12V–transformed mouse embryonic fibroblasts (MEF) deficient in endogenous STAT1 (STAT−/−) with wild-type (WT) STAT1 resulted in the upregulation of p27^Kip1 as previously described (24) and in downregulation

**Materials and Methods**

**Animals, cell culture, and luciferase reporter assays.** Urethane treatment of STAT−/− and STAT+/+ mice and immunohistochemistry analysis of lung tumors were done as previously described (24). The generation and characterization of Ras-transformed MEFs lacking STAT1 or reconstituted with STAT1 proteins were previously described (24). The maintenance of the human colon carcinoma HCT116 and HK2-8 cells was previously reported (26). The pGL2 vectors containing the luciferase reporter gene under the control of full-length or truncated mouse Skp2 promoter were previously described (27). Luciferase reporter assays were done with the Dual-Luciferase Reporter Assay System (Promega) using Renilla luciferase as an internal control as previously described (24).

**Protein extraction and immunoblotting.** Protein extraction and immunoblotting were done as described (24). For immunoblot analysis, the following antibodies were used: anti–STAT1α p91(C-111) antibody was purchased from Santa Cruz Biotechnology; anti-Skp2 antibody was from Zymed Laboratories; anti-KPC1/2 antibodies were as described (28); anti-p27^Kip1 antibody was from BD Transduction Laboratories; phospho-ERK1/2 (Thr202/Tyr204) antibody, anti-cyclin D1 monoclonal antibody (BD Biosciences), and pan-specific ERK1/2 antibody were from Cell Signalling; and antiactin antibody (C4) was from Biosource International. All antibodies were used at a final concentration of 0.1–1 μg/mL. After incubation with anti-mouse IgG or anti-rabbit IgG antibodies conjugated to horseradish peroxidase, proteins were visualized with enhanced chemiluminescence reagent (Thermo Scientific) detection system according to the manufacturer’s instructions. Quantification of protein bands was done by densitometry using Scion Image from NIH.

**Northern blotting and electrophoretic mobility shift assays.** RNA isolation and Northern blot analysis were done as described (24). Electrophoretic mobility shift assays (EMSA) were done based on a previous protocol (24) using an oligonucleotide encompassing the STAT binding site at position −196(S2) of the mouse Skp2 promoter. The sequences of the oligonucleotides were as follows: forward primer, 5′-GATCTGACATTTCCAGCCGG-3′; reverse primer, 5′-GATCCCGGCTGGCTGGGGAATGTCAGTTGC-3′. The sequences of the S2 mutant oligonucleotides were as follows: forward primer 5′-GATCTGACATAAGCCAGCCAGCAGG-3′ and reverse primer 5′-GATCCCGGCTGGCTGGCTATGTCAGTTGAAGAGGAG-3′.

**Chromatin immunoprecipitation.** Chromatin immunoprecipitation (ChIP) assays were carried out based on a protocol described elsewhere (24). For PCR, the following primers were used: forward primer 5′-GGACCTCGGCA-GACGG-3′ and reverse primer 5′-CGGGCTGGTTATGTCAGTTGAAGAGGAG-3′. The amplification conditions were: 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, 59°C for 1 minute, 72°C for 30 seconds, and a final elongation of 72°C for 10 minutes.

**Results and Discussion**

**STAT1 decreases Skp2 expression and stabilizes p27^Kip1 in Ras-transformed cells.** We previously showed an important role of STAT1 in the induction of p27^Kip1 in Ras-transformed cells (24). Specifically, we found that p27^Kip1 upregulation occurred at the transcriptional level, and it was both necessary and sufficient to mediate the antitumor properties of STAT1 in Ras-transformed cells (24). However, we have obtained evidence for a different mechanism of p27^Kip1 induction by STAT1 in Ras-transformed cells that implicates Skp2. For example, reconstitution of Ha-RasG12V–transformed mouse embryonic fibroblasts (MEF) deficient in endogenous STAT1 (STAT−/−) with wild-type (WT) STAT1 resulted in the upregulation of p27^Kip1 as previously described (24) and in downregulation
of Skp2 (Fig. 1A). Unlike Skp2, expression of KPC2, a subunit of the Kip1 ubiquitination-promoting complex (KPC) that is also involved in p27Kip1 degradation (29), was not affected by STAT1 (Fig. 1A). Downregulation of Skp2 by STAT1 most likely is a property of Ras-transformed MEFs because STAT1 had no effect on either Skp2 or p27Kip1 level in reconstituted immortalized STAT1−/− MEFs (Fig. 1B). We previously showed that STAT1 increases p27Kip1 levels in mouse lung tumors caused by an activating K-Ras mutation due to urethane treatment (24). As such, we examined whether upregulation of p27Kip1 by STAT1 in the lung tumors was associated with downregulation of Skp2. To this end, lung tumors from urethane-treated STAT1+/+ and STAT1−/− mice were subjected to immunohistochemistry. Skp2 staining of the areas in rectangles is shown in higher magnification (bottom).

D, HCT116 and HK2-8 were serum starved for 18 hours followed by stimulation with 10% calf serum for the indicated time points. Protein extracts (50 μg) were subjected to immunoblotting for STAT1 (a), Skp2 (b), anti-p27Kip1 (c), KPC1 (d), KPC2 (e), cyclin D1 (f), phospho-Thr202/Tyr204 ERK 1/2 (g), ERK1/2 (h), or actin (i).

To address the function of STAT1 in Skp2 expression in human cells, we used the human colon carcinoma HCT116 cells, which contain an activated form of K-Ras, and the HK2-8 cells, which are isogenic derivatives of HCT116 cells engineered to express a normal allele of K-Ras (26). We observed that mitogenic stimulation of the cells with serum resulted in the upregulation of Skp2 in HCT116 cells but not in HK2-8 cells (Fig. 1D, b). Interestingly, upregulation of Skp2 in HCT116 cells was accompanied by a downregulation of p27Kip1. Contrary to this finding with HCT116 cells, p27Kip1 levels remained stable during the serum stimulation of HK2-8 cells, which contained lower levels of Skp2 than HCT116 cells (Fig. 1D, c). Unlike Skp2, expression of the KPC1 and KPC2 subunits of the KPC ubiquitin ligase complex did not significantly differ between the two cell types, suggesting a specific effect of the K-Ras.
mutation on Skp2 (Fig. 1D, d,e). Serum stimulation resulted in the upregulation of cyclin D1 levels in both HCT116 and HK2-8 cells, indicating cell progression (Fig. 1D, f). The presence of an activated form of K-Ras in HCT116 cells was confirmed by immunoblot analysis for phosphorylated ERK1/2, which was more highly induced in HCT116 cells than in HK2-8 cells (Fig. 1D, g, h). Consistent with this interpretation, cyclin D1 levels were elevated in HCT116 cells compared with HK2-8 cells (Fig. 1D, f). We also observed that activation of K-Ras was associated with a downregulation of STAT1 in HCT116 cells compared with HK2-8 cells (Fig. 1D, a). This finding is in line with previous data showing a negative effect of activated K-Ras on STAT1 expression in human colon cancer cells (30).

The above data implied that downregulation of Skp2 by STAT1 results in the stabilization of p27Kip1. To verify this matter, we assessed the stability of p27Kip1 in Ras-transformed MEFs lacking endogenous STAT1 or reconstituted with either STAT1WT or STAT1Y701F. Protein stability of p27Kip1 was assessed by immunoblotting after cell treatment with the protein synthesis inhibitor cycloheximide. We found that STAT1WT increased the half-life of p27Kip1 in Ras-transformed cells from 3 to 6 hours compared with control cells lacking STAT1 or cells reconstituted with STAT1Y701F (Fig. 2). These data showed the ability of STAT1 to promote the stabilization of p27Kip1 in Ras-transformed cells.

**STAT1 diminishes Skp2 gene transcription in Ras-transformed cells.** Next, we sought to explain the mechanism of Skp2 inhibition by STAT1. To this end, we performed Northern blot analysis to assess the levels of Skp2 mRNA in Ras-transformed cells. Because the effect of STAT1 on Cdkn1b gene transcription in Ras-transformed MEFs was proportional to cell density (24), we measured the levels of Skp2 mRNA in cells maintained at different levels of confluence. We found that Stat1WT decreased Skp2 mRNA levels by 50% in Ras-transformed cells compared with cells lacking STAT1 or reconstituted with Stat1Y701F under conditions of low or high cell density (Fig. 3A). Contrary to Ras-transformed cells, Skp2 mRNA was assessed by immunoblotting after cell treatment with the protein synthesis inhibitor cycloheximide. We found that STAT1WT increased the half-life of p27Kip1 in Ras-transformed cells from 3 to 6 hours compared with control cells lacking STAT1 or cells reconstituted with STAT1Y701F (Fig. 2). These data showed the ability of STAT1 to promote the stabilization of p27Kip1 in Ras-transformed cells.

**FIGURE 2.** STAT1 increases p27Kip1 stability in Ras-transformed cells. A, Ha-Ras G12V–transformed MEFs lacking STAT1 (Control) or reconstituted with either Stat1WT or Stat1Y701F were treated with cycloheximide (CHX; 20 μg/mL) for the indicated time points. Protein extracts (50 μg) were subjected to immunoblotting for p27Kip1 (top panels) or actin (bottom panels). B, the graphs indicate the quantification of the half-life of p27Kip1 after normalization to actin. Error bars show the SD from two separate experiments.
was equally expressed in immortalized STAT1−/− MEFs in the absence or presence of either Stat1WT or STAT1Y701F (ref. 24; Fig. 3B). This result suggested that the ability of STAT1 to inhibit Skp2 mRNA expression is a property of Ras-transformed cells.

To substantiate the transcriptional role of STAT1, we assessed the activity of the mouse Skp2 gene promoter in Ras-transformed cells (27). First, we noticed that the mouse Skp2 promoter contains four potential STAT binding sites at positions −226 (S1), −196 (S2), −74 (S3), and −25 (S4) as identified by TRANSFAC database analysis (Fig. 3C). Next, we performed luciferase reporter assays using a pGL2 vector containing either the full-length sequence of the mouse Skp2 promoter (−2275 to +94 bp) or a truncated form of it (−318 to +94 bp), which maintains the four putative STAT binding sites.

**FIGURE 3.** STAT1 inhibits Skp2 gene transcription in Ras-transformed cells. Northern blot analysis of Ras-transformed MEFs (A) or immortalized MEFs (B) lacking STAT1 (lane 1) or reconstituted with either STAT1 WT (lane 2) or STAT1Y701F (lane 3). Ras-transformed MEFs were maintained at different levels of density (50-90%), whereas immortalized MEFs were maintained at 90% confluence before RNA extraction. Total RNA (15 μg) was subjected to Northern blot analysis using [α-32P]dCTP-labeled probe representing the entire mouse Skp2 cDNA. For normalization, [α-32P] dCTP-labeled mouse β-actin cDNA was used as probe. The radioactive bands were detected by autoradiography, and normalization was done at the linear range of exposure using the NIH image software. The ratio of Skp2 to actin mRNA for each lane is indicated.

As a control, a piece of Skp2 gene (+6) lacking promoter activity was used. Results are expressed as ±SD from three experiments done in triplicate.

*P < 0.01.
active in Ras-transformed STAT\(^{−/−}\) MEFs (Fig. 3C, Control). Specifically, we noticed that the truncated \(Skp2\) promoter was more active than the full-length promoter, most likely due to the presence of both positive and negative transcription regulatory elements in the full-length promoter as previously described (27). We also noticed that the presence of STAT1WT resulted in a significant inhibition of the activity of both forms of the \(Skp2\) promoter, clearly indicating that STAT1WT is an inhibitor of \(Skp2\) gene transcription (Fig. 3C). It is of interest, however, that \(Skp2\) promoter activity was slightly lower in cells expressing STAT1Y701F than in Ras-transformed cells lacking STAT1 (Fig. 3C). Nevertheless, this modest inhibition of \(Skp2\) promoter activity by STAT1Y701F in the transactivation reporter assays may be of little biological relevance given that \(Skp2\) mRNA expression is not inhibited by STAT1Y701F as indicated by Northern blot analysis (Fig. 3A). Although STAT1Y701F is defective in DNA binding, the possibility remains that it may affect promoter activity in the transient transactivation assays through interactions with other transcription factors implicated in \(Skp2\) promoter activity. Consistent with this interpretation, nonphosphorylated STAT1 has been found to control gene transcription in various systems through interactions with other transcription factors (31).

**STAT1 interacts with \(Skp2\) promoter DNA.** To further substantiate the transcriptional role of STAT1, we examined the binding of STAT1 to \(Skp2\) promoter sequence. First, we tested the ability of STAT1 to bind one or more of the four STAT binding sites within the \(Skp2\) promoter sequence (Fig. 3C). EMSAs identified the binding of STAT1 to the mouse \(Skp2\) promoter DNA (S2) within the mouse \(Skp2\) promoter, the presence of \(Skp2\) promoter DNA was detected by semiquantitative PCR using a set of primers that amplified a 232-bp fragment of DNA containing the STAT binding at position −196. Input DNA refers to PCR amplification of the fragments using genomic DNA as template. Bottom, quantification of STAT1 binding activity from three independent experiments is shown with ±SD. **, \(P < 0.01\).
STAT1WT to the STAT binding site located at position -196 within the mouse Skp2 promoter (Fig. 4A). The specificity of binding was tested by the inclusion of a mutant STAT binding site as well as by cold competition assays with wild-type STAT binding site (Fig. 4A). Binding of STAT1 to the other three putative STAT binding sites was not possible in EMSAs (data not shown). Next, we examined the ability of STAT1 to interact with the Skp2 promoter DNA in vivo by ChIP (Fig. 4B). We found that STAT1WT but not STAT1Y701F binds to the Skp2 promoter sequence encompassing the STAT binding site at position -196 (Fig. 4B). Collectively, these data supported a direct role of STAT1 in the inhibition of Skp2 promoter activity.

By promoting the degradation of p27\(^{kip1}\), Skp2 is considered a significant player in cell transformation and tumor progression by oncogenic Ras (4, 5, 32). Our findings support the notion that downregulation of Skp2 contributes to the antitumor activity of STAT1 in Ras-transformed cells. We previously identified STAT1 as an important suppressor of Ras transformation and tumorigenesis through its ability to induce the expression of p27\(^{kip1}\) (24). STAT1 does so by involving two different pathways that converge on p27\(^{kip1}\). One involves the induction of p27\(^{kip1}\) at the transcriptional level (24), whereas the other uses a posttranslational arm that leads to p27\(^{kip1}\) stabilization through the downregulation of Skp2. The significance of our findings is further underscored by the ability of STAT1 to impede Skp2 expression in Ras-transformed MEFs but not in immortalized MEFs. This indicates that the transcriptional function of STAT1 is profoundly affected by the oncogenic environment, which can determine the tumor-suppressor function of STAT1. The antitumor properties of STAT1 have largely been explained by its ability to promote tumor immunosurveillance as a component of IFN signaling (17). However, in Ras-transformed cells, STAT1 exhibits a tumor site-specific suppressor function independent of its role in IFN signaling (24). We have not detected any downregulation of Skp2 by STAT1 in response to IFN treatment in various cells (data not shown), indicating that regulation of Skp2 may be relevant to STAT1 function as an inhibitor of oncogenic Ras signaling rather than as a universal inducer of IFN signaling.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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