Transforming Growth Factor β1 (TGF-β1) Suppresses Growth of B-cell Lymphoma Cells by p14<sup>ARF</sup>-dependent Regulation of Mutant p53*<sup>†</sup>

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**Background:** TGF-β1 suppresses growth of B-cell lymphoma cells.

**Results:** TGF-β1-induced down-regulation of mutant p53 via p14<sup>ARF</sup> renders B-cell lymphoma cells sensitive to TGF-β1.

**Conclusion:** Overexpression of p14<sup>ARF</sup> possibly causes TGF-β1 resistance.

**Significance:** p14<sup>ARF</sup> is a potential therapeutic target for B-cell lymphoma.

Previously we reported that TGF-β1-induced growth suppression was associated with a decrease in mutant p53 levels in B-cell lymphoma cells. The goal of the present study was to understand the mechanism involved in TGF-β1-mediated down-regulation of mutant p53. In RL and CA46, two B-cell lymphoma cell lines, TGF-β1 treatment caused down-regulation of E2F-1 transcription factor resulting in the down-regulation of both p14<sup>ARF</sup> and mutant p53, leading to growth arrest. Experimental overexpression of E2F-1 increased p14<sup>ARF</sup> level and blocked TGF-β1-induced down-regulation of p14<sup>ARF</sup>. Overexpression of p14<sup>ARF</sup> blocked the down-regulation of mutant p53 and prevented growth arrest. p14<sup>ARF</sup> also attenuated TGF-β1-induced p21<sup>Cip1/WAF1</sup> induction, which was reversible by p53 siRNA, indicating the involvement of mutant p53 in controlling the TGF-β1-induced expression of p21<sup>Cip1/WAF1</sup>. The interaction observed between phospho-Smad2 and mutant p53 in the nucleus could be the mechanism responsible for blocking the growth-suppressive effects of TGF-β1. In RL cells, p14<sup>ARF</sup> is present in a trimer consisting of mutant p53-Mdm2-p14<sup>ARF</sup> and in a dimer consisting of Mdm2-p14<sup>ARF</sup>. Because it is known that Mdm2 can degrade p53, it is possible that, in its trimeric form, p14<sup>ARF</sup> is able to stabilize mutant p53 by inhibiting Mdm2. In its dimeric form, p14<sup>ARF</sup> may be sequestering Mdm2, limiting its ability to degrade p53. Collectively, these data demonstrate a unique mechanism in which the inhibition of TGF-β1-mediated growth suppression by mutant p53 can be reversed by the down-regulation of its stabilizing protein p14<sup>ARF</sup>. This work suggests that the high levels of p14<sup>ARF</sup> often found in tumor cells could be a potential therapeutic target.

Members of the TGF-β1 family have pleiotropic functions, including proliferation, differentiation, migration, and apoptosis, in a broad range of cell lineages (1, 2). TGF-β1 signals through a heteromeric receptor complex on the cell surface and downstream intracellular signal-transducing Smad complexes activated by phosphorylation. Activated Smad complexes translocate into the nucleus and, in conjunction with other nuclear cofactors, regulate the transcription of target genes (3). The signaling of TGF-β1 is finely regulated by negative feedback, including inhibitory Smads (4, 5) and PPM1A phosphatase (6).

p53 is a transcription factor that mediates several cellular processes, including regulation of the cell cycle, apoptosis, DNA damage repair, and angiogenesis (7). Approximately 50% of human cancers have inactivating mutations of p53, and most of the remaining malignancies deactivate the p53 pathway either by blunting its activity, reducing its activators, or inactivating its downstream targets (8). It has been shown that mutant p53-expressing tumors are aggressive and associated with poor prognosis (9). Various p53 mutants confer different gain-of-function phenotypes such as increased cell growth (10, 11), enhanced tumorigenicity (11–15) and invasiveness (11, 13, 16), disturbed spindle checkpoint (17, 18), and resistance to cytotoxic agents (19).

p53 can have different effects on TGF-β1-induced growth suppression depending on its wild type or mutant status. In mink Mv1Lu epithelial cells, TGF-β1-mediated G1 growth arrest is heavily dependent on wild type p53 inhibiting the translation of CDK4 (20). It is also reported that p53 is required for TGF-β1-mediated growth arrest in certain mammalian cell types (21). It appears that p53 functions in this regard by cooperating with Smads to up-regulate the expression of the CDK inhibitor p21<sup>Cip1/WAF1</sup>. However, it was shown that the expression of mutant p53 caused cells to become resistant to TGF-β1-mediated growth suppression (12, 22). Recently, it was shown that a mutant p53-Smad complex contributes to TGF-β1-induced cell migration, invasion, and metastasis (23), confirming that p53 and Smad physically interact.

**Significance:** p14<sup>ARF</sup> is a potential therapeutic target for B-cell lymphoma.

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2The abbreviations used are: CDK, cyclin-dependent kinase; TβRII, TGF-β1 receptor II; PMA, phorbol 12-myristate 13-acetate; hCAR, human coxsackie adenovirus receptor; m.o.i., multiplicity of infection; EGFP, enhanced GFP.
In primary tissues, p53 is expressed at very low levels because of its rapid degradation. For p53 to effectively function in growth arrest, its level must be stabilized. The primary mechanism involved in stabilizing p53 is through inhibition of the interaction between p53 and Mdm2, which targets p53 for degradation (24–26). Among several mechanisms involved in disrupting the Mdm2-p53 interaction, up-regulation of the tumor-suppressor protein p14ARF is an important one (27). Activation of p14ARF disrupts the physical interaction between p53 and Mdm2 resulting in the rapid degradation of Mdm2 and consequently more stable expression of p53. p14ARF tumor suppressor is the product of the alternative reading frame of the Ink4a locus, which also codes for p16^INK4a^, an inhibitor for cyclin D-dependent kinases (28–30). In primary tissue, p14ARF (p19ARF in the mouse) is expressed at low levels. However, it can be induced by oncogenes such as Ras (31), Myc (32), and v-Abl (33) to cause p53-dependent growth arrest or apoptosis. In addition, p14ARF is able to inhibit cell growth through p53-independent pathways. For example, it has been shown that p14ARF is able to inhibit DNA synthesis in p53-null cells (34, 35). NFκB activity has been shown to be inhibited by p14ARF through interacting with RelA and repressing its transcriptional activity (36). p14ARF is also involved in inhibiting the function of proproliferative factor B23 through direct interaction with B23 and promoting its polyubiquitinylation and proteosomal degradation (37). It has been suggested that p53-independent functions of p14ARF may include its ability to promote sumoylation of several p14ARF-interacting proteins (38).

We have previously reported the effect of TGF-β1 on a human B-lymphoma cell line, RL, which expresses a mutant form of p53 having a single point mutation, A138P. We found that TGF-β1 causes growth inhibition in these cells that occurs simultaneously with a decrease in the level of mutant p53 (39). In this study, we examine the role and mechanism of the down-regulation of mutant p53 level caused by TGF-β1 treatment. We provide evidence suggesting that the decrease in mutant p53 level upon exposure to TGF-β1 mediates the growth-suppressive effect of this cytokine in B-cell lymphoma cell lines RL and CA46. The decrease in mutant p53 level is likely to be the result of a reduction in p14ARF levels because overexpression of p14ARF blocked TGF-β1-induced down-regulation of mutant p53 and subsequent growth arrest. Moreover, siRNA-mediated knockdown of p14ARF resulted in the down-regulation of mutant p53 and rendered cells more sensitive to TGF-β1-mediated growth suppression. Collectively these data demonstrate a unique mechanism in which the inhibition of TGF-β1-mediated growth suppression by mutant p53 is relieved by a TGF-β1-mediated signaling pathway that results in the down-regulation of the p53-stabilizing protein p14ARF. They also suggest that p14ARF antagonists may have an inhibitory effect on lymphoma proliferation.

**EXPERIMENTAL PROCEDURES**

**Reagents**—For Western blot analysis and immunoprecipitation, anti-TGF-β1 receptor II (TBRII) (sc-400), Smad2 (sc-6200), p14ARF (sc-8613), and p53 (sc-126) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); rabbit polyclonal phosho-Smad2 and anti-E2F-1 antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA); mouse monoclonal anti-p27^kip1^ (WAF1) antibody was from Upstate (Charlottesville, VA); anti-β-actin was purchased from Abcam (Cambridge, UK); and anti-Nucleoprin p62 was from BD Biosciences. All HRP-conjugated secondary antibodies were purchased from GE Healthcare. Recombinant TGF-β1 (240-B) was purchased from R&D Systems (Minneapolis, MN). Phorbol 12-myristate 13-acetate (PMA) was from Sigma. Anti-lgM was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Cell Culture**—Lymphoma cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 1,000 units of penicillin/ml, and 100 μg of streptomycin/ml. No exogenous growth factors were added. Cells were grown at 37 °C in 5% CO₂. Fresh growth medium was added to cells every 3–4 days. For growth inhibition, cells were stimulated with 2 ng/ml recombinant TGF-β1 in RPMI 1640 medium with 5% FBS.

**Cell Lysis and Western Blot Analysis**—Cytoplasmic and nuclear extracts were prepared according to the procedure reported earlier (40). Whole cell lysates were prepared according to the following procedure. Harvested cells were resuspended in lysis buffer (10 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1 mM MgCl₂, 5 μg/ml E-64, 1 mM PMSF, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 mM NaF, and 1% Triton X-100) and incubated on ice for 30 min. Samples were then homogenized and centrifuged, and the supernatants were collected. Protein concentrations were measured with Bio-Rad Protein Assay Dye reagent. Gel electrophoresis was carried out using 4–12% SDS-PAGE under reducing conditions. After membrane transfer, bound antibodies were detected using a chemiluminescence detection system (GE Healthcare).

**WST-1 Assay**—The quantification of cell proliferation was evaluated by WST-1 assay (Roche Applied Science), a colorimetric assay based on the cleavage of tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. 1 × 10⁴ cells in 100 μl of culture medium were loaded in each well of a 96-well plate and incubated with 2 ng/ml TGF-β1 for 48 h at 37 °C in 5% CO₂. 10 μl of WST-1 reagent was added to each well, and cells were incubated for 1 h at 37 °C. The absorbance was measured with a microplate reader (Bio-Rad Model 680XR) with a 450-nm wavelength filter and a 655-nm reference wavelength filter.

**Immunoprecipitation**—Lysates were pre-cleared with protein A/G Plus-agarose (sc-2003, Santa Cruz Biotechnology Inc.) for 30 min at 4 °C, and the pre-cleared lysates were incubated overnight with the primary antibody and A/G Plus-agarose beads. The agarose beads were washed three times with extraction buffer containing 25 mM MOPS (pH 7.2), 15 mM MgCl₂, 137 mM NaCl, 1 mM PMSF, 15 mM EGTA, 5 μg/ml E-64, 1 mM Na₃VO₄, 1 mM NaF, and 0.1% Triton X-100. The immune complexes were dissociated with lithium dodecyl sulfate buffer and boiled for 5 min. Electrophoresis was carried out by 4–12% SDS-PAGE under reducing conditions.

**Northern Blotting**—Total RNA was extracted from RL cells with an RNeasy Mini kit (Qiagen, Valencia, CA) and purified...
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according to the manufacturer’s specifications. Up to 25 μg of total RNA from each sample was electrophoresed under denaturing conditions on a 1% agarose gel in glyoxal (Ambion, Austin, TX), blotted onto Hybond-N+ membrane (Amersham Biosciences), and cross-linked by UV irradiation. The membrane was prehybridized overnight at 42 °C in Hybrisol I (Chemicon, Millipore) followed by overnight hybridization with 1 × 10^6 cpm/ml specific cDNA probe labeled with ^32P by random priming of a 450-bp BamHI/EcoRV fragment from the p14ARF/pcDNA3 using High Primer DNA labeling reagent (Roche Diagnostics). Probes were purified by a G-50 column (Amersham Biosciences) before use. The membranes were then washed and exposed to x-ray film at −70 °C.

Statistical Analysis—Values were obtained from three independent experiments and were expressed as means ± S.D. Statistical analysis was performed using Student’s t test and analyzed by two-tailed test of paired samples. Values were considered significant (∗) if p values were <0.05.

DNA Constructs and Transfection—pSuper/p14ARFs was obtained from Dr. Bert Vogelstein, The Johns Hopkins University, Baltimore, MD; and pCMV-Bam-Neo/p53 was obtained from Dr. Didier Trono, Global Health Institute, Switzerland) was carried out with minor modification (44). After cross-linking with formaldehyde, soluble chromatin from the samples was obtained by sonication (Bronson sonicator 350). The chromatin was incubated overnight with 2 μg of either normal IgG or anti-Smad2/3 or anti-E2F-1 antibody. After washing and elution, the immunocomplexed DNA was isolated with Chelex beads (Bio-Rad) and analyzed by PCR. Primers used to amplify DNA fragments corresponding to a Smad binding region (SBR1) on human p14ARF promoter were 5'-GAGGAAAAAGCATCTTGGAG-3' (forward) and 5'-AATAGACGGGAGCAACG-3' (reverse) (45). Primers for p14ARF promoters were 5'-GCTGAGGTTGGAAAGATGG-3' (forward) and 5'-AGACTGGGACCCAGGCACC-3' (reverse).

RESULTS

Down-regulation of p14ARF and Mutant p53 in B-cell Lymphoma Cells in Response to TGF-β1—We have shown previously that RL cells are unresponsive to TGF-β1-mediated growth suppression, whereas in the presence of a low dose of PMA, RL cells can be rendered responsive to TGF-β1 (43) (Fig. 1A). We have also reported that treatment of RL cells with PMA/TGF-β1 results in the down-regulation of mutant p53 (Ala-138 to Pro), the only form of p53 present in RL cells (39, 46) (Fig. 1B). Because p14ARF is involved in the stability of p53 protein, we wanted to determine the status of p14ARF in PMA/TGF-β1-treated cells. As shown in Fig. 1B, down-regulation of the p14ARF level was observed only with PMA/TGF-β1 treatment, conditions that also decrease mutant p53 levels, but PMA or TGF-β1 treatment alone had the effect on p14ARF or mutant p53 levels. TGF-β1-induced down-regulation of p14ARF and mutant p53 was also observed in the presence of physiologically relevant stimulation delivered by anti-IgM treatment (Fig. 1C). To further elucidate the relative kinetics of the down-regulation of both p14ARF and mutant p53, RL cells were treated with either PMA alone, TGF-β1 alone, or with PMA/TGF-β1 for various periods of time, and the levels of p14ARF, mutant p53, and Mdm2 were measured by Western blot analysis. As shown in Fig. 1D, a reduction in p14ARF and mutant p53 levels occurred at 16 h (lane 7 versus lane 5 or lane 6), although the degree of down-regulation was greater in the

p21Cip1/WAF1 Promoter-Reporter Assay—RL/hCAR-EGFP cells were infected with TβRII/PAd and p14ARF/PAd for 24 h. Then the cells were transfected with p53 expression construct (a gift from Dr. Bert Vogelstein, The Johns Hopkins University), p21Cip1/WAF1 promoter-luciferase construct pWWP-Luc (p21Cip1/WAF1) (a gift from Dr. Weiguo Zhu, Peking University Health Science Center, China), and β-galactosidase expression construct by electroporation as described previously (43). For mutant p53 knockdown experiments, RL cells were infected with pWPXL-p53si for 24 h before adenovirus infection. Transfected cells were further cultured for 24 h before activation with TGF-β1 for 16 h. β-Galactosidase and luciferase activity was measured with the Beta-Glo and Bright-Glo (Promega, Madison, WI) assays according to the instructions of the manufacturer. p21Cip1/WAF1 promoter-luciferase activity was normalized with the β-galactosidase assay.

Chromatin Immunoprecipitation (ChIP) Assay—This assay was carried out according to the protocol described previously with minor modification (44). After cross-linking with formaldehyde, soluble chromatin from the samples was obtained by sonication (Bronson sonicator 350). The chromatin was incubated overnight with 2 μg of either normal IgG or anti-Smad2/3 or anti-E2F-1 antibody. After washing and elution, the immunocomplexed DNA was isolated with Chelex beads (Bio-Rad) and analyzed by PCR. Primers used to amplify DNA fragments corresponding to a Smad binding region (SBR1) on human p14ARF promoter were 5'-GAGGAAAAAGCATCTTGGAG-3' (forward) and 5'-AATAGACGGGAGCAACG-3' (reverse) (45). Primers for p14ARF promoters were 5'-GCTGAGGTTGGAAAGATGG-3' (forward) and 5'-AGACTGGGACCCAGGCACC-3' (reverse).
case of p14ARF compared with mutant p53. The level of Mdm2 was unaffected at the 16-h time point. We next wanted to assess whether the down-regulation of p14ARF was linked to the down-regulation of mutant p53.

**Effect of p14ARF on TGF-β1-induced Down-regulation of Mutant p53**

We have observed that the resistance of RL cells to TGF-β1-induced growth arrest was due to the ligand-induced down-regulation of TβRII, which can be prevented by low dose PMA treatment (47). In accordance with this finding, transfection of TβRII DNA leading to forced receptor expression rendered RL cells responsive to TGF-β1-induced growth suppression in the absence of PMA (Fig. 2A, left panel). However, transfection of p14ARF into TβRII-overexpressing cells rendered these cells resistant to TGF-β1-induced growth suppression (Fig. 2A, right panel). To investigate the relationship between the status of p14ARF and mutant p53, levels of p14ARF and mutant p53 in cell lysates from TβRII-overexpressing cells were analyzed by Western blot analysis in the presence or absence of experimentally overexpressed p14ARF. As shown in Fig. 2B, TGF-β1-induced down-regulation of p14ARF coincided with the down-regulation of mutant p53 (lane 2 versus lane 1), and this down-regulation was blocked by overexpression of p14ARF (lane 2 versus lane 4). Interestingly, down-regulation of mutant p53 coincided with the up-regulation of p21Cip1/WAF1 (lane 1 versus lane 2), and the induction of p21Cip1/WAF1 was blocked in the presence of p14ARF (lane 2 versus lane 4). The inhibition of TGF-β1-induced up-regulation of p21Cip1/WAF1 by overexpressed p14ARF was not due to the blockage in TGF-β1 signaling because TGF-β1-induced phosphorylation of Smad2 was unaffected by the p14ARF overexpression (lane 2 versus lane 4). These data suggest that p14ARF is involved in TGF-β1-induced down-regulation of mutant p53 and subsequent growth suppression of RL cells.

Next we wanted to investigate the relationship between p14ARF and mutant p53 by knocking down p14ARF with an siRNA construct and test whether down-regulation of p53 by p14ARF was able to cause growth suppression in the absence of TGF-β1 treatment. As shown in Fig. 2C (upper left panel), down-regulation of p14ARF by the siRNA construct caused down-regulation of the mutant p53 level, and this down-regulation was specific to the siRNA construct because we were able to rescue the mutant p53 by overexpressing p14ARF in the presence of p14ARF siRNA (Fig. 2C, lower left panel). Although the p14ARF siRNA construct down-regulated mutant p53, this siRNA construct alone did not affect the growth of RL cells (right panel). TGF-β1 signaling was still required for growth suppression. Interestingly, the degree of growth suppression was higher in the presence of the p14ARF siRNA construct as compared with the vector control, suggesting that growth inhibition is enhanced if p14ARF is suppressed before exposure to...
TGF-β1. We have also shown functionally the specificity of the p14ARF siRNA by rescuing the TGF-β1-mediated growth suppression in the presence of both p14ARF siRNA and overexpressed p14ARF (Fig. 2C, lower right panel).

It was of interest to determine whether this novel down-regulation of p14ARF and mutant p53 by TGF-β1 occurs in other B-cell lymphoma cell lines. As shown in Fig. 2D (right panel), both p14ARF and mutant p53 were also down-regulated upon TGF-β1 treatment in the TGF-β1-sensitive Burkitt lymphoma cell line CA46 (lane 2 versus lane 1), and the down-regulation of mutant p53 was blocked by overexpression of p14ARF (lane 2 versus lane 4). The down-regulation of p14ARF and mutant p53 by TGF-β1 correlated with the induction of p21Cip1/WAF1 expression and growth suppression, which were blocked by overexpression of p14ARF. Both RL and CA46 are derived from tumors of germinal center B-cells.

Next we wanted to investigate how p14ARF and mutant p53 were connected to TGF-β1-induced p21Cip1/WAF1 expression in RL cells. There are binding sites for Smads and p53 in the DNA binding domain, the mutant lacks transcriptional activity (supplemental Fig. 1) (41). To explore whether the A138P p53 mutant affected p21Cip1/WAF1 transcription induced by Smad...
complexes, we analyzed p21^{Cip1/WAF1} promoter activity in the presence of either p14^{ARF}, wild type p53, or A138P mutant p53. As shown in Fig. 2E, TGF-β1-induced promoter activity of p21^{Cip1/WAF1} was inhibited by p14^{ARF} and mutant p53, whereas wild type p53 expression caused a slightly higher activity compared with TGF-β1 treatment alone. Collectively, these data suggest that the inhibition of TGF-β1-induced p21^{Cip1/WAF1} promoter activity by p14^{ARF} could be mediated by mutant p53.

Inhibition of p21^{Cip1/WAF1} Promoter Activity Mediated by p14^{ARF} Can Be Blocked by p53 siRNA—If the inhibitory effect of p14^{ARF} on p21^{Cip1/WAF1} promoter activity was mediated by the mutant p53, knocking down mutant p53 in the presence of overexpressed p14^{ARF} should reverse the inhibitory effect of p14^{ARF}. As shown in Fig. 3A, the siRNA construct against p53 (p53si) induced down-regulation of mutant p53 in RL cells in a time-dependent manner. Using this siRNA, we were able to show that the inhibition of p21^{Cip1/WAF1} promoter activity by p14^{ARF} was reversed by the down-regulation of mutant p53 (Fig. 3B). Consistent with these data, lysates from the cells harboring different expression constructs showed the TGF-β1-induced up-regulation of p21^{Cip1/WAF1} expression upon down-regulation of mutant p53 (Fig. 3C, compare lane 5 with lane 4). These data strongly suggest that p14^{ARF} plays an important role in blocking TGF-β1-induced growth suppression by controlling the expression of mutant p53 in these B-cell lymphomas.

To further examine the involvement of mutant p53 and TGF-β1 signaling in controlling p21^{Cip1/WAF1} expression, we performed ChIP analysis using anti-Smad2/3 antibody to detect the recruitment of Smad2/3 to p21^{Cip1/WAF1} promoter. As shown in Fig. 3D, recruitment of Smad2/3 to p21^{Cip1/WAF1} promoter was observed as early as 1 h and increased substantially after 24 h of PMA/TGF-β1 treatment. The increase in Smad2/3 recruitment at the 24-h time point correlated with the down-regulation of mutant p53 upon PMA/TGF-β1 treatment (Fig. 3D). To examine whether mutant p53 is involved in blocking TGF-β1-induced Smad2/3 recruitment to p21^{Cip1/WAF1} promoter, a ChIP assay was performed in the cells where mutant p53 was down-regulated by siRNA. Interestingly, an increased level of Smad2/3 recruitment was observed only after 1 h of PMA/TGF-β1 treatment when mutant p53 was down-regulated by siRNA, suggesting a role of mutant p53 in blocking TGF-β1 signaling.
Mutant p53 Physically Interacts with Phospho-Smad2 in the Nucleus—To gain more insight into the mechanism underlying mutant p53-mediated blockage of TGF-β1-mediated p21Cip1/WAF1 promoter activity, we investigated whether mutant p53 interacts with phospho-Smads and prevents phospho-Smads from binding to the promoter. RL cells were treated with TGF-β1 for different time periods, and the nuclear extracts were used for immunoprecipitation with anti-Smad2 antibody. As shown in Fig. 4, anti-Smad2 antibody was able to pull down mutant p53, and the interaction was increased upon TGF-β1 treatment in a time-dependent manner. This experiment suggests that by interacting with phospho-Smad2 mutant p53 might have interfered in the TGF-β1-induced activation of p21Cip1/WAF1 promoter.

Down-regulation of p14ARF Protein by TGF-β1 Was Due to a Reduction in Its mRNA Level—To investigate whether the down-regulation of p14ARF mRNA level for mutant p53 was unaffected by TGF-β1 throughout the time points tested. These data along with the data shown in Fig. 1C indicate that TGF-β1 down-regulated p14ARF and mutant p53 expression by two different mechanisms: transcriptional or mRNA stability in the case of p14ARF and at the protein level in the case of mutant p53.

Next we wanted to understand the mechanism underlying the down-regulation of p14ARF upon TGF-β1 treatment. It has been shown that TGF-β1-mediated growth arrest in CA46 cells is regulated by transcriptional repression of E2F-1 (49). It also has been reported previously that E2F-1 transcriptionally activates p14ARF expression (50). So we tested whether E2F-1 is involved in TGF-β1-mediated down-regulation of p14ARF expression in RL cells. To elucidate the relative kinetics of the down-regulation of E2F-1, p14ARF, and mutant p53, RL cells were treated with PMA/TGF-β1 for various periods of time,
FIGURE 4. Mutant p53 physically interacts with Smad2. RL cells were treated with either medium alone or TGF-β1 for 1 or 6 h. Nuclear extracts were prepared, and equal amounts of extracts were used for immunoprecipitation (IP) with anti-Smad2. Left panel, the immunocomplexes were analyzed by Western blot analysis. Right panel, equal amounts of nuclear extracts were analyzed by Western blot analysis. One representative experiment of two is shown here. The quality of nuclear extracts was shown by probing with β-actin. pSmad2, phospho-Smad2.

FIGURE 5. Down-regulation of p14ARF, but not mutant p53, by TGF-β1 was due to a reduction in its mRNA level. A, RL Cells were treated with either medium alone or TGF-β1 in the presence of PMA for various time periods. Total RNA was extracted, and the mRNA levels of p14ARF and p53 were determined by Northern blot analysis. One representative experiment of two is shown here. B, left panel, RL cells were treated with either medium alone or PMA/TGF-β1 for various time periods, and equal amounts of nuclear extracts were analyzed by Western blot analysis. Right panel, quantitation of the bands is presented as fold change. C, TβRII-overexpressing RL cells (RL/pWPI/HA-TβRII) and CA46 cells were treated with either medium alone or TGF-β1, and equal amounts of nuclear extracts were analyzed by Western blot analysis. D, RL cells were transfected with either the empty vector or the E2F-1 expression vector, and after 48 h, cells were treated with either medium alone or PMA/TGF-β1 for 24 h. Equal amounts of nuclear extracts were analyzed by Western blot analysis. E, as described under “Experimental Procedures,” immunoprecipitated soluble chromatin complexes were isolated from RL cells treated with either medium alone or PMA/TGF-β1 for the indicated time points. The level of DNA enrichment was assessed by PCR followed by analysis of equal volumes of PCRs on an agarose gel. One representative experiment of two is shown here. IP, immunoprecipitation.
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and the levels of all three proteins were analyzed by Western blot analysis. The time points were chosen based on the slow kinetics of E2F-1 down-regulation observed in CA46 cells after TGF-β1 treatment (49). As shown in Fig. 5B, PMA/TGF-β1-induced down-regulation of E2F-1 was observed as early as 6 h, and this down-regulation preceded the down-regulation of p14ARF and mutant p53, indicating the possible involvement of E2F-1 in PMA/TGF-β1-induced down-regulation of p14ARF. We also observed that TGF-β1 treatments of TβRII-overexpressing RL cells and CA46 cells caused down-regulation of E2F-1 levels (Fig. 5C). To test the effect of E2F-1 expression on the levels of p14ARF and mutant p53, RL cells were transfected with either the empty vector or the E2F-1 expression vector, and the cells were treated with medium alone or with PMA/TGF-β1. As shown in Fig. 5D, ectopic expression of E2F-1 resulted in increased levels of both p14ARF and mutant p53 (lane 2 versus lane 1). Moreover, overexpression of E2F-1 blocked the down-regulation of p14ARF and mutant p53 by PMA/TGF-β1 treatment (lane 3 versus lane 2). To further demonstrate the role of E2F-1 in controlling the expression of p14ARF, we performed ChIP analysis to assess the binding of E2F-1 to the p14ARF promoter. As shown in Fig. 5E, E2F-1 was found to constitutively occupy the p14ARF promoter, and this promoter binding of E2F-1 was inhibited upon TGF-β1 treatment in a time-dependent manner. Collectively, our data suggest that PMA/TGF-β-induced down-regulation of p14ARF was mediated via E2F-1.

The relation between p14ARF, p53, and Mdm2 is well established (27). To monitor the interaction among these proteins in response to TGF-β treatment, TβRII-overexpressing cells (RL/pWPI/HA-TβRII) were treated with TGF-β1 for 24 h in the presence or absence of a proteasome inhibitor, MG132, for the last 8 h. Nuclear extracts were prepared and subjected to immunoprecipitation analysis using anti-Mdm2 antibody. As shown in Fig. 6, whereas anti-Mdm2 antibody was able to pull down both p14ARF and mutant p53 from untreated cells (lane 2), no p14ARF and mutant p53 were observed in the immunoprecipitated complex from TGF-β1-treated samples (lane 3 versus lane 2). This was due to the down-regulation of p14ARF and mutant p53 expression upon TGF-β1 treatment (Input panel, lane 2). Blocking of TGF-β1-induced degradation of mutant p53, but not p14ARF, by MG132 resulted in an immunoprecipitated complex that contained mutant p53 (lane 5 versus lane 3). These data suggest that TGF-β1-induced down-regulation of mutant p53 might be via Mdm2-mediated degradation, whereas down-regulation of p14ARF was not at the protein level.

DISCUSSION

TGF-β1 blocks growth in a large number of cell types. Mutants of the tumor suppressor p53 have been shown to increase cell proliferation and resistance to TGF-β1-mediated growth inhibition (12, 22). In this study, we have shown a unique mechanism whereby TGF-β1 treatment down-regulated the E2F-1 level, leading to the down-regulation of p14ARF and mutant p53 to allow the expression of p21Cip1/WAF1 and subsequent growth suppression of B-cell lymphoma cell lines (Fig. 7). Experimental overexpression of E2F-1 not only up-regulated p14ARF and mutant p53 but also blocked TGF-β1-induced down-regulation of p14ARF and mutant p53. Overexpression of p14ARF blocked TGF-β1-induced growth suppression by preventing down-regulation of mutant p53 and up-regulation of p21Cip1/WAF1. p14ARF-mediated blockage of p21Cip1/WAF1 expression was reversed by siRNA-mediated reduction of mutant p53, indicating the involvement of mutant p53 in controlling TGF-β1-induced expression of p21Cip1/WAF1. We have also shown by ChIP analysis that TGF-β1-induced Smad2/3 recruitment to p21Cip1/WAF1 promoter was increased when the mutant p53 level was down-regulated by siRNA (Fig. 3D). Collectively, these data indicate a critical role for p14ARF in stabilizing mutant p53 and possibly conferring TGF-β1 resistance in B-cell lymphoma cell lines.

In a gene expression profile study of diffuse large B-cell lymphomas, Lenburg et al. (51) observed increased levels of p14ARF transcript expression in four of seven lymphomas classified as aggressive lymphomas. The levels of p14ARF transcript expression in these four aggressive lymphomas ranged from ∼18- to 140-fold greater than the average level of transcript expression.
observed in normal resting and activated B-cells. When the levels of p14ARF transcript expression in these four aggressive lymphomas were compared with the average level observed in normal resting and activated B-cells, it was reported that the expression of p14ARF (murine homologue of human p19ARF) induced by TGF-β1 contributes to growth arrest in mouse keratinocytes (54).

In primary cells, the level of wild type p53 is usually very low, whereas the level of mutant p53 is abundant in some tumor cell lines, including RL and CA46. These high levels of mutant p53 not only give these tumor cells a proliferative advantage but also confer resistance to TGF-β1-induced growth suppression. We have shown here that mutant p53 by interacting with phospho-Smad2 interferes with TGF-β1-induced p21Cip1/WAF1 promoter activity. The level of p14ARF is critical in maintaining the level of mutant p53 and subsequent blockage in TGF-β1-induced p21Cip1/WAF1 expression because siRNA knockdown of mutant p53 reverses not only the p14ARF-mediated down-regulation of p21Cip1/WAF1 promoter activity but also increases p21Cip1/WAF1 protein expression.

These changes in the tumor cells are not restricted to TGF-β signaling. A similar sequence of cellular events occurs after signal transduction through the surface immunoglobulin molecule. Prior work has demonstrated that anti-idiotypic antibodies may kill tumor cells in vivo through cell signaling events (56). It is likely that activation-induced cell death from a variety of stimuli require the down-regulation of mutant p53 through this or a closely related mechanism.

In summary, we have demonstrated that in RL and CA46 lymphoma cells an altered TGF-β1-induced signaling pathway has developed that reduces levels of p14ARF via transcriptional repression or mRNA stability. In normal cells, wild type p53 and p14ARF are expressed at very low levels. When p53 is mutated, it cannot feed back and down-regulate p14ARF levels as the wild type protein does (57). Therefore, mutant p53 and p14ARF levels build to high levels. These high p14ARF and mutant p53 levels block TGF-β1-mediated growth suppression by attenuating p21Cip1/WAF1 expression. The data presented here also demonstrate that TGF-β1 reduces levels of p14ARF via down-regulating E2F-1 transcription factor. It will be interesting to see whether E2F-1 overexpression is also observed in aggressive non-Hodgkin lymphomas where elevated levels of p14ARF expression have been reported (52). Our data suggest that the high levels of p14ARF often found in tumor cells may be a potential therapeutic target in that reducing them may make other treatments more effective by compromising the capacity of mutant p53 to promote proliferation.

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