Oncogenic Ki-ras Confers a More Aggressive Colon Cancer Phenotype through Modification of Transforming Growth Factor-β Receptor III*

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 Transforming growth factor-β1 (TGF-β1) can act as a tumor suppressor or a tumor promoter depending on the characteristics of the malignant cell. Each of three Ki-rasG12V transfectants of HD6-4 colon cancer cells had been shown to be more aggressive in vivo than controls in earlier studies (Yan, Z., Chen, M., Peruchò, M., and Friedman, E. (1997) J. Biol. Chem. 272, 30928–30936). We now show that stable expression of oncogenic Ki-rasG12V converts the HD6-4 colon cancer cell line from insensitive to TGF-β1 to growth-promoted by TGF-β1. Each of three Ki-rasG12V transfectants responded to TGF-β1 by an increase in proliferation and by decreasing the abundance of the Cdk inhibitor p21 and the tumor suppressor PTEN, whereas each of three wild-type Ki-ras transfectants remained unresponsive to TGF-β1. The wild-type Ki-ras transfectants lack functional TGF-β receptors, whereas all three Ki-rasG12V transfectants expressed functional TGF-β receptors that bound 125I-TGF-β1. The previous studies showed that in cells with wild-type Ki-ras, TGF-β receptors were not mutated, and receptor proteins were transported to the cell surface, but post-translational modification of TGF-β receptor III (TβRIII) was incomplete. We now show that the betaglycan form of TβRIII is highly modified following translation when transiently expressed in Ki-rasG12V cells, whereas no such post-translational modification of TβRIII occurs in control cells. Antisense oligonucleotides directed to Ki-Ras decreased both TβRIII post-translational modification in Ki-rasG12V cells and TGF-β1 down-regulation of p21, demonstrating the direct effect of mutant Ras. Therefore, one mechanism by which mutant Ki-Ras confers a more aggressive tumor phenotype is by enhancing TβRIII post-translational modification.

The TGF-β1 family of proteins number >25 and regulate cell growth and differentiation as well as morphogenesis and angiogenesis (1, 2). There are three mammalian isofoms, TGF-β1, TGF-β2, and TGF-β3, which are structurally very similar with nine conserved cysteines. The TGF-βs belong to a superfamily of structurally related proteins including the activins, inhibins, and bone morphogenic proteins (3). The TGF-βs induce diverse biological responses by binding to the high affinity receptors TβRI (53 kDa) and TβRII (75 kDa), which function as a heterodimer. Both receptors have a cysteine-rich extracellular domain, one transmembrane segment, and a cytoplasmic tail that includes a serine/threonine kinase domain (4, 5). Constitutively phosphorylated TβRII binds TGF-β1, which then recruits TβRI into the complex. TβRI is transphosphorylated by TβRII and propagates the signal by its kinase activity to downstream substrates (6). Two other cell-surface TGF-β-binding proteins are the type III receptors betaglycan and endoglin, which mediate cellular responses to TGF-β, but have no signaling sequences. Betaglycan and endoglin may function by regulating TGF-β access to TβRII (7–9). TβRIII receptors are not found in every TGF-β-responsive cell and are down-regulated during myoblast differentiation into myotubes (10).

The chief mediators of TGF-β signaling are the SMAD family of structurally related proteins. Receptor-activated SMAD proteins form heterotrimeric complexes and translocate to the nucleus, where they interact with other transcription factors to drive TGF-β1-induced transcription (11). Transcription factors interacting with SMAD proteins include FAST-1 and FAST-2 (12, 13) and the c-Jun/c-Fos heterodimer (14). The cooperation of SMAD proteins with other transcription factors suggests that TGF-β1 induces multiple parallel signaling pathways. Additional reported members of the TGF-β signaling pathways include the SMAD-interacting protein SARA and related proteins (15), Ras (16–18), the Raf homolog TAK-1 (19), TAK-1-associated proteins TAB-1 and TAB-2 (20), and the FK506- and rapamycin-binding protein FKBP-12 and the α-subunit of farnesyltransferase (21–23).

Several investigators have reported that expression of oncogenic Ras confers resistance to the growth inhibitory properties of TGF-β (24–27). In mouse mammary epithelial cells, oncogenic Ras was shown to constitutively activate the MAPKs ERK1 and ERK2. Activated ERK1/ERK2 phosphorylate multiple sites within the linker regions of SMAD2 and SMAD3, decreasing, but not totally eliminating, SMAD translocation into the nucleus following TGF-β stimulation (28). Oncogenic Ras, by this mechanism, eliminates the antimitogenic activity of TGF-β. However, treatment of the same oncogenic Ras-transformed cells with TGF-β1 causes an epithelial-to-fibroblastoid conversion to a more invasive phenotype (29), showing that these cells maintained some TGF-β signaling pathways, which mediated tumor aggressiveness. In the mouse skin model of chemical carcinogenesis, one Ha-ras gene is activated by mutation and the normal is allele lost, causing the cells to undergo a morphological transformation to a highly invasive

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§ The abbreviations used are: TGF-β, transforming growth factor-β; TβR, transforming growth factor-β receptor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; TdR, thymidine; bp, base pair(s); DMEM, Dulbecco’s modified Eagle’s medium; RT-PCR, reverse transcription-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

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spindle phenotype that can be induced in synergy with TGF-β1 (30).

We now report another system in which oncogenic Ras and TGF-β1 work together to enhance tumorigenicity and also report initial studies on the mechanism of this response. Goblet cells compose ~20% of the colon epithelial cell population and are resistant to growth inhibition by TGF-β1 in vitro, becoming enriched relative to other colon epithelial cells in mice injected with recombinant TGF-β1 (31). Colon goblet cell lines are also resistant to the growth inhibitory effects of TGF-β1 (32, 33). We now show that oncogenic Ras up-regulates post-translational modification of the type III TGF-β receptor in the HD6-4 colon goblet cell line. Unexpectedly, TGF-β1 induces cell proliferation in the presence of oncogenic Ki-ras and, in parallel, down-regulates the Cdk inhibitor p21cip1/Waf1 and the tumor suppressor phosphatase PTEN.

EXPERIMENTAL PROCEDURES

Materials—125I-TGF-β1 and [3H]TdR were obtained from PerkinElmer Life Sciences. Human recombinant TGF-β1 was from R&D Systems. Protein A-Sepharose was obtained from Amersham Pharmacia Biotech. Polyvinylidene difluoride transfer paper (Immobilon-P) was from Millipore Corp. Antibody TED1 to PTEN was the kind gift of Dr. Hong Sun (Department of Genetics, Yale University School of Medicine). Antibodies to TβRI/IIa5, TGF-β1, and TβRIII were purchased from Santa Cruz Biotechnology; antibodies to phospho-SMAD2 and total SMAD2 were from Upstate Biotechnology, Inc.; antibody to betaglycan/TβRIII was from R&D Systems; and mouse monoclonal IgG2a clone 70 to p21cip1 and anti-phospho-polyamine monoclonal antibody PY99 were purchased from Transduction Laboratories. Phosphorylated oligodeoxynucleotides were a gift of Dr. Brett Monia (Isis Pharmaceuticals, Carlsbad, CA). Isis-13177 is a 20-mer of random sequence, and Isis-6957 is a 20-mer targeted to the 5’-translated region of Ki-Ras (CAT-GTC-CTG-CCG-GCT-GC). This sequence is found within the promoter region of the c-Ki-ras gene cloned into pMIKVa12 (see below) ~60 bp upstream of the translational start site. Endoglycosidase F (N-glycosidase F-free), also known as endo-β-N-acetylglucosidase F, was purchased from Roche Molecular Biochemicals. All other reagents, including chondroitinase ABC and heparitinase III, were from Sigma.

Antisense Oligonucleotide Treatments—Treatment of Ki-ras transfec-
tants was as described (34). Colon cells were plated at 2 × 104/well in six-well plates. 1 h later, the cells were washed with prewarmed serum-free insulin transferrin/selenious acid-supplement-
dem DMEM and then incubated in this medium with a fixed ratio of oligonucleotide to Lipofectin (2.4 μl oligonucleotide/100 μl of Lipofectin). The oligonucleotide-containing medium was then replaced with recombinant TGF-β1 and, in parallel, down-regulates the Cdk inhibitor p21cip1/Waf1 and the tumor suppressor phosphatase PTEN.

RESULTS

Oncogenic Ki-ras Transfectants Gain Response to TGF-β1—The Ki-rasG12V transfectant cloned sublines V, V1, and V2 were isolated following stable transfection of HD6-4 colon carcinoma cells with a mini-gene construct of the wild-type Ki-ras4B gene mutated at codon 12 to aspartate. Three stable sublines (V1, G1, and G3) were isolated following transfection with the wild-type mini-gene construct of cellular Ki-ras4B (35, 36). The Ki-
rasG12V transflectant lines expressed oncogenic Ki-Ras protein, whereas the wild-type Ki-ras transfectant G lines expressed more wild-type Ki-Ras protein as shown by Western blotting with Ki-Ras-specific and pan-Ras antibodies (35, 36).

The parental line is not responsive to TGF-β1 (31–33), but
Mutant Ki-Ras Modification of TβRIII

FIG. 1. Ki-rasG12V transfectant cells respond to TGF-β1, but wild-type ras cells do not. A, parallel cultures of Ki-rasG12V transfectant V cells and parental wild-type ras cells (RasWT) were treated with 8 ng/ml TGF-β1 for 0, 5, 15, or 30 min, and cell lysates were prepared and analyzed by Western blotting with anti-phosphotyrosine (P-Tyr) antibody. The arrow marks a protein exhibiting an increase in phosphotyrosine content only in the Ki-rasG12V transfectant V cells. A 200-kDa protein exhibited similar phosphotyrosine content at all time points in both cell types. B, parallel cultures of Ki-rasG12V transfectant V cells and wild-type Ki-ras transfectant G cells were treated with 1 ng/ml TGF-β1 for 30 min before cell lysis and Western blotting with antibody directed to SMAD2 phosphorylated at serines 465 and 467 (P-Smad2) and with antibody to total SMAD2/SMAD3. The lower panel shows cellular proteins of 55–60 kDa after the Western blot was stained with Coomassie Blue to demonstrate equal loading and transfer. IB, immunoblotting.

unexpectedly became TGF-β1-responsive following expression of oncogenic Ki-Ras protein. Ki-rasG12V transfectant V cells exhibited a rapid increase in tyrosine phosphorylation of an unknown cellular protein of ~60 kDa after addition of TGF-β1, whereas no increase in tyrosine phosphorylation of this or any other protein was found in control cells treated with TGF-β1 (Fig. 1A). The 60-kDa protein showed increased tyrosine phosphorylation following 5 and 15 min of TGF-β1 treatment (Fig. 1A, arrow), whereas the abundance of phosphotyrosine in a 200-kDa protein remained constant, demonstrating equal loading. The chief mediators of TGF-β signaling are the SMAD family of structurally related proteins. Upon TGF-β receptor activation, TβRI activates phosphorylation of SMAD2 and/or SMAD3, each of which can form an association with the common mediator SMAD4 and then translocate to the nucleus and mediate transcription (reviewed in Refs. 11 and 37). SMAD2 is a mediator SMAD4 and then translocate to the nucleus and SMAD3, each of which can form an association with the common mediator SMAD4. Their mechanism of this unexpected proliferative response to TGF-β1 was explored by assaying two proteins associated with tumorigenicity: the Cdk inhibitor p21Cip1/Waf1 (43, 44) and the tumor suppressor phosphatase PTEN (45, 46). p21Cip1/Waf1 inhibits cell cycling by targeting most Cdk-cyclin complexes, binding to them and inhibiting their kinase activity. PTEN is a phospholipid phosphatase, deleted or mutated in a wide variety of tumors (46). Loss of PTEN activity would up-regulate the activity of the serine/threonine kinase Akt, which is known to play an important role in generating cell survival signals (47–49). Ki-rasG12V transfectant V cells and control cells were treated for 2 days with 0, 1, 2, 4, or 8 ng/ml TGF-β1, and then cell lysates were blotted for abundance of p21 and PTEN (Fig. 2A, lower panel). In control cells with wild-type Ki-ras, TGF-β1 caused no alteration in the levels of either protein. However, in duplicate experiments, TGF-β1 induced a dose-dependent decrease in the abundance of the Cdk inhibitor p21Cip1/Waf1, with the optimal growth-stimulating concentration of 4 ng/ml TGF-β1 reducing p21 levels to 16% of the control. PTEN levels were decreased 6-fold by 2, 4, or 8 ng/ml TGF-β1 only in the mutant Ki-Ras transfectant V cells. Coomassie Blue staining of the blot demonstrated equal loading (Fig. 2A, lower panel).

The generality of these responses was determined by assaying several more cell lines. Levels of p21Cip1/Waf1 and PTEN were examined in two other oncogenic Ki-ras transfectant lines (V1 and V2), two wild-type Ki-ras transfectant lines (G2 and G3), and the parental line with endogenous wild-type ras. Treatment with the optimal concentration of 4 ng/ml TGF-β1 decreased p21 levels in V1 and V2 cells expressing mutant Ki-Ras by 52 and 90%, respectively, whereas no decreases in p21 levels were observed in G1 and G3 lines with wild-type ras.
Mutant Ki-Ras Modification of TβRIII

Fig. 2. TGF-β1 stimulates proliferation of Ki-ras<sup>G12V</sup> transfectant cells and down-regulates levels of the Cdk inhibitor p21 and the tumor suppressor PTEN. A: upper panel, HD6-4 colon carcinoma cells stably transfected with Ki-ras<sup>G12V</sup> transfectant V cells and parental cells expressing only wild-type ras (Ras<sup>WT</sup>) were treated for 54 h with increasing concentrations of TGF-β1 in serum-free transferrin/selenium-supplemented acid-supplemented DMEM and then incubated with [³H]TdR for 3 h before analysis to measure cell proliferation. Both attached cells and loosely adherent cells were collected for this assay. All data are the mean of two experiments, each performed in triplicate. The t test demonstrated that the proliferation of TGF-β1-treated V cells was statistically greater than that of TGF-β1-treated control cells, with p values of <0.01, 0.001, and 0.001 for TGF-β1 levels of 1, 2, or 4 ng/ml, respectively. Error bars are shown only if the S.E. is >5%. Lower panel, TGF-β1 decreases the abundance of p21 and PTEN in Ki-ras<sup>G12V</sup> transfectant V cells. ras<sup>G12V</sup> and wild-type ras cells were treated for 54 h with increasing concentrations of TGF-β1 of 0, 1, 2, 4, and 8 ng/ml, and then the abundance of p21<sup>Cip1/Waf1</sup> and PTEN in cell lysates was determined by Western blotting. The lower panel shows cellular proteins of ~44 kDa detected in this immunoblot by staining with Coomassie Blue to demonstrate equal loading. B: upper panel, TGF-β1 stimulates proliferation of two other Ki-ras<sup>G12V</sup> transfectants. Four other stable ras transfectants, two with mutant Ki-ras<sup>G12V</sup> (V1 and V2 cells) and two with wild-type Ki-ras (G2 and G3 cells), and the parental line were treated with 4 ng/ml TGF-β1 or left untreated, and [³H]TdR incorporation for each cell line was determined as described for A. All data are the mean of two experiments, each performed in triplicate. The t test demonstrated that the proliferation of TGF-β1-treated V1 and V2 cells was statistically greater than that of untreated V1 and V2 cells, with p values <0.0005 for each line. Lower panel, Western blots for the Cdk inhibitor p21 and the tumor suppressor PTEN and a double protein band of ~44 kDa detected by Coomassie Blue staining of the Western blot to demonstrate equal loading.

(Fig. 2B, lower panel). Treatment with 4 ng/ml TGF-β1 decreased PTEN levels in V1 and V2 cells expressing mutant Ki-Ras by 50% and 30%, respectively, in duplicate experiments. In contrast, no decreases were observed in PTEN levels in G2 and G3 cells expressing only wild-type Ki-ras. Thus, TGF-β1 acts like a mitogen in mutant Ki-ras-transformed colon carcinoma cells, possibly by decreasing the abundance of the Cdk inhibitor p21<sup>Cip1/Waf1</sup>. The loss of PTEN expression with TGF-β1 treatment may contribute to the aggressive growth of each Ki-ras<sup>G12V</sup> transfectant cell line in vivo, as cells can respond to TGF-β1 from autocrine or paracrine sources (35). These experiments demonstrate that oncogenic Ki-ras can confer a more aggressive, tumorigenic phenotype in colon cancer cells by altering their response to TGF-β1.

The Cdk inhibitor p21 is induced by TGF-β1 acting at the transcriptional level in several cell types, including (most if not all) epithelial cells (50, 51), but decreases in p21 abundance are seen when cells are treated with a mitogen. TGF-β1 has also been reported to rapidly down-regulate expression of PTEN at the transcriptional level in human keratinocyte HaCaT cells (45). Ongoing studies are exploring the mechanisms for these changes. In the following studies, we provide the mechanism for the gain of response to TGF-β1 by oncogenic ras.

Functional TGF-β Receptors Induced in Ki-ras<sup>G12V</sup> Transfectants without Changes in TβRI, TβRII, or TGF-β1 Abundance or Mobility—Functional TβRI, TβRII, and TβRIII receptors had not been detected in parental cells expressing wild-type Ki-ras in earlier studies using cross-linking of <sup>125</sup>I-TGF-β1 (33, 31). TGF-β1-binding proteins on the surface of viable cells were chemically cross-linked with <sup>125</sup>I-TGF-β1 and then molecularly sized by SDS-PAGE and detected by autoradiography. Functional TβRI and TβRII and a polydisperse band of higher molecular mass TGF-β1-binding proteins of the size of TβRII were detected in each Ki-ras<sup>G12V</sup> transfectant line (V, V1, and V2). In contrast, the parental line HD6-4 expressing only endogenous wild-type Ki-ras (Fig. 3, left panel, WT lane) and the wild-type Ki-ras transfectant G cell line (right panel) exhibited no detectable TGF-β1 binding. Identical amounts of labeled TGF-β1 were detected on each lane in the autoradiograph, demonstrating equal loading. The parental cells displayed no binding of <sup>125</sup>I-TGF-β1, although neither the TβRII nor TβRI receptor was mutated, and these cells exhibited cell-surface TβRII and TβRIIIs and TβRIIIs at levels equal to those seen in TGF-β1-responsive cells, indicating that receptor localization was not altered (31). Thus, functional TβRI, TβRII, and TβRIII were detected in each of three Ki-ras<sup>G12V</sup> transfectant lines, but in neither control line.

Possibly oncogenic Ki-Ras could increase the ability of TGF-β receptors to bind <sup>125</sup>I-TGF-β1 by altering the level of autocrine TGF-β1. However, no difference in the level of mature TGF-β1 was discerned between the Ki-ras<sup>G12V</sup> transfectant V cells and parental cells (Fig. 4). Thus, oncogenic Ki-Ras protein did not alter the abundance or electrophoretic mobility of autocrine TGF-β1. No differences in cellular abundance, cell surface levels, or electrophoretic mobility of TβRI or TβRII were detected in the two cell types, expressing either wild-type or mutant Ki-Ras (Fig. 5). Total TβRI levels were assayed by Western blotting (Fig. 5A), and cell-surface TβRI levels were assayed by
immunoprecipitation following biotinylation (Fig. 5C). Total TβRII levels were assayed by immunoprecipitation (Fig. 5B), and cell-surface TβRII levels were assayed by immunoprecipitation following biotinylation (Fig. 5C). Each experiment was controlled by absorption of the antibody with specific peptides corresponding to either TβRI or TβRII. The biotinylation experiment demonstrated that equal amounts of the TβRI and TβRII proteins reached the cell surface of cells expressing either wild-type or mutant Ki-Ras. Thus, the increased binding of 125I-TGF-β1 in the oncogenic Ki-ras transfectant cells (Fig. 3) was not a result of increased transport of receptor proteins to the cell surface. These results, taken together, demonstrate that expression of oncogenic Ki-Ras protein did not alter the abundance, cell-surface expression, or electrophoretic mobility of either TβRI or TβRII, although these receptors regained the ability to bind exogenous 125I-TGF-β1 when oncogenic Ki-Ras protein was expressed.

Restoration of TβRII function by itself might explain the ability of TβRI and TβRII to bind TGF-β1 since the parental cells exhibit incomplete post-translational modification of TβRII (31). TβRII functions by concentrating TGF-β in the cell periphery and presenting it to TGFRII (8, 9, 52). TGFRII then forms a complex with TGFRI and phosphorylates TGFRII, transmitting the TGF-β1 signal (53). Therefore, expression of mutant Ki-Ras was closely correlated with gain of TGF-β receptor function.

Increased Post-translational Modification of Endogenous Betaglycan/TβRIII in Oncogenic Ki-rasG12V Transfectants—The major form of TβRII found in HD6-4 colon carcinoma cells is betaglycan, whose core protein migrates at ~110 kDa (31). Very little post-translational modification of betaglycan occurs in parental cells with wild-type ras (31). In TGF-β1-responsive colon carcinoma cells, in contrast, betaglycan migrates as more modified forms at 120 and 140 kDa as well as a heterogeneous diffuse group of species above 200 kDa (31). Post-translational glycosylation of the integrin β1-chain was found to be aberrant in each of the oncogenic Ki-ras transfectants (35), suggesting that post-translational glycosylation of betaglycan might also be altered. Oncogenic forms of ras genes have been implicated in modulating various Golgi acetylglucosaminyltransferases in different cell types (54, 55). Western blotting for TβRIII revealed new high molecular mass bands for each of the three Ki-rasG12V transfectants, but not for the wild-type Ki-ras G cells (data not shown), indicating greater post-translational modification of betaglycan. Thus, oncogenic Ki-Ras might up-regulate an acetylglucosaminyltransferase or a galactosyltransferase, which would modify betaglycan and restore its biological function.

The post-translational modifications of endogenous betaglycan were analyzed in Ki-rasG12V transfectant V cells by immunoprecipitation after prelabeling cellular proteins with [35S]methionine. Betaglycan forms were size-fractionated on gradient SDS-PAGE and detected by autoradiography. In control cells with wild-type ras, betaglycan/TβRIII migrated as a core peptide of 110 kDa (Fig. 6A, left panel) and one higher molecular mass, but low abundant diffuse band (arrows). Thus, little post-translational modification occurred in control cells, as reported previously (31). In contrast, three higher molecular mass betaglycan species in addition to the core protein were detected in lysates from Ki-rasG12V transfectant cells (Fig. 6A, left panel, arrowheads). Therefore, at least some of the high molecular mass 125I-TGF-β1-binding proteins detected in each Ki-rasG12V transfectant line (Fig. 3) were post-translational modified betaglycan/TβRIII.

We next questioned whether the post-translational modification of betaglycan induced by mutant Ki-Ras was similar to that seen in other TGF-β1-responsive cells. In such cells, the betaglycan core protein is heavily modified by glycosaminoglycan groups and by N-glycosylation (56). The betaglycan in V cells and in parental cells was chemically deglycosylated with N-glycosidase F, and proteoglycan groups were removed by digestion with heparitinase and chondroitinase ABC (Fig. 6A, right panel). These treatments have been shown to reduce betaglycan to its protein core (31). After treatment, equal amounts of betaglycan core protein were immunoprecipitated from prelabeled V cells and control cells, and the core proteins displayed equivalent electrophoretic migration rates (Fig. 6A, left and right panels). Thus, mutant Ki-Ras functioned at the level of post-translational modification of betaglycan, just as mutant Ki-Ras functioned in the same cells to modulate post-translational modification of β1 integrin (35).

These data indicated that betaglycan mRNA levels should be similar in mutant Ki-Ras and control cells. Similar amounts of betaglycan mRNA were detected in Ki-rasVal12 transfectant and control cells by semiquantitative RT-PCR (Fig. 6B), con-
sistent with the equal levels of betaglycan core peptide exhibited in each cell line. These data suggest that mutant Ki-Ras functions to increase betaglycan modification, not to increase the abundance of the betaglycan core protein.

More Abundant Post-translational Modification of Transiently Transfected Betaglycan in Ki-rasG12V Transfectant Cells—To confirm that betaglycan was highly modified following translation in Ki-rasG12V transfectant cells, betaglycan tagged with a c-Myc epitope was inserted into a retroviral vector. Both Ki-rasG12V transfectant V cells and control cells expressing wild-type Ki-ras were infected with this recombinant retrovirus. The predominant betaglycan species immunoprecipitated from control cells was the core peptide (Fig. 7). Therefore, very little post-translational modification of betaglycan occurred in cells with wild-type ras, consistent with the data on endogenous betaglycan shown in Fig. 6A. Moreover, highly modified, polydisperse betaglycan was synthesized in the Ki-rasG12V transfectant V cells (Fig. 7), again confirming the increased post-translational modification of endogenous betaglycan (Fig. 6A). Control experiments were carried out with the retroviral vector alone (data not shown) or without the c-Myc antibody in immunoprecipitations (−lanes). Note that equal amounts of IgG were found in each antibody lane, confirm-
and then treated for 2 days with 4 ng/ml TGF-β thiolated random sequence oligonucleotides as described above.

Thiolated antisense oligonucleotides to Ki-Ras or phosphorothiolated antisense oligonucleotides directed to mutant Ki-Ras block the abnormal glycosylation of p21 by TGF-β.

The possibility was investigated that betaglycan/TβRIII, whether transfected or endogenous, was highly modified following translation in the Ki-rasG12V transfectant cells, but not in control cells expressing only wild-type Ki-ras.

Antisense Oligonucleotides to Ki-Ras Decrease Post-translational Modification of Betaglycan and Block Down-regulation of p21 by TGF-β—The possibility was investigated that betaglycan modifications occurred as a result of adaptation of Ki-rasG12V transfectants to culture and were not directly related to expression of mutant Ki-Ras protein. Therefore, the expression of mutant Ki-rasG12V in V2 cells was decreased by treatment with phosphorothiolated antisense oligonucleotides targeted to Ki-Ras. This treatment almost completely eliminated the expression of the mutant Ki-Ras protein in V2 cells compared with cells treated in parallel with random sequence oligonucleotides, as shown by Western blotting with a pan-Ki-rasG12V-specific antibody (Fig. 8A, left panel). Phosphorothiolated antisense oligonucleotides directed to mutant Ki-Ras block the abnormal glycosylation of the integrin β1-chain in V2 cells (35), so we expected that blocking mutant Ki-Ras would alter betaglycan modification. The sharp decrease in mutant Ki-Ras protein caused a marked decrease in the abundance of the most highly modified betaglycan species in the Ki-rasG12V transfectant cells. The decrease in betaglycan modification was dependent on the concentration of phosphorothiolated antisense oligonucleotides, with the greatest effect seen at 200 nM. Each of the lysates exhibited similar amounts of core protein, demonstrating equal loading. Therefore, the increased post-translational modification of betaglycan/TβRIII found in each of the oncogenic ras transfectant lines was due to expression of the mutant Ki-ras gene.

We next tested whether blocking expression of the mutant Ki-ras gene would also block response to TGF-β. V2 cells were treated with equal concentrations (200 nM) of either phosphorothiolated antisense oligonucleotides to Ki-Ras or phosphorothiolated random sequence oligonucleotides as described above and then treated for 2 days with 4 ng/ml TGF-β or left untreated. TGF-β down-regulated the abundance of the Cdk inhibitor p21Cip1/Waf1 in V2 cells pretreated with random sequence oligonucleotides in duplicate experiments analyzed by Western blotting (Fig. 8B). However, the TGF-β1 response was blocked in V2 cells treated with antisense oligonucleotides to down-regulate mutant Ki-Ras, and the abundance of p21Cip1/Waf1 was unchanged (Fig. 8B). The abundance of a cellular protein slightly larger than p21Cip1/Waf1 is also shown to demonstrate equal loading and blotting. These experiments demonstrate that the increased post-translational modification of betaglycan/TβRIII observed in each Ki-rasG12V transfectant line and the response of the Ki-rasG12V transfectant cells to TGF-β1 were caused by expression of the mutant Ki-ras gene.

Discussion

TβRIII or betaglycan is the most abundant TGF-β-binding protein in a number of cell types. Betaglycan has a small cytoplasmic domain with no consensus signaling motif (7, 9), so it is not believed to directly transduce TGF-β signaling. However, in the absence of TβRIII, only a small fraction of TβRII in myoblasts binds TGF-β1 with high affinity, whereas expression of TβRIII in these cells converts the majority of TβRII molecules to high affinity TGF-β1 receptors (52). Thus, the major role of TβRIII is believed to be its ability to concentrate TGF-β1 and present it to the signaling receptors, TβRII and TβRI. The role of TβRIII is not limited to binding exogenous TGF-β1. Stable transfection of TβRIII in MCF-7 breast cancer cells restores autocrine TGF-β1 signaling as well (57).

In this study, we have shown that both functional, post-translationally modified TβRIII and the biological response to TGF-β1 were restored by stable expression of oncogenic Ki-rasV12 in HD6-4 colon carcinoma cells. Both responses were blocked by phosphorylated antisense oligonucleotides, which markedly reduced the abundance of mutant Ki-Ras protein, proving that the changes in TGF-β receptors and response were caused by the mutant Ki-Ras protein. The oncogenic Ki-rasV12 transfectant cell lines had been shown in earlier studies to have altered post-translational glycosylation of β1 integrin (35), so the post-translational modification of TβRIII shown in this study may also be glycosylation. TGF-β mediates glycosylation of β1 integrin in colon carcinoma cells by activating Ras proteins (58). The TGF-β1/Ras pathway functions to convert a partially glycosylated β1 integrin precursor into the mature, fully glycosylated form and is blocked by dominant-negative N17Ras (58). In like fashion, constitutive activation of Ki-Ras in stable oncogenic Ki-ras transfectants may up-regulate glycosyltransferases that modify TβRIII. β1–6-N-Acetlyglucosaminyltransferase V is increased severalfold in activity in rodent fibroblast lines transfected with the oncogenic T24 Ha-ras gene (54). In addition, NIH3T3 cells expressing the N-ras proto-oncogene exhibit 5–7-fold increases in the activities of β1-galactosyltransferase and β3-N-acetylgalcosaminyltransferase, both of which synthesize polyglycosaminoglycan chains (55). What is the biological significance of these observations? ras genes are mutated in about half of all colon cancers. The colon goblet cell lineage comprises 20% of the cells in the colonic crypt, so some of the ras mutations that occur in the development of colon cancer must take place in this lineage.

The parental HD6-4 cell line used in this study was shown earlier to respond to very high levels of TGF-β1 (100 ng/ml), 20–100 times more than used in this study, by induction of p21Cip1/Waf1 (31). Lower concentrations of 1–5 ng/ml TGF-β1, which initiated responses in other colon carcinoma cells and in the mutant Ki-Ras transfectants in this study, were ineffective on the parental HD6-4 cells (31). Very high TGF-β1 levels of 100 ng/ml could bypass the need for a functional TβRIII to bind and concentrate TGF-β1 and to present it to the signaling receptors, leading us to hypothesize that, under these conditions, TGF-β1 bound directly to TβRII. Supporting this hypoth-

**Fig. 7.** Exogenous betaglycan is highly modified in rasG12V transfectants. rasG12V (V clone) and parental wild-type ras (rasWT) cell lines were infected with either a recombinant retrovirus encoding a Myc-tagged betaglycan gene (LXSN-Myc-BG) or a recombinant retrovirus encoding only the c-Myc expression tag (LXSN-Myc). After 48 h of culture to allow expression and post-translational modification of betaglycan, the expressed betaglycan was immunoprecipitated (IP) by its c-Myc tag and analyzed by Western blotting with anti-TβRIII antibody. The core betaglycan peptide was synthesized in both cell types (myc-BG core), but was highly modified only in rasG12V cells (myc-BG). Similar levels of IgG were detected in each immunoprecipitate as controls. Ab, antibody.
and this TGF-β1, decreasing levels of p21 Cip1/Waf1 in response to TGF-β1 was also blocked by inducible dominant-negative TβRII (31). Therefore, before transfection with mutant Ki-ras, HD6-4 cells had intact TGF-β signaling pathways. These intact pathways mediated induction of p21Cip1/Waf1 in response to TGF-β1 and maturation of p15, integrin in response to autocrine TGF-β1.

These results demonstrate that cells expressing mutant Ki-Ras became much more sensitive to added TGF-β1. Thus, signaling did occur through the TβRII-mediated pathway. HD6-4 cells also responded to autocrine TGF-β1 by β1, integrin maturation, and this TGF-β response to autocrine TGF-β1 was also blocked by inducible dominant-negative TβRII (31). Therefore, before transfection with mutant Ki-ras, HD6-4 cells had intact TGF-β signaling pathways. These intact pathways mediated induction of p21Cip1/Waf1 in response to TGF-β1 and maturation of p15, integrin in response to autocrine TGF-β1.

These results demonstrate that cells expressing mutant Ki-Ras became much more sensitive to added TGF-β1, and their response to TGF-β1 was altered. TGF-β1 was no longer capable of up-regulating levels of p21Cip1/Waf1 in mutant Ki-Ras cells as it did in the parental HD6-4 cell line (31) and, in fact, displayed the opposite response to TGF-β1, decreasing levels of p21Cip1/Waf1. It is not yet known whether the TGF-β1 effects on p21Cip1/Waf1 are mediated at the transcriptional or post-translational level. TGF-β1 is mitogenic in osteoblasts; and in these cells, TGF-β1 stimulated degradation of the Cdk inhibitors p57, p27, and p21 by the proteasome (59). Thus, TGF-β1 may induce rapid turnover of p21Cip1/Waf1 in oncocgenic Ki-ras transfectants and, in so doing, increase their growth rate. In fact, Ki-ras transfectants grow much more quickly than control cells in athymic mice (35).

What is the basis for this switch in response to TGF-β1? The general picture has emerged that TGF-β1 has a dual role in carcinoma development: TGF-β1 inhibits the proliferation of normal cells (60, 61) and benign tumor cells (62), but enhances malignancy at later stages of tumorgenesis in cells that retain some response to TGF-β (40, 63–66). Supporting this model are studies showing that TGF-β1 inhibits benign skin tumor formation, but enhances progression of skin tumors to invasive spindle carcinomas in carcinogen-treated transgenic mice with keratinocyte-targeted expression of TGF-β1 (67). The tumor-promoting effects of TGF-β1 are more evident when tumor cells are highly aggressive. The metastatic potential of mammary adenocarcinoma cells is increased in vitro treatment with TGF-β1 before injection into syngeneic rats (68). Highly aggressive and metastatic U9 colon cancer cells use autocrine TGF-β1 to mediate their growth and invasion (40). Decreasing TGF-β1 protein levels in the metastatic U9 colon cancer cell line by antisense methodology decreases both U9 cell metastasis to the liver and subcutaneous tumor formation in a nude mouse system, and the tumors that do arise regain TGF-β1 expression (69). Also, levels of TGF-β1 protein within colon cancers and in serum are enhanced in patients whose disease progresses (70–73). Many other types of carcinomas, including pancreatic cancer, gastric cancer, endometrial cancer, breast cancer, gliomas, and osteosarcoma, also respond to TGF-β1 with disease progression (2).

The mechanism of this switch in response to TGF-β1 is unknown, but many include alterations in Ras. ras genes are mutated in ~30% of all cancers and about half of all colon cancers. In some cases, residual TGF-β signaling pathways may remain functional after some TGF-β signaling pathways are blocked by mutations in TβRII or SMAD proteins (74–76). Such residual pathways may mediate epithelial-to-mesenchymal transition, a critical process in many cancers (77–79). In addition, the TGF-β1 signaling pathway is involved in fibroblast growth factor (FGF)-mediated transformation of MCF10A breast epithelial cells (80). TGF-β1 activates the FGF-2 promoter in these cells, and the TGF-β1-induced FGF-2 is required for the transformation process (81, 82). The TGF-β1-FGF-2 interaction is thought to contribute to the development of fibroblasts in the breast, which is a major component of breast cancer (83). Therefore, it is clear that the role of TGF-β1 in breast cancer development is complex and multifaceted, and understanding its mechanisms of action is essential for developing effective therapeutic strategies.
transformation (77). In ras-transformed cells, the TGF-β1 signaling cascade, which leads to growth inhibition, is blocked by MAPK phosphorylation of signal-transducing SMAD proteins (28). However, the ras-transformed cells retain TGF-β response, demonstrating that these signals are relayed either by residual SMAD activity or by a SMAD-independent pathway. In ras-transformed IEC cells, TGF-β1 growth resistance is lost, but the cells retain some TGF-β1 signaling systems, as the cells remain responsive to TGF-β stimulation of fibronectin synthesis (78). Likewise, in ras-transformed mammary epithelial cells, TGF-β1 growth inhibition is lost, but is replaced by the capacity to undergo epithelial-to-mesenchymal transdifferentiation with increased cell invasiveness (29).

Autocrine TGF-β1 (Fig. 4) may mediate an epithelial-to-mesenchymal transdifferentiation in the ras-transformed HD-64 colon carcinoma cells used in this study. ras transformation of colon carcinoma cells disrupts their basolateral polarity and decreases their lateral adherence by down-regulation of N-cadherin (36). The cells become rounded and less differentiated, are less adherent to laminin and collagen I because of blocked maturation of integrin β1, form multicellular aggregates, and grow much more rapidly in athymic mice (35). TGF-β1 initiates several parallel signaling pathways with cellular responses determined by the set of transcription factors that are activated by separate and interacting TGF-β pathways (79). After SMAD signaling is inhibited by mutant Ki-Ras, the TGF-β1 pathways left functioning may interact with pathways activated by mutant Ki-Ras to increase tumorigenicity. Such oncogenic ras-activated pathways include the post-translational modification of TβRIII described in this study, the post-translational modification of β1 integrin (35), increase in growth rate through constitutive activation of MAPKs (35), and decreases in intercellular adhesion through up-regulation of carcinomaembryonic antigen (36).

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