A Role for *K-ras* in Conferring Resistance to the MEK Inhibitor, CI-1040

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Abstract

PD184352/CI-1040 is a potent and selective MEK1/2 inhibitor that represents the first MEK-targeted agent to enter clinical trials. Here, we report the development and molecular characterization of CI-1040 resistance in the murine colon 26 (C26) carcinoma cell line. The growth rate of the resistant line (C26/CI-1040^r) in the presence of 2 μM CI-1040 is comparable to that of parental C26 cells in the absence of CI-1040. C26/CI-1040^r cells are approximately 100-fold more resistant than the parental line to CI-1040 inhibition in soft agar and are less sensitive to the induction of apoptosis that normally occurs in response to CI-1040 treatment. *K-ras* expression is significantly elevated in C26/CI-1040^r cells. We confirmed a causative role for *K-ras* in conferring resistance to CI-1040 by transfecting *K-ras* into parental C26 cells, whereupon an elevation in the levels of phosphorylated ERK1/2 was observed in addition to resistance to CI-1040. Furthermore, an *in vivo*–derived MEK inhibitor–resistant line also shows increased *K-ras* expression. Our data suggest that increasing activated *K-ras* expression represents one potential mechanism by which tumor cells that initially are responsive to blockade of the MAP kinase pathway can overcome their sensitivity to MEK inhibition.

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Introduction

Ras is one of the most frequently mutated oncogenes in human cancers. Approximately 30% of all human cancers contain an activating *Ras* mutation [1]. The incidence of *K-ras* mutations is particularly high in pancreatic and colon cancers (90% and 44%, respectively) [1,2]. Active *Ras* directly binds to and regulates the function of numerous downstream targets, including Raf kinase, phosphotidylinositol 3-kinase (PI3K), RalGDS, MEKK1, and others. Raf is the best-characterized downstream effector of *Ras* and plays a critical role in *Ras* biology [3]. How *Ras* activates Raf is not completely understood. However, it is believed that *Ras* binding results in membrane localization of Raf and thus leads to subsequent phosphorylation and activation of Raf [4]. Active Raf directly phosphorylates and activates the MAP kinase kinase referred to as MEK. MEK phosphorylates ERK on both threonine and tyrosine residues in the activation loop, resulting in an approximate 3000-fold activation of ERK. The Raf–MEK–ERK cascade is a signaling paradigm for many MAP kinase cascades, which regulate a wide array of cellular activities in diverse species ranging from yeasts to humans [5,6].

The Raf–MEK–ERK kinase cascade plays an essential role in cell proliferation. Inhibition of this pathway can block oncogenic transformation by *Ras* [5,6]. The importance of this pathway in human carcinogenesis is further supported by the recent observation that *B-Raf* is a human oncogene [7]. Activating mutations of *B-Raf* have been found in approximately 66% of human melanomas [7]. Given the central function of the Raf–MEK–ERK pathway in cell proliferation, extensive efforts have been devoted to developing inhibitors of this pathway in the hope of developing improved molecular-targeted anticancer therapies [1,8,9]. We previously reported the identification and evaluation of a potent and selective MEK inhibitor, PD184352 (CI-1040) [10]. This compound is orally active and has been shown to suppress ERK phosphorylation *in vivo*, thereby resulting in broad-spectrum activity against a diverse panel of human and murine tumor xenografts. Interestingly, CI-1040 did not exhibit any overt signs of clinical toxicity [10]. CI-1040 was advanced into clinical testing in cancer patients and represents the first MEK inhibitor to enter clinical development [11–13]. In this report, we have isolated a CI-1040–resistant clone (C26/CI-1040^r) from the mouse colon carcinoma cell line C26, which is known to contain a *K-rasV12* mutation. Resistance was obtained by culturing cells in the presence of gradually increasing concentrations of CI-1040 over a 6-month time period. The growth rate of C26/CI-1040^r in the presence of 2 μM CI-1040 is similar to parental C26 cells grown in its absence. C26/CI-1040^r cells are resistant to cell cycle arrest and apoptosis in response to CI-1040 treatment. RNA expression profiling indicates that the resistant cells have

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a high level of *K-rasV12* expression. Furthermore, a CI-1040–resistant line was also derived from C26 tumors treated *in vivo* with a CI-1040 analog (PD0325901), and these resistant cells similarly display an elevation in *K-rasV12* expression. Consequently, studies were carried out to overexpress *K-rasV12* in C26 parental cells, whereupon resistance to CI-1040 was conferred. Our data suggest that elevated expression of *K-ras* is at least partially responsible for the resistance of murine C26 colon carcinoma cells to the MEK inhibitor CI-1040 reported here.

Materials and Methods

Cell Culture

The C26 mouse colon carcinoma cell line was cultured in DMEM/F12 medium supplemented with 10% FBS and 20 μg/ml gentamicin. C26/CI-1040^r cells were grown in the same growth medium as parental C26 cells but were continuously maintained in the presence of 2 μM CI-1040. All cells were incubated at 37°C with 5% CO_2.

Creating the Resistant C26 Cell Line

Exponentially growing C26 cells were initially exposed to 0.1 μM CI-1040. The concentration of CI-1040 was gradually increased to a final concentration of 2 μM over a 6-month time period. Cells were then serially diluted in a 96-well plate until a single colony isolate could be obtained. Selective pressure for CI-1040 resistance was maintained by continuous exposure of this isolate (referred to as C26/CI-1040^r) to 2 μM CI-1040.

Soft Agar Assays

Cells were plated in 2× DMEM-F12 growth medium supplemented with 20% fetal bovine serum at a density of 2×10^4 cells/well in six-well plastic dishes. A two-layer agar system was used, in which the final concentrations of Bacto-Agar (Difco Laboratories, Detroit, MI) were 0.6% and 0.3% in the bottom and top layers, respectively. After incubation of the samples for 7 days, 1 ml of 1 mg/ml *p*-iodonitrotetrazolium violet (Sigma, St. Louis, MO) was added to each well for an additional 24 hours to visualize the colonies. Colonies containing more than 50 cells were quantitated by phase contrast microcopy (Nion, Meville, NY) using the public domain NIH program developed at the US National Institutes of Health (available at http://rsb.info.nih.gov/nih-image/).

[^{14}C]Thymidine Incorporation

Five hundred cells were plated per well in a 96-well CytoStar plate (cat no. RPNQ 0162; Amersham, Piscataway, NJ) in 100 μl of DMEM/F12 with 10% FBS and 20 μg/ml gentamicin. On the next day, cells were fed with 100 μl of fresh medium with the indicated concentration of CI-1040 and 0.1 μCi of [^{14}C]thymidine and cultured in a 37°C incubator with 5% CO_2 for 7 days. Quantitation of counts was determined daily for 6 days after [^{14}C]thymidine addition, using a Wallac microbeta counter.

Apoptosis Assay

Roche's Cell Death Detection ELISAplus kit (cat no. 1 774 425) was used for apoptotic measurement. The 96-well plate assay detects the amount of fragmented DNA, a hallmark of apoptosis. Briefly, 5000 C26 parental or C26/CI-1040^r cells were plated per well in 96-well tissue culture plates or polyHEMA-coated plates. One day after plating, cells were treated with the indicated concentration of CI-1040. Cells were harvested for the apoptosis assay 24 hours after CI-1040 treatment according to procedures provided by the manufacturer.

Cell Cycle Analysis

C26 parental or C26/CI-1040^r cells were plated in six-well plates at 1×10^5 cells/well in DMEM/F12 with 10% FBS and 20 μg/ml gentamicin. On the following day, the cells were treated with the indicated concentration of CI-1040 for 24 hours. At the time of cell harvesting, both floating and attached cells were combined. The cells were first centrifuged at 2000 rpm for 5 minutes to remove the medium. Pellets were washed once with PBS and then resuspended in 0.5 ml of PBS with 0.1% FBS. The cells were added dropwise to 75% ice-cold ethanol. After fixing for 1 hour at 4°C, the cells were then centrifuged at 2000 rpm for 5 minutes, washed once in PBS with 0.1% FBS, and then treated with 0.5 ml of PBS with 0.2 mg/ml RNAse A and 50 μg/ml propidium iodide (PI) for 30 minutes at 37°C. The RNase A/PI–treated cells were passed through a 5-ml strainer capped tube and the cell cycle distribution was determined using the BD LSR as described previously [14]. The cell cycle distribution was analyzed with ModFit LT (AMPL Software Pty Ltd., Turramurra, NSW, Australia) and the pre-G1 population was analyzed with CellQuest (BD Sciences, San Jose, CA).

Immunoblotting, Immunoprecipitation, and Kinase Assays

For immunoblots, cells were plated in 12-well or 6-well plates as described above. One day after plating, cells were treated with the indicated concentration of CI-1040 or DMSO for 1 hour. The cells were quickly washed once with PBS containing 0.1 μM Na_3VO_4 and lysed in 200 μl of lysis buffer (50 mM glycerol phosphatate, 10 mM Hepes, pH 7.4, 1% Triton X-100, 70 mM NaCl, 1 mM sodium vanadate, and 40 μl of Roche's complete proteinase cocktail per milliliter) per well for 12-well plates or 500 μl/well for six-well plates. After transferring the lysate to a 1.5-ml eppendorf tube, incubation at 4°C occurred for an additional 15 minutes. The cells were then centrifuged at 10,000 rpm for 10 minutes to collect the supernatant. Immunoblots were performed as described previously [14] with 1% BSA and 1% ovalbumin in 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween-20. For *Ras* immunoprecipitation, equal amounts of each cell lysate were incubated with 40 μl of agarose-conjugated pan-*Ras* antibody (sc-35AC; Santa Cruz Biotechnology, Santa Cruz, CA) in a final volume of 0.5 ml for 2 hours at 4°C with rotation. The immunoprecipitated samples were then washed five times with cell lysis buffer and each sample was resuspended in 30 μl of SDS sample buffer. The immunoprecipitated samples were resolved on 4% to 20% Novex

SDS gradient gel and blotted with *K-ras*–specific antibody (sc-30; Santa Cruz Biotechnology). For MEK1/2 immunoprecipitation kinase assay, equal amounts of C26 parental or resistant cell lysates were incubated with 20 μl of anti-MEK1 antibody (sc-219; Santa Cruz Biotechnology) or 20 μl of agarose-conjugated anti-MEK2 antibody (sc-524AC; Santa Cruz Biotechnology). After a 2-hour incubation at 4°C, 40 μl of protein A/G plus agarose was added to the MEK1-immunoprecipitated cell lysate and incubated for another hour. The immunoprecipitates were then washed five times with cell lysis buffer and twice with kinase buffer (25 mM Hepes, pH 7.5, 5 mM MgAc2). Each immunoprecipitated sample was resuspended in kinase buffer with 250 μM cold ATP, 2 μCi of [r-^{32}P] ATP (the indicated concentration of CI-1040), and 0.1 μM GST-ERK1K71R for 30 minutes at room temperature. The reaction samples were then resolved on 8% Novex SDS gel and subjected to X-ray radiography exposure.

Ras Activation Assay

Upstate's *Ras* Activation kit (cat no. 17-218) was used for *Ras*-GTP measurements. Briefly, agarose-conjugated Raf-1 RBD (*Ras* binding domain, or RBD) was employed to specifically bind and precipitate the GTP bond form of *Ras* from cell lysates. The precipitated *Ras*-GTP was then resolved on an SDS gel and detected with anti-*Ras* antibody. The cells were first lysed in lysis buffer as described above and 500 μg of each cell lysate in 100 μl of lysis buffer was incubated with 400 μl of MLB buffer provided in the kit. The remaining steps were carried out according to the procedure recommended by the manufacturer.

Selection of K-rasV12 Stable Clones

C26 parental cells were transfected with pZip *K-rasV12* with lipofectamine 2000 according to the protocol suggested by the manufacturer (cat no. 11668-027; Invitrogen, Carlsbad, CA). Specifically, 4 μg of pZip *K-rasV12* in 250 μl of Opti-MEM was mixed with 10 μl of Lipofectamine 2000 in 250 μl of Opti-MEM. After a 20-minute incubation, the DNA/Lipofectamine 2000 mixture was added to 90% confluent C26 parental cells. The transfection was stopped 24 hours later by feeding cells with fresh medium. The cells were then replated 1 day after transfection as 10-, 100-, and 1000-fold dilutions in 150-mm cell culture dishes with 750 μg/ml G418 in the presence or absence of 2 μM CI-1040. The cells were fed with fresh medium with G418 twice during a 17-day period for colony formation. Individual clones were picked up with cloning cylinders and grown in the same manner as described previously. CI29 refers to the *Ras*-transfected colonies selected in the presence of 2 μM CI-1040, and 33 designates the colonies selected in the absence of CI-1040.

Cell Pictures and Cell Counting

A total of 50,000 cells/well was plated in a six-well plate. On the next day, the plated cells were treated with DMSO or 2 μM CI-1040 for 2 days. Pictures were taken 2 days after compound treatment. Cell counts were determined on a Coulter Counter (Beckman Coulter, Inc., Fullerton, CA) on days 1, 2, and 3 after cell plating.

RNA Preparation

Approximately 2 × 10^6 C26 parental and resistant cells were seeded into T150 flasks, respectively. Once cells reached approximately 80% confluence, they were treated with either DMSO or 0.3 μM CI-1040 for 1 or 24 hours. The total RNA was isolated from both the parent and resistant lines by first washing the cells in PBS, and then adding 3 ml of a reagent consisting of 25 mM sodium citrate, 0.5% Sarkosyl, 4 M guanidium thiocyanate, and 0.7% 2-mercaptoethanol to solubilize the cell monolayer. Solutions were transferred to 14-ml snap-cap tubes and another 3 ml of the above reagent was added to the flask for a final rinse. The solution in each 14-ml tube was then split into two tubes and 3 ml of phenol and 600 μl of chloroform IAA were added and mixed vigorously, and the tube was placed on ice for 15 minutes. All the tubes were centrifuged at 8000 rpm for 20 minutes at 4°C. The aqueous phase was transferred to a second 14-ml tube, 3 ml of chloroform IAA was added, and the tubes were mixed vigorously and then centrifuged at 8000 rpm for 10 minutes at 4°C. Again, the aqueous phase was transferred to another clean tube, an equal volume of isopropyl alcohol (~3 ml) was added, and tubes were mixed and placed at −20°C overnight. Tubes were removed from −20°C and centrifuged at 8000 rpm for 10 minutes at 4°C. Immediately following the centrifugation, the liquid was aspirated and the pellet was dissolved in 300 μl of the abovementioned reagent. The RNA was transferred to an autoclaved 1.5-ml centrifuge tube, and the 14-ml tube was rewashed with another 300 μl of the reagent mentioned above and then combined with the first wash. An equal volume of isopropyl alcohol was added to the RNA; the tubes were mixed and placed at −20°C for 1 hour. Tubes were then centrifuged at 2000*g* for 3 minutes at 4°C. Following centrifugation, the liquid was aspirated and 500 μl of 70% ethanol was added. Tubes were then vortexed and centrifuged at 2000*g* for 3 minutes at 4°C. Pellets were dissolved in 75 μl of DEPC–water.

Affymetrix Gene Chip Profiling

cRNA was synthesized from the total RNA and then subjected to chip hybridization as detailed previously [15]. The Affymetrix (Santa Carla, CA) mouse genome U74Av2 array was used for RNA expression profiling. Briefly, double-stranded cDNA was prepared from the RNA template using a modified oligo-dT primer containing a 5′ T7 RNA polymerase promoter sequence and the Superscript Choice System for cDNA Synthesis (Life Technologies, Inc., Gaithersburg, MD). Following phenol–chloroform extraction and ethanol precipitation, one-half of the cDNA reaction (0.5–1.0 μg) was used as the template in an *in vitro* transcription reaction containing T7 RNA polymerase;, a mixture of unlabeled ATP, CTP, GTP, and UTP; and biotin-11-CTP and biotin-16-UTP (BioArray High Yield Kit; ENZO, Inc., Farmingdale, NY). The resulting biotinylated cRNA "target" was purified on an affinity resin (RNeasy; Qiagen, Valencia, CA) and quantified using the convention that 1 OD_{260} corresponds to

40 μg/ml RNA. Fifteen micrograms of biotinylated cRNA was randomly fragmented to an average size of 50 nucleotides by incubating at 94°C for 35 minutes in 40 mM Tris–acetate, pH 8.1, 100 mM potassium acetate, and 30 mM magnesium acetate. The fragmented cRNA was hybridized in a solution containing 100 mM MES, pH 6.6, 1 M [Na^+], 20 mM EDTA, 0.01% Tween 20, 50 pM of control oligonucleotide B2 (Affymetrix), 0.1 mg/ml of sonicated herring sperm DNA, and 0.5 mg/ml BSA for 16 hours at 45°C on the MG-U74Av2 genechips. Scanned output files were analyzed using Affymetrix's software Microarray Suite 5.0.

Data Extraction and Comparison Analysis

All data sets from each gene chip were normalized to a target intensity of 600. The new statistical algorithms were used to generate signal and detection in the Microarray Suite 5.0. The fold change was calculated between parent and resistant samples using the average of the signals between the duplicates. A two-fold change cutoff was used to filter out genes with insignificant changes. The list of common genes with changes of at least two-fold between resistant and parental cell lines treated in the presence or absence of CI-1040 at 1- and 24-hour time point was generated.

Development of In Vivo Resistance to MEK Inhibition

During the course of an extended treatment regimen (28-day treatment) of C26-bearing mice with PD0325901, a structurally related analog of CI-1040, tumors initially regressed to below the limit of palpation, but then regrew while still under treatment. The tumor from one mouse that

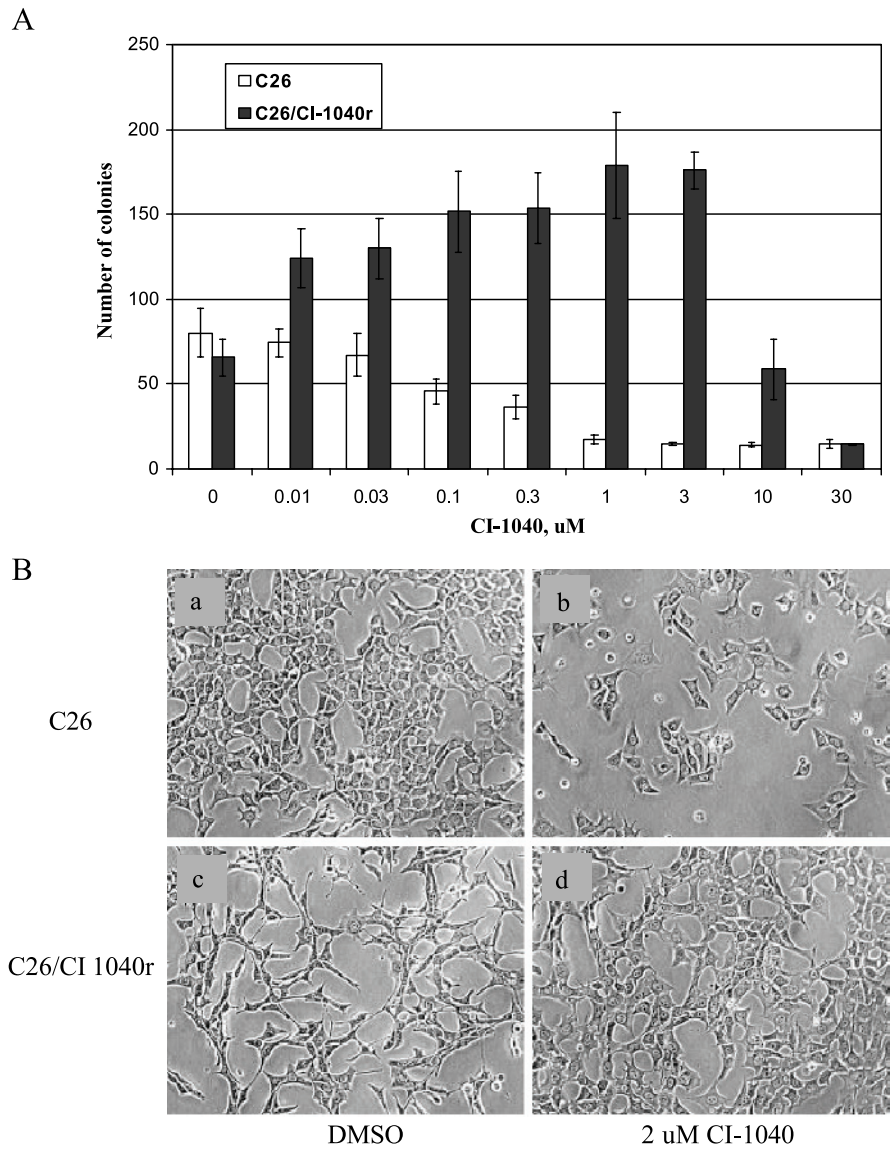

Figure 1. *C26/CI-1040^r cells are resistant to the growth-inhibitory effects of CI-1040. (A) Dose response of CI-1040 in C26/CI-1040^r and its parental cell line C26 in a soft agar growth assay. (B) Cell morphology and cell density of C26 and C26/CI-1040^r in cell culture. Cells were seeded in six-well plates at 50,000 cells/well. One day after cell plating, the cells were treated with DMSO or 2 μM CI-1040 for 2 days. (C) Effect of CI-1040 on cell monolayer growth. After pictures were taken as shown in (B), both floating and attached cells were combined and then counted with a Coulter Counter. (D–F) Effect of CI-1040 on C26 parental cell growth was measured by [^{14}C]thymidine incorporation.*

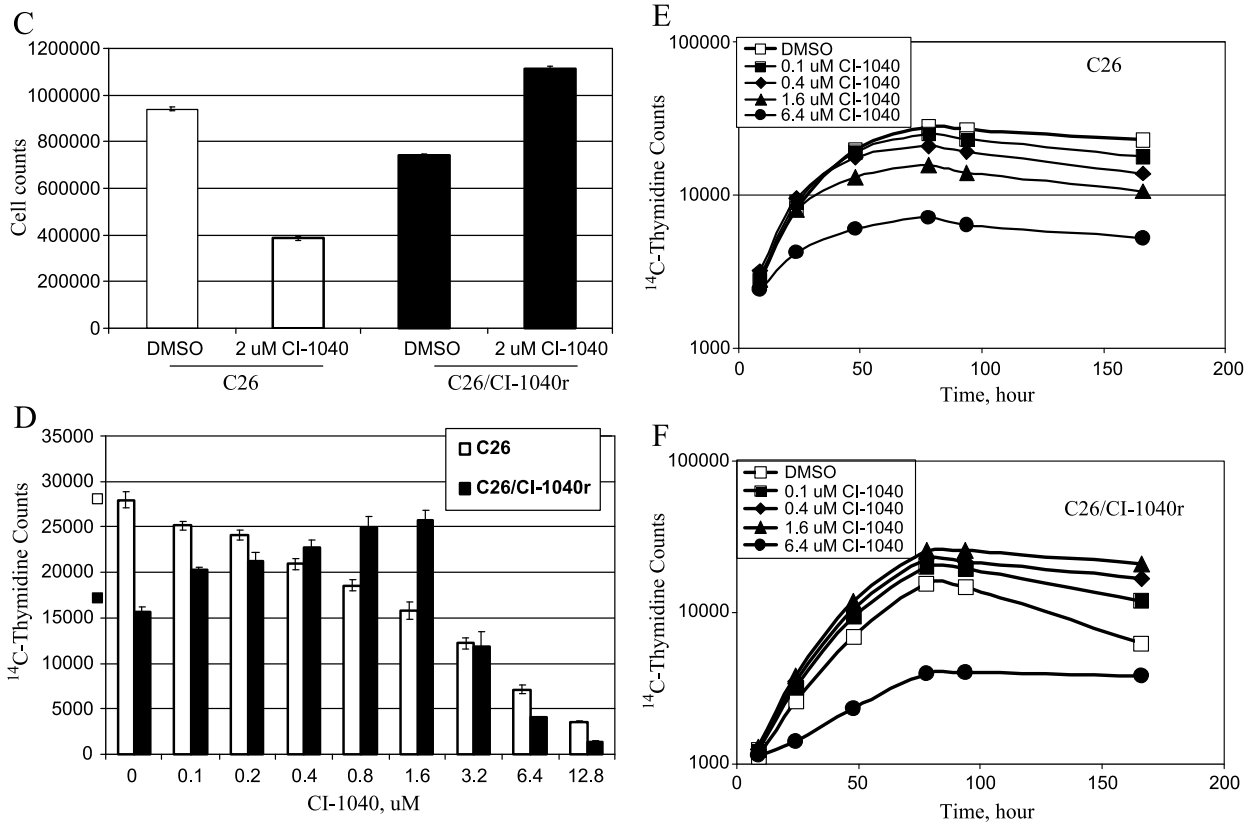

Figure 1. *(Continued)*

had received the highest dose (25 mg/kg, which is equivalent to the maximum tolerated dose of PD0325901) was transplanted into recipient mice. Daily dosing with PD0325901 at 25 mg/kg was initiated 10 days after tumor implantation. Several of the most drug-resistant tumors were excised and transplanted again. This selection process was repeated, becoming more stringent (beginning dosing 8 days, and then 4 days after tumor implantation) for four more passages in BALB/c mice. This tumor line was serially transplanted eight more times without drug pressure before being used in the study reported here. This line exhibited a tumor doubling time comparable to that of the parental C26 line.

Animal Efficacy Studies

Tumor fragments (approximately 3 mm^3 in size) of either parental C26 or C26/PD0325901^r were implanted subcutaneously into the right subaxillary region of young adult, female, BALB/c mice. Treatment administered by oral gavage was initiated when tumors reached a mass of approximately 200 mg. CI-1040 was prepared at 300 mg/kg in a vehicle of 0.5% hydroxypropylmethylcellulose, plus 0.2% Tween 80 in water. Tumor size was evaluated periodically by caliper measurements, generally three times per week. A complete response is defined as a tumor that decreased in mass to below the limit of palpation (62 mg) during the study. A partial response represents a tumor that decreased by a least 50% of its initial mass during the study. *T* and *C* are defined as the times required for treated and control tumors, respectively, to reach 750 mg.

Results

Isolation and Characterization of a CI-1040–Resistant Cell Line

We have previously reported that the MEK inhibitor CI-1040 inhibited both human and mouse colon carcinoma growth. Growth of C26 tumors *in vivo* was inhibited up to 79% with no signs of toxicity, and tumor growth inhibition correlated with a reduction in the levels of phosphorylated ERK (pERK) in excised tumors [10]. CI-1040 inhibited anchorage-independent growth of C26 cells with an IC_{50} of 0.15 μM, similar to the degree of MEK inhibition observed when these cells were treated with CI-1040. To isolate drug-resistant cells, C26 parent cells were cultured in the presence of gradually increasing concentrations of CI-1040 starting at 0.1 μM. Six months after initiation of these studies, a single clone, C26/CI-1040^r, was isolated from a cell population growing at the parental growth rate in the presence of 2 μM CI-1040.

Soft agar assays confirmed the resistance of C26/CI-1040^r to the MEK inhibitor, CI-1040 (Figure 1*A*). The anchorage-independent growth of parental C26 cells was impaired

in a dose-dependent manner. At a concentration of 1 μM, CI-1040 inhibited colony formation of parental C26 cells by >80% (Figure 1*A*). In contrast, no inhibition of colony formation was observed when resistant C26/CI-1040^r cells were plated at concentrations <3 μM (Figure 1*A*). Surprisingly, we consistently observed a significant increase in the number of CI-1040–resistant colonies (approximately 100% increase) when the inhibitor concentration was lower than 3 μM. A much higher concentration of the inhibitor (30 μM) was required to inhibit the growth of the C26/CI-1040^r line.

Morphologic assessment also revealed that C26/CI-1040^r cells show a more transformed phenotype than parental cells (Figure 1*B*). C26/CI-1040^r cells are more reflective, elongated, and less attached—properties that are also characteristic of *Ras*-transformed cells. Treatment with CI-1040 resulted in morphologic reversion of the resistant cells to the parental phenotype (compare panels a and d in Figure 1*B*). Quantification of cell counts showed that 2 μM CI-1040 inhibited the cell growth of parental cells by 60%, whereas the same concentration of inhibitor stimulated the growth of resistant cells by 45% relative to DMSO controls (Figure 1*C*), consistent with observations obtained in the anchorage-independent growth assays.

To determine the proliferation rate, DNA synthesis was measured by [^{14}C]thymidine incorporation (Figure 1, *D*–*F*). CI-1040 inhibited the DNA synthesis of parental cells in a dose-dependent manner, even at the lowest concentration (0.1 μM) tested (Figure 1*D*). In contrast, CI-1040 slightly enhanced DNA synthesis in the resistant line when the inhibitor concentration was low. Higher concentrations of CI-1040 eventually inhibited the DNA synthesis of C26/CI-1040^r cells. These results suggest that the proliferation of C26/CI-1040^r cells is less sensitive to inhibition by CI-1040 than parental cells. In fact, C26/CI-1040^r cells grow more favorably when low concentrations of CI-1040 are present in the growth medium.

C26/CI-1040^r Cells Are Resistant to Apoptosis Induced by the MEK Inhibitor, CI-1040

We also investigated the effect of CI-1040 on apoptosis. Cell death was measured in both the parental and resistant cell lines grown in the presence of varying concentrations of CI-1040. In parental cells, significant apoptosis was observed at CI-1040 concentrations exceeding 0.4 μM, with maximum apoptosis observed at 1.6 μM (Figure 2*A*). In comparison, low concentrations of CI-1040 did not cause a significant increase of apoptosis in C26/CI-1040^r cells. The concentration of CI-1040 required to elicit an equivalent degree of apoptosis in the resistant cell line relative to the parental line was approximately 10-fold higher (Figure 2*A*). We followed up on this finding by testing for sensitivity to apoptosis induction by CI-1040 under anchorage-independent growth conditions. Due to the difficulty in isolating cells from soft agar, C26 cells were grown on polyHEMA-coated plates to prevent cell adherence. As shown in Figure 2*B*, C26 cells were much more sensitive to CI-1040–induced apoptosis than C26/CI-1040^r cells. In addition, we found that non-adhering conditions enhanced the sensitivity of C26 cells to CI-1040–induced apoptosis (compare Figure 2, *A* and *B*). The difference was particularly evident at low concentrations of the inhibitor (i.e., 0.1 μM).

The effect of CI-1040 on cell proliferation and apoptosis was further determined by flow cytometry analysis. CI-1040 treatment for 24 hours caused a G1 cell cycle arrest in C26 cells concordant with a decrease of S and G2/M phase cells (Figure 3). Similarly, the apoptotic cell population (pre-G1 cells) was increased in cells treated with CI-1040 at concentrations ≥0.5 μM. In contrast, C26/CI-1040^r cells were much less sensitive to CI-1040 with respect to both growth arrest and apoptosis under the same treatment conditions (Figure 3).

C26/CI-1040^r Cells Exhibit Elevated ERK Pathway Activation But Are Still Sensitive to Inhibition by CI-1040

To determine the mechanism of CI-1040 resistance, we examined the activation status of ERK. Western blotting with an antiphosphoERK antibody demonstrated that the resistant cells have a much higher level of active ERK than the parental cells (Figure 4, *A* and *C*). Treatment with CI-1040 inhibited phosphoERK expression in both the parental and resistant cells. In fact, both the parental and resistant cells showed a similar dose response to CI-1040

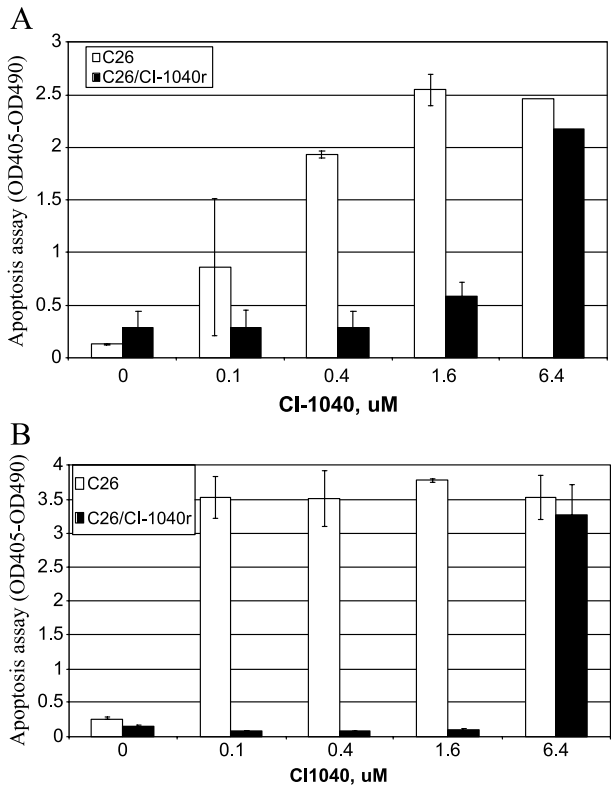

Figure 2. *The C26/CI-1040^r cell line is less sensitive to CI-1040–induced apoptosis. Cells were plated on regular or polyHEMA-coated 96-well tissue culture plates. One day after cell plating, the cells were treated with the indicated concentration of CI-1040. The cells were then harvested for the apoptosis assay 24 hours after compound treatment. (A) Apoptosis assay for C26 and C26/CI-1040^r cells grown on regular tissue culture plates. (B) Apoptosis assay for C26 and C26/CI-1040^r cells grown on polyHEMA-coated 96-well plates.*

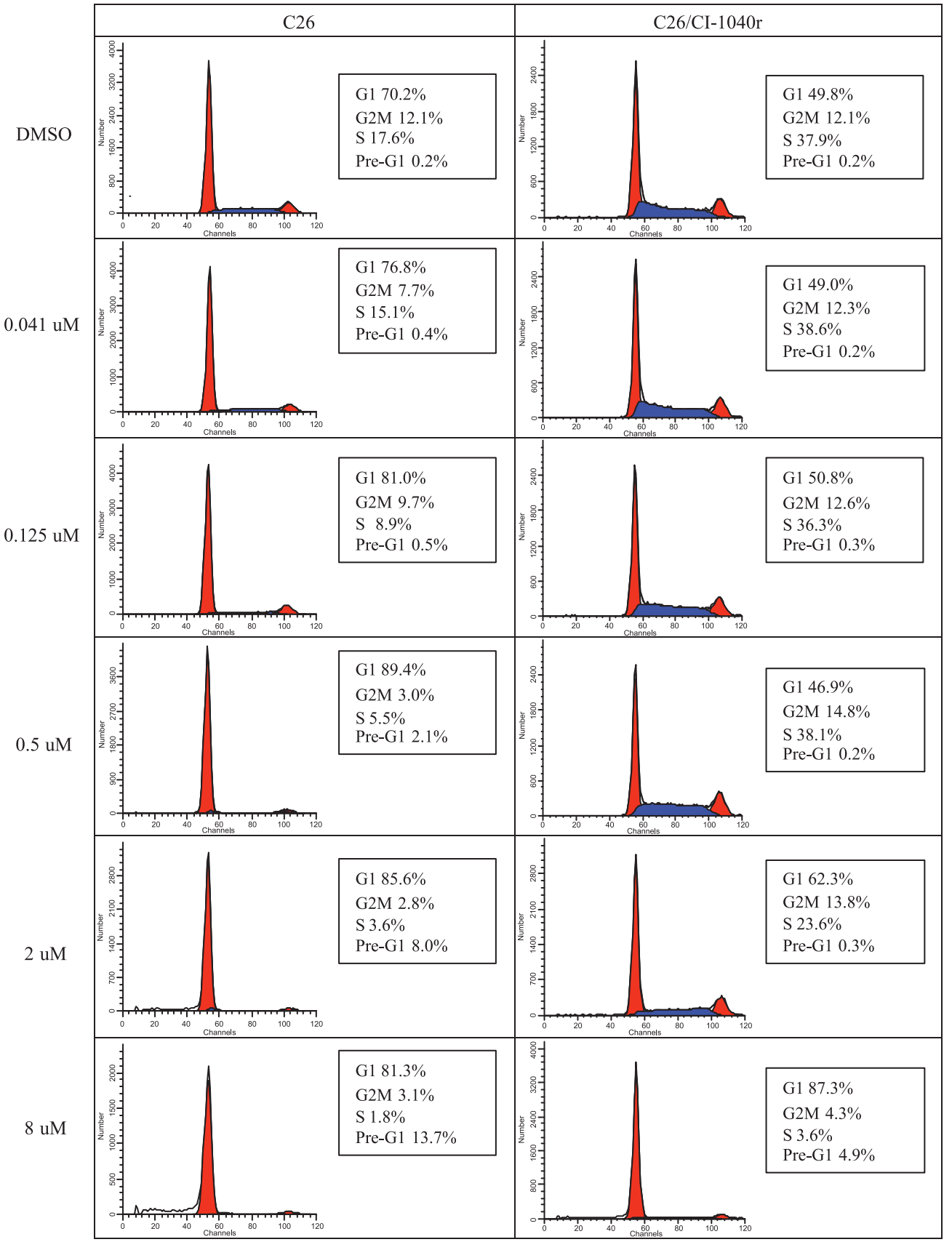

Figure 3. *C26/CI-1040^r is less sensitive to CI-1040–induced cell cycle arrest than the parental C26 cell line. Cells were treated for 24 hours with CI-1040 and then cell cycle analysis was run on the BD LSR flowcytometer. A total of 25,000 events was collected per analysis.*

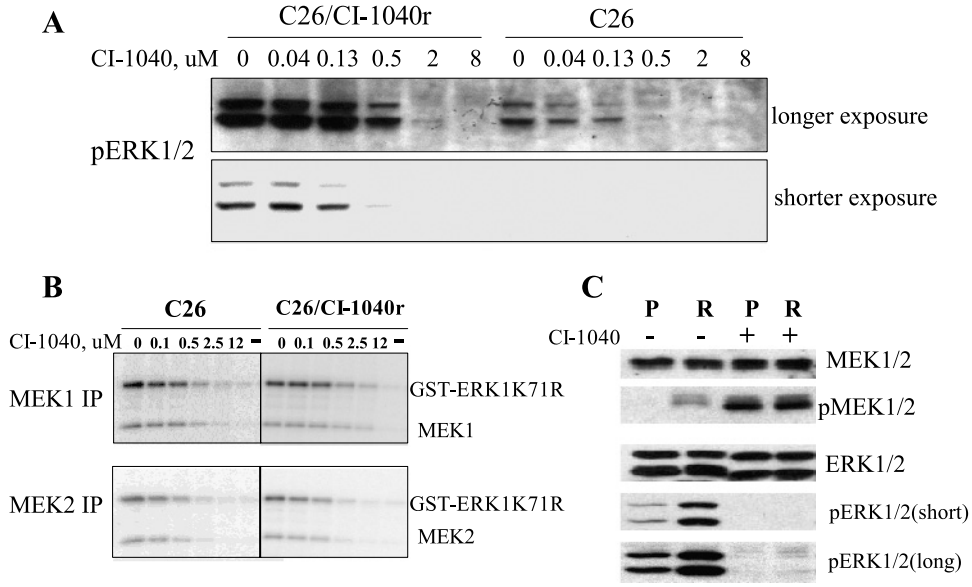

Figure 4. *The basal level of ERK1/2 phosphorylation increases in C26/CI-1040^r cells, but the sensitivity of MEK1/2 to CI-1040 inhibition is not significantly altered. (A) Effect of CI-1040 on ERK phosphorylation in C26 and C26/CI-1040^r cells. Cells were treated with the indicated concentration of CI-1040 for 1 hour and then harvested for Western analysis. Two different exposures of the same immunoblots are shown. (B) Effect of CI-1040 on MEK1 and MEK2 in C26 and C26/CI-1040^r cells. MEK1 or MEK2 from C26 or C26/CI-1040^r cells was immunoprecipitated with the corresponding antibody. The kinase activities were assayed with GST-ERK1K71R as substrate as described in the Materials and Methods section. The exposure time was adjusted to give a similar basal level of intensity (without compound treatment). (C) Effect of CI-1040 treatment on ERK and MEK phosphorylation. C26 (P) and C26/CI-1040^r (R) cells were treated with DMSO (−) or 2 μM CI-1040 (+) as described in the Materials and Methods section.*

(see Figure 4*A* for comparison of the longer exposure of C26 parent with shorter exposure of C26/CI-1040^r). These results suggest that the alterations in the resistant cells likely occur at a step upstream of MEK. To further exclude the possibility that the resistance is due to a MEK mutation in C26/CI-1040^r cells, MEK1 and MEK2 proteins were independently immunoprecipitated with the corresponding antibody, and the dose response of the immunoprecipitated kinases to CI-1040 inhibition was evaluated with purified kinase inactive ERK1, GST-ERK1K71R, as a substrate. CI-1040 resulted in a similar dose-dependent inhibition of both MEK1 and MEK2 *in vitro* regardless of whether MEK was isolated from the parental or resistant cells (Figure 4*B*).

MEK activation status is determined by phosphorylation in its activation loop and this can be indirectly determined using a phospho-specific MEK antibody. As shown in Figure 4*C*, resistant cells have higher MEK activity than parental cells. These results suggest that the mutations/alterations in the resistant cells likely occur at a step before MEK. CI-1040 treatment did not inhibit MEK phosphorylation, supporting the concept that the MEK inhibitor does not block signal transduction upstream of MEK. Interestingly, treatment with the MEK inhibitor increased phosphorylation of MEK (Figure 4*C*). This observation could be explained by the possibility that the *Ras*–ERK pathway is subjected to feedback regulation [16]. By blocking ERK activation and signaling, treatment with CI-1040 could block any feedback regulation resulting from ERK activation. Such a lack of feedback inhibition may contribute to the increased phosphorylation of MEK shown in Figure 4*C*.

K-ras Is Elevated in CI-1040–Resistant Cells

In an attempt to identify gene(s) that potentially contribute to the CI-1040–resistant phenotype, we performed RNA expression profiling analysis. C26 and C26/CI-1040^r cells were treated with 0.3 μM CI-1040 or DMSO for 1 and 24 hours, respectively. The cells were harvested and total RNA was prepared. As expected, treatment with CI-1040 for 1 hour did not cause a significant effect on gene expression in either C26 parental or resistant cells (data not shown). Interestingly, over 2000 genes showed a change in expression of two-fold or greater in C26 cells 24 hours after treatment with 0.3 μM CI-1040. In contrast, only 10 genes were found to exhibit a two-fold change in C26/CI-1040^r cells under the same conditions (data not shown). A baseline comparison of C26 parental *versus* C26/CI-1040^r cells grown under the same treatment condition yielded a list of 25 genes that changed two-fold (Table 1). Twenty-three of these 25 genes are known. The expression level of *K-ras* (the C26 parental colon cancer cell line contains an active *K-rasV12* mutation; unpublished observation) in the resistant cell line is approximately 2.5-fold higher than that observed in the parental cell line (Figure 5*A*). To further confirm the differential expression at the protein level, *K-ras* was immunoprecipitated and detected by an anti–*K-ras* Western blot. Our results confirmed that *K-ras* protein levels were elevated in the resistant cells (Figure 5*B*). To determine the amount of *K-ras* in the GTP-bound or active form, a *Ras* activation assay was performed. The principle of this assay is that the activated form of *Ras* can associate with the RBD of Raf-1. C26 parental and resistant cell lysates were normalized for protein and

Table 1. *K-ras* and EGF-Like Growth Factors Are Upregulated in C26/CI-1040^r Cells.

Gene Name	DMSO Control		0.3 μM CI-1040	
	1 hr	24 hr	1 hr	24 hr
K-ras	2.197	2.767	2.515	5.04
Epiregulin	5.044	3.493	7.633	11.438
Amphiregulin (schwannoma-derived growth factor)	6.89	7.156	12.973	18.572
Betacellulin	2.827	9.282	4.667	5.397
Transforming growth factor, beta-induced, 68 kDa	8.039	2.277	7.758	3.256
Inhibitor of DNA binding 3, dominant negative helix–loop–helix protein	−10.084	−3.716	−8.994	−6.368
Wingless-type MMTV integration site family, member 10A	2.492	2.216	2.687	3.649
Interferon-stimulated protein, 20 kDa	−2.39	−3.52	−2.788	−11.962
T cell–specific GTPase	−4.314	−12.942	−5.102	−22.143
Immunoglobulin heavy chain 6 (heavy chain of IgM)	−2.032	−3.125	−3.073	−2.533
Transportin-SR	2.961	2.07	2.099	5.682
Pleckstrin homology, Sec7 and coiled/coil domains, binding protein	−3.287	−3.243	−3.831	−2.75
Keratin 18	−21.004	−11.989	−28.629	−2.98
Testis-derived transcript (three LIM domains)	2.95	2.717	2.606	18.536
Parathyroid hormone–like hormone	4.149	2.663	7.965	4.149
Cellular retinoic acid–binding protein 1	−10.132	−27.598	−19.095	−103.292
High-mobility group (nonhistone chromosomal) protein isoform I–C	2.643	2.959	2.935	22.683
Amyloid beta	3.031	2.731	2.738	6.443
Osteoglycin (osteoinductive factor, mimecan)	−2.23	−3.337	−2.079	−142.462
Leucine-rich protein, B7 gene	13.253	5.892	9.417	6.874
Carboxylesterase 1 (hydrolyzes drugs containing ester or amide bonds)	−2.142	−9.127	−2.831	−25.545
Fatty acid–binding protein 4, adipocyte	−2.319	−2.208	−3.26	−3.712
Xlr-related, meiosis regulated	−2.196	−2.004	−2.531	−11.276
Gene 33/Mig-6	2.764	2.634	4.126	26.297
AA606367	2.959	2.388	2.549	3.633

Fold change (C26/CI-1040^r *vs* C26).*
*Positive and negative values indicate fold change of upregulation and downregulation of C26/CI-1040^r *vs* C26, respectively.

precipitated with Raf-1 RBD agarose. The precipitated samples were then subjected to SDS gel electrophoresis and probed with the *Ras* antibody. As shown at Figure 5*C*, overexpressed *K-ras* in C26/CI-1040^r cells was also active.

Increased K-ras Expression in an In Vivo–Derived Resistant Clone

To further investigate the mechanism of resistance to MEK inhibition, we performed parallel experiments in C26 tumor-bearing mice to isolate a resistant line. BALB/c mice implanted with C26 cells uniformly develop tumors. Treatment with PD0325901, a structurally related analog of CI-1040 and a potent MEK inhibitor, typically causes regression in this tumor model [17]. During an extended treatment period (28 days), regrowth of tumors was observed in some animals and a resistant line (C26/PD0325901^r) was isolated as described in the Materials and Methods section. As shown in Table 2, the C26/PD0325901^r line was significantly resistant to CI-1040 treatment *in vivo*. Growth of C26/PD0325901^r tumors was not inhibited in response to CI-1040 treatment. In contrast, growth of parental tumors was significantly delayed in response to the same CI-1040 treatment regimen (Table 2). Subsequent examination of the expression level of *K-ras* revealed that this protein was elevated in the *in vivo*–derived C26/PD0325901^r cells (Figure 5*D*). Consistent with previous *in vitro* findings, these observations suggest a possible role of *K-ras* in conferring MEK resistance to MEK inhibitors.

Expression of K-ras Confers C26 Resistance to CI-1040

Elevated expression of *K-ras* or EGF-like growth factors could contribute to the resistance phenotype of C26/CI-1040^r cells. It has been reported that *Ras* activation can induce the expression of betacellulin, epiregulin, and amphiregulin [18–20]. The induction of these EGF-like growth factors may contribute to an autocrine loop and stimulate cell growth in *Ras*-transformed cells. Therefore, we focused on *K-ras* because the elevated expression of EGF-like growth factors could be a consequence of *K-ras* overexpression. *K-rasV12* was transfected into the parental C26 cells and stable clones were isolated under two different conditions. Clones CI29-2, CI29-13, and CI29-16 were selected in the presence of 2 μM CI-1040 and G418, which selects for the presence of *K-ras* expression plasmids. In a separate group, clones 33-4, 33-10, and 33-11 were selected for G418 alone in the absence of CI-1040. These separately derived stable clones were tested for their ability to grow on soft agar in the presence of CI-1040. As shown in Figure 6*A*, the three CI29 clones exhibited complete resistance to 2 μM CI-1040 and the three 33 clones also displayed significant resistance to 2 μM CI-1040 when compared to C26 parental cells. Interestingly, the MEK inhibitor significantly enhanced the growth of both the CI29-13 and CI29-16 clones. These results demonstrated that *K-ras* expression may lead to CI-1040 resistance.

The effect of *K-ras* expression on ERK activation was determined. We found that ERK was activated to varying degrees among the *K-ras* expression clones. The degree of ERK activation did not linearly correlate with the degree of CI-1040 resistance. However, it is worth noting that CI29-13 has the highest ERK activity. This clone also displays a significant growth advantage in the presence of MEK inhibitor. Similarly, clones 33-4 and 33-11 both showed a moderate increase in ERK activity and less resistance to CI-1040 (Figure 6*B*). Collectively, our data provide a qualitative correlation between ERK activation by *K-ras* and resistance to MEK inhibition.

Discussion

Previous studies have shown that the MEK inhibitor CI-1040 has promising potential to inhibit cancer cell growth [10]. In

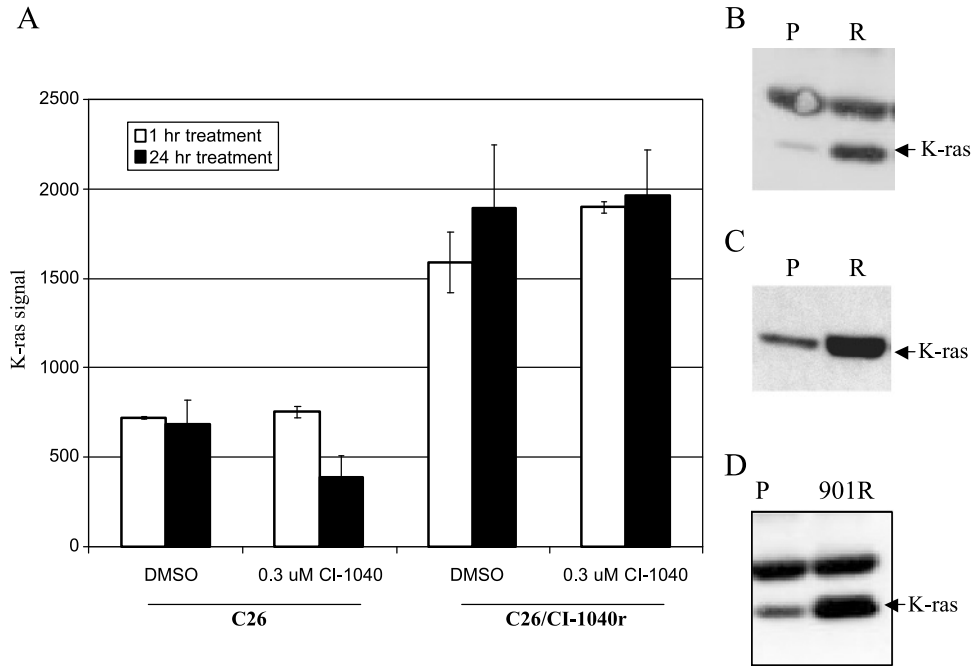

Figure 5. *K-ras is activated and overexpressed in CI-1040–resistant cells. (A) K-ras mRNA is upregulated in C26/CI-1040^r cells. K-ras signal data are derived from genechip experiments (Table 1). (B and D) K-ras protein is overexpressed in both in vitro– and in vivo–derived resistant cells. The K-ras from C26 parental or resistant cells was immunoprecipitated with a pan-ras antibody (SC-35AC) and blotted with K-ras antibody (SC-30). (C) K-ras is activated. The Ras activation assay was performed with parental and C26/CI-1040^r cell lysate using Upstate's ras activation assay kit.*

this study, we have isolated and characterized CI-1040–resistant clones derived from the C26 mouse colon carcinoma cell lines after long-term culture in the presence of increasing concentration of CI-1040. The drug resistance of C26/CI-1040^r cells is likely due to a combined effect of resistance to both growth inhibition and apoptosis in response to CI-1040 treatment. Our results further demonstrate that C26/CI-1040^r cells exhibit elevated expression of activated *K-ras*. Consistently, *K-ras* expression was also shown to increase in MEK inhibitor–resistant lines derived from *in vivo* experiments. Furthermore, overexpression of active *K-ras* in C26 parental cells indeed conferred resistance to CI-1040. Collectively, our studies demonstrate that high-level expression of active *K-ras* may provide a possible molecular mechanism for resistance to the MEK inhibitor.

A surprising and interesting observation in our investigation is that a low concentration of CI-1040 (<2 μM) significantly stimulates the growth of the resistant cells but inhibits the parental cells. The growth-stimulatory effect of CI-1040 is even more dramatic in clones that have the highest level of ERK activation (Figure 6). These results demonstrate that the very high level of ERK activity in the drug-resistant cell lines may have a growth-inhibitory effect. Partial inhibition of ERK in these cells may be beneficial for cell growth. Our data are consistent with previous observations that superactivation of *Ras* and Raf could be growth-inhibitory [21–23]. Treatment with 2 μM CI-1040 significantly inhibited ERK activation in C26/CI-1040^r cells (Figure 4*A*), whereas this concentration of MEK inhibitor did not inhibit cell growth of the resistant cells (Figure 1). These results demonstrated that ERK inhibition is not linearly correlated with the growth inhibition. Other signaling pathway(s) might contribute to C26 cell proliferation in the presence of 2 μM CI-1040.

Although ERK activity is required for cell growth, superactivation of the *Ras*–ERK pathway can also cause growth-inhibitory effects. It has been well documented that sustained high-level ERK activation in PC12 cells does not stimulate cell growth. In contrast, such sustained ERK activation stimulates differentiation of PC12 cells [24], which can be blocked by treatment with a MEK inhibitor [25]. Furthermore, a high level of activation of Raf can cause fibroblast cell growth arrest through induction of p21 [26,28]. In primary human and mouse fibroblasts, activation of the *Ras*–ERK pathway does not induce oncogenic transformation and instead induces cellular senescence [22]. Therefore, a moderate inhibition of ERK in the drug-resistant cells that

Table 2. Tumor Growth of C26/PD0325901^r Is Resistant to CI-1040.

	Complete Response*	Partial Response†	*T*–*C* (Days)‡
C26 parental	7/10	3/10	12.7
C26/PD0325901^r	0/10	0/10	–1.4

*A complete response is defined as a tumor that decreased in mass to below the limit of palpation (62 mg) during the study.
†Partial response represents a tumor that decreased by at least 50% of its original mass during the study.
‡*T*–*C* is the difference, in days, for the median-treated and control tumors to reach a fixed evaluation size of 750 mg.

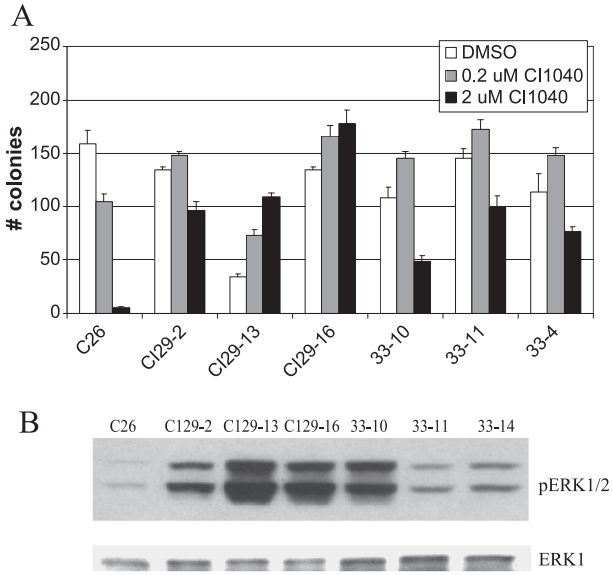

Figure 6. *Overexpression of K-ras in C26 cells confers CI-1040 resistance. CI29-2, CI29-13, and CI29-16 are single clones from C26 parental cells transfected with K-rasV12 selected in the presence of 2 μM CI-1040. 33-10, 33-11, and 33-4 are single clones from C26 parental cells transfected with K-rasV12 selected in the absence of CI-1040. (A) Soft agar assay. (B) Overexpression of K-ras increased ERK1/2 phosphorylation.*

have high level ERK activity may contribute to the growth-stimulating effect of CI-1040 in the resistant cell lines.

One characteristic of cancer cells is their capability to grow in an anchorage-independent manner. Both the clonogenic and apoptosis assays suggest that C26/CI-1040^r cells are about 100-fold more resistant to CI-1040 inhibition than parental C26 cells. In contrast, the sensitivity difference between C26 and C26/CI-1040^r cells is significantly reduced when cells are grown as monolayers.

Although it has been reported that MEK1/2 activity can protect cells from apoptosis [27,28], the main function of MEK revolves around cell growth and proliferation [5,6]. However, in this report, we clearly observed that CI-1040 treatment induces apoptosis in addition to cell cycle arrest. These observations serve to further demonstrate the functional importance of ERK in cell survival.

Many human cancers contain mutations in *Ras* and exhibit activation of the ERK pathway [2,29]. Furthermore, mutations in many other oncogenes including several growth factor receptor tyrosine kinases also result in high-level activation of the ERK pathway. The expectation that all tumors with activating *Ras* mutations would be sensitive to MEK inhibition is debatable in light of the data reported here. Clearly, resistance to MEK inhibition in C26/CI-1040^r cells is mediated in part by overexpression of *Ras*. However, it should be pointed out that exquisite sensitivity to MEK inhibition has been found for a number of human tumor xenograft models known to contain *ras* mutations, such as the MiaPaCa-2 pancreatic model. In this regard, it is noteworthy that Hamad et al. [30] reported that the mechanism of *Ras* transformation in humans might be distinctly different from that in mice. Thus, observations reported here on the molecular mechanism of resistance to MEK inhibition should be viewed within the context of the murine background of C26/CI-1040^r cells. Further studies are warranted in human tumors to obtain a better understanding of the relationship of *ras* mutations to MEK inhibition sensitivity in the context of the complex interrelationships of key signaling pathways and molecules in a given tumor. Derived commonalities from such studies could well lead to the ability to predict which patient population would derive the greatest benefit of treatment with a MEK inhibitor.

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