Role of the Mitogen-Activated Protein Kinase Signaling Pathway in the Regulation of Human Melanocytic Antigen Expression

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Abstract

Heterogeneous expression of melanocytic antigens occurs frequently in melanomas and represents a potent barrier to immunotherapy. We previously showed that coordinated losses of several melanocytic antigens are generally attributable to down-regulation of antigen gene expression rather than irreversible mutation. Treatment of melanoma cells with mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitors blocks ERK activation and increases steady-state levels of mRNAs and corresponding protein expression for the melanocytic antigens Melan-A/MART-1, gp100, and tyrosinase. Although the degree of MEK inhibitor enhancement of antigen expression varied among different cell lines irrespective of their antigen expression status, all showed detectable responses. Notably, the antigen-enhancing effects of the MEK inhibitors could not be attributed to the master melanocytic regulator MITF-M. Because MAPK pathway activation via constitutively active mutant forms of BRAF is common in melanomas, correlation between BRAF function and antigen expression was investigated. No simple correlation of endogenous BRAF mutational status and antigen levels was observed, but transient overexpression of V600E BRAF increased ERK activation and reduced Melan-A/MART-1 levels in antigen-positive cell lines. These data indicate that whereas multiple factors may regulate antigen expression in melanomas, enhancement of MAPK signaling can act as a negative influence. Blocking such signaling with MEK inhibitors accordingly augments antigen levels, thereby enhancing Melan-A/MART-1–specific cytotoxic T-cell responses to antigen-negative cells following MEK inhibition treatment. Consequently, MAPK inhibition may assist targeting of melanomas for immunotherapy. (Mol Cancer Res 2006;4(10):779–92)

Introduction

Melanocytic cells express a set of genes specific to that lineage. The products of many of these genes are localized in the melanosome, and some are directly linked to melanin synthesis (1-3). Importantly, with respect to immunotherapy, several melanocytic gene products have been directly shown to function as target antigens for CTLs (4-9). Loss of such antigen expression by target melanoma cells is thus a mechanism for escape from immune recognition (10-15). We reported previously that antigen gene expression in melanoma cell lines can be reversibly down-regulated by cytokines (16, 17). In a panel of 22 melanoma cell lines, including 11 that are nominally Melan-A/MART-1 antigen negative, all show measurable melanocytic antigen mRNAs and proteins if sensitive assays are applied (16, 17), indicating that the expression defects are most likely due to gene regulatory changes rather than from irreversible gene deletion or mutation. Moreover, a defect in a cell lineage–specific master transcription factor, such as MITF-M (1, 3, 18), could simultaneously down-regulate a set of coordinated melanocytic antigens. This type of event is suggested by the observation that antigen-negative melanomas commonly have low-level expression of multiple melanocytic genes rather than selective losses (19). Loss of multiple targets for cytotoxic T cells at the same time would confer significant survival benefit for a tumor cell.

Recently, the *BRAF* gene has been found to be frequently mutated in melanomas, particularly at a specific codon (V600E; ref. 20). Activation of BRAF kinase activity transmits a mitogen-activated protein kinase (MAPK) pathway signal through BRAF-mediated phosphorylation of its substrates, the MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) proteins. Mutations within the BRAF kinase domain associated with human tumors frequently result in constitutive BRAF kinase activation, and the V600E mutation is a potent activator (20, 21). These observations, and our own data with MEK inhibitors, prompted us to examine whether BRAF mutational status was associated with melanocytic antigen

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expression. The relationship between melanocytic gene expression and *BRAF* mutational status is further complicated by additional tumor-specific MAPK alterations, including activating mutations in NRAS (noted among melanomas that are wild type for *BRAF*). To examine this relationship directly, we transiently transfected melanoma cells with mutant or wild-type *BRAF* to modulate MAPK signaling and to allow the assessment of subsequent effects on antigen expression. Indeed, transfected mutant *BRAF* increased ERK activation and reduced antigen levels. In contrast, transfection with wild-type *BRAF* decreased phospho-ERK (pERK) levels with concomitant augmentation of antigen expression, suggesting that transient high levels of non-activated BRAF protein confer a negative MAPK signal. Although there may be many influences on pERK levels in tumor cells, our current data indicate that blocking MAPK signaling results in elevation of melanoma antigen gene expression, which could have potential significance in immunotherapy.

Results

Antigen Expression Enhancement by MAPK Inhibitors

In a search for agents able to modulate antigen expression, we screened melanoma cell lines with a diverse set of kinase pathway modifiers. By fluorescence-activated cell sorting analyses, no appreciable effects on Melan-A/MART-1 or gp100 expression were seen with LY294002 and wortmannin (phosphatidylinositol 3-kinase inhibitors), olomoucine (cyclin-dependent kinase inhibitor), PP2 (src kinase inhibitor), AG957 (tyrosine kinase inhibitor), and rottlerin (protein kinase C inhibitor; data not shown). In contrast, agents that down-regulate the MAPK pathway (MEK inhibitors PD98059 and U0126) consistently enhanced protein levels for both Melan-A/MART-1 (Fig. 1A; shown for U0126) and gp100 (Fig. 1B; shown for U0126 and PD98059) in both antigen-positive and antigen-negative melanoma lines after 3 days of treatment. By both Western blotting and flow analysis, the MEK inhibitors blocked downstream ERK1/2 phosphorylation in the treated cells (Fig. 1C and D, respectively), suggesting that MAPK signaling inhibition was indeed the primary cause of the induced augmentation of antigen expression.

In viable MU89 cells, treatment with U0126 augmented Melan-A/MART-1 levels in a dose-dependent manner. A 3-day assay showed the optimum dose of U0126 was 20 μmol/L (Fig. 2). Induction of apoptosis by U0126 also increased with dose, with ~20% apoptosis observed at 20 μmol/L after 3 days of treatment. With the latter dose of U0126, elevation of Melan-A/MART-1 protein continued up to 96 hours. However, because apoptosis also increased to 96 hours with levels exceeding 25% (Fig. 2), we used 3-day assays as the best

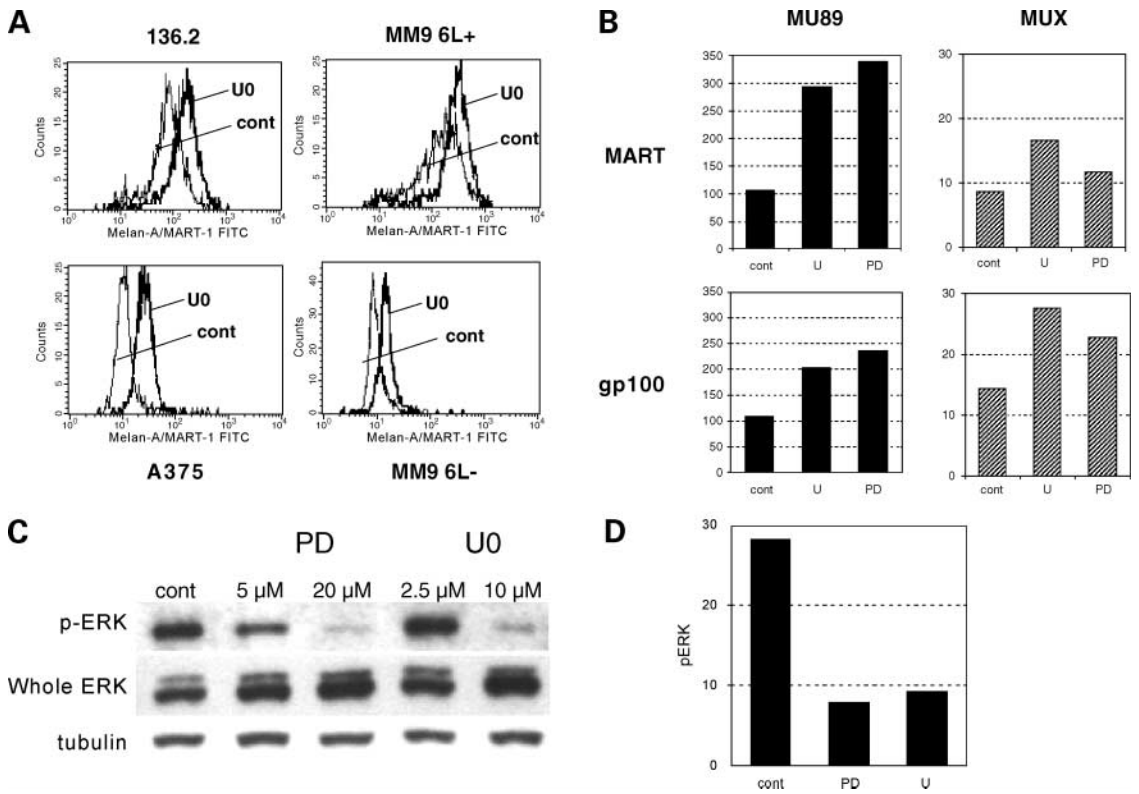

FIGURE 1. Effect of MEK inhibitors on melanoma antigen expression and activated ERK levels. **A.** Fluorescence-activated cell sorting analysis of indicated melanoma cells stained for Melan-A/MART-1 expression, with and without U0126 for 3 days (*U0*; all 20 μmol/L U0126 except 15 μmol/L for 136.2 cells). **B.** Comparison of mean channel fluorescence values for MU89 and MUX cells stained for Melan-A/MART-1 or gp100, with and without 3-day treatment by 20 μmol/L U0126 (*U*) or 40 μmol/L PD98059 (*PD*). **C.** Western blot analysis for levels of activated ERK1/2 (*pERK*) in MU89 cells before and after indicated treatments with MEK inhibitors. Whole ERK, total protein recognized by antibodies against ERK protein epitopes. **D.** Flow analysis for activated ERK1/2 (*pERK*) in MU89 cells before and after 10 μmol/L treatments with U0126 and PD98059.

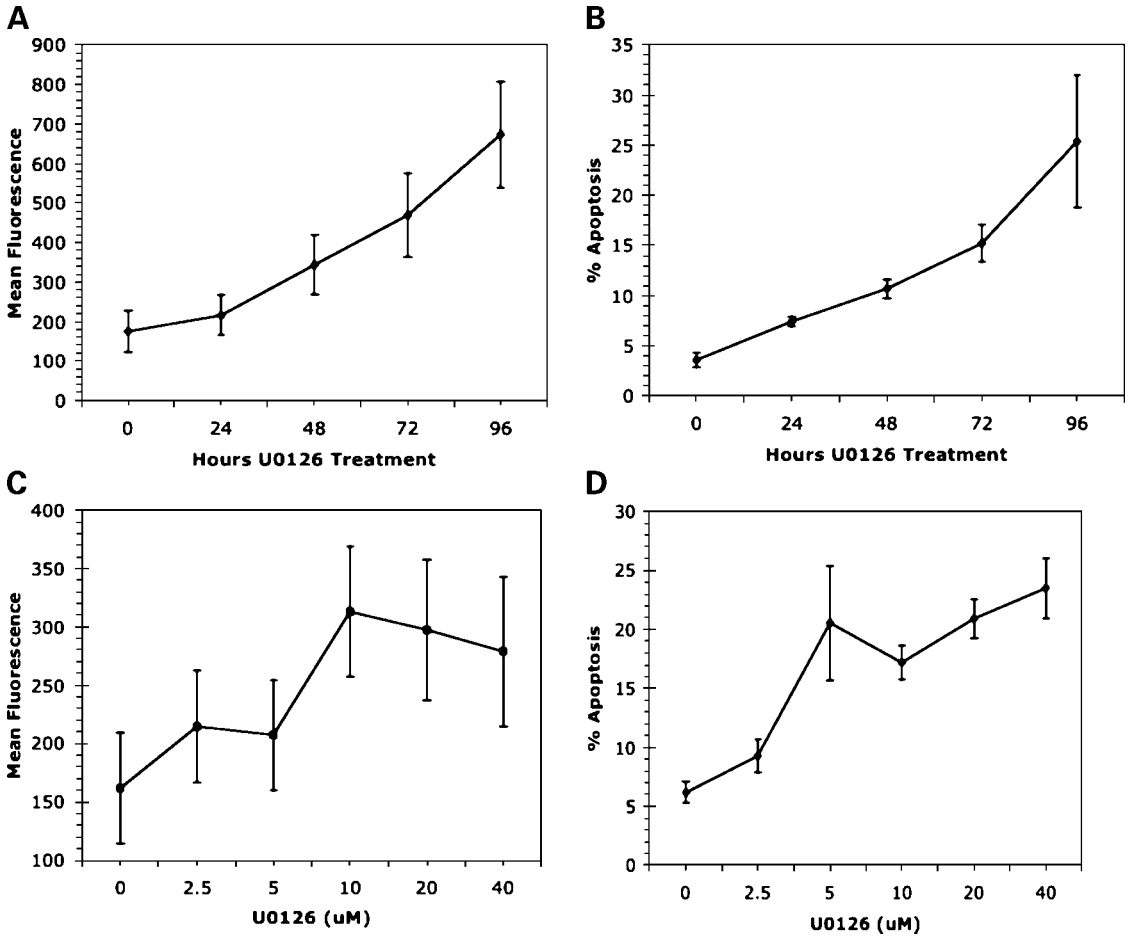

FIGURE 2. Dose-response and time-course of Melan-A/MART-1 and apoptosis induction by MEK inhibitor U0126. **A.** Flow cytometry of MU89 cells treated with 10 μmol/L U0126. Points, mean channel fluorescence for Melan-A/MART-1 plotted over time; bars, SD. **B.** MU89 cells were treated with 10 μmol/L U0126 for indicated times. Points, % apoptotic cells (intact Annexin V–positive, propidium iodide–negative cells); bars, SD. **C.** MU89 cells were treated with the indicated concentrations of U0126 for 3 days. Points, mean channel fluorescence for Melan-A/Mart-1; bars, SD. **D.** Percent apoptosis was derived from flow cytometric analysis of MU89 cells treated with increasing concentrations of U0126. Points, % intact Annexin V–positive, propidium iodide–negative cells; bars, SD.

compromise for antigen induction and cell viability. (Only viable cells show enhanced antigen expression, as apoptotic cells lose antigen expression along with cell viability). Similar response patterns were seen with PD98059, but with an optimal dose of 40 μmol/L (data not shown). At doses where effects on antigen expression were prominent, both MEK inhibitors also produced marked changes in cellular morphologies, including spindling, spreading, and flattening.

Subsequently we screened an extended panel of 22 melanoma cell lines for their responses to MEK inhibitors. These lines were grouped according to their antigen expression status, where "antigen-positive" denotes cells with readily detectable expression of the representative melanocytic antigens Melan-A/MART-1, tyrosinase, and gp100, as assessed at the protein level by flow cytometry. "Antigen-negative" indicates cells whose expression of these three markers is undetectable in this manner, although their expression at the mRNA level always has been detectable by sensitive PCR (16, 17). In the majority of cases, expression of these and other melanocytic markers was highly correlated, but some exceptions were found and are included among our surveyed cells as noted below.

The cell line panel included 11 antigen-positive lines and 8 nominally antigen-negative lines. Three of the cell lines were positive for gp100 but were also nominally negative for Melan-A/MART-1. Expression of Melan-A/MART-1 and gp100 was measured by flow cytometry, with results expressed as a response index equivalent to the % increase of peak channel fluorescence relative to the original control values. Of 22 cell lines, all showed elevated Melan-A/MART-1 expression in response to at least one of the two MEK inhibitors used, including lines that were nominally negative for this antigen by flow cytometry (Fig. 3A). However, the cell lines differed in the magnitudes of their responsiveness; 3 of 22 lines showed weak Melan-A/MART-1 responses. The MEK inhibitors also enhanced gp100 in all but one of the 22 cells lines (ML). In some cases, one of the two MEK inhibitor treatments gave minimal stimulation or actually decreased antigen levels (indicated by negative stimulation indices) even when the other

A

Melanoma Cell Line	Antigen status	BRAF status	Relative pERK level	Relative MITF-M level	MART U0126 Index	MART PD98059 Index	gp100 U0126 Index	gp100 PD98059 Index
MU89	+	M / W	0.79	1.10	248	109	193	121
453A	+	M / W	ND	ND	110	112	114	252
MA	+	M	0.74	1.17	100	93	74	61
CHOLLAR	-	M	1.07	1.41	97	110	27	44
H59-44T	+	W	0.08	1.35	97	99	61	91
136.2	+	M / W	0.73	2.11	95	29	90	97
MUX	-	M	0.95	0.68	89	34	91	58
MM96L+	+	M / W	0.82	1.65	89	58	269	241
MO	+	W	1.41	1.58	69	7	79	14
Mel-Juso	M-/G+	W	0.41	0.32	61	-3	85	72
MM96L -	-	M	0.53	0.21	60	17	63	24
EW	-	M / W	0.14	0.01	52	32	19	6
SKMEL28	+	M	0.63	1.37	49	-5	40	9
LH	-	W	0.09	0.38	43	5	30	17
IGR.39D	-	M / W	0.58	0.38	43	29	31	26
K2	+	M / W	0.68	0.17	42	62	-5	36
WM164	+	M	0.45	2.64	38	42	54	70
A375	-	M	0.37	0.08	36	-5	125	48
MM455	M-/G+	M / W	0.94	0.22	34	50	19	35
ML	-	W	0.04	0.22	32	-7	-5	-30
MALME-3M	+	M / W	1.09	2.23	28	-49	73	42
Roth	M-/G+	W	1.58	0.09	-33	19	16	88

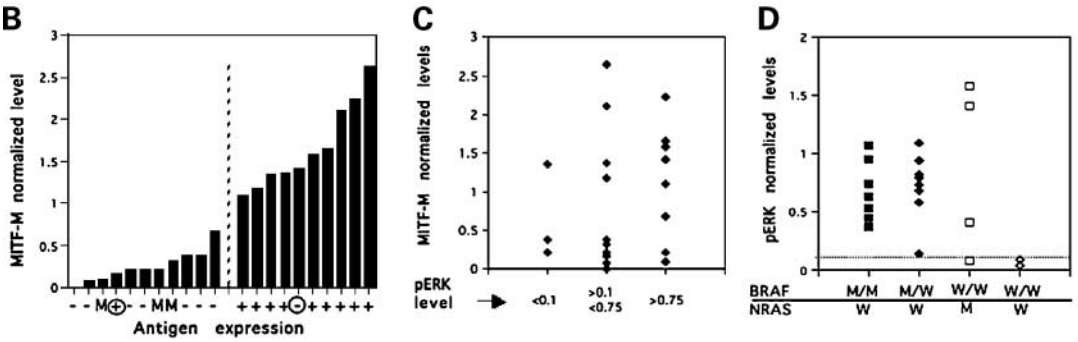

FIGURE 3. Characteristics of a panel of melanoma cell lines and their responses to MEK inhibitors. **A.** Nominal antigen expression status of a panel of cell lines (+ and −, positive and negative for Melan-A/MART-1, gp100, and tyrosinase; M−/G+, nominally negative for Melan-A/MART-1 but positive for gp100). *BRAF* gene mutational status denoted as follows: M, homozygous for V600E BRAF mutation; W, homozygous for wild-type *BRAF*; M/W, V600E/wild-type *BRAF* heterozygous. Relative pERK level indicates relative expression of activated ERK normalized to β-tubulin, as assessed by Western blotting. Relative MITF-M levels indicate expression of MITF-M protein normalized to β-tubulin, as assessed by Western blotting. MART and gp100 indexes denote relative changes (at 3 days) in cellular expression of Melan-A/MART-1 and gp100 in response to U0126 (20 μmol/L) or PD98059 (40 μmol/L). Index values are equivalent to the percent increase in antigen expression over untreated controls. The index is calculated using the formula: index = [(geometric mean of treated cells − geometric mean of control untreated cells) / geometric mean of control untreated cells] × 100. Shaded bars in U0/PD columns indicate magnitudes of responses ranked in descending order of response. Code for Index ranges: Black bars, ≥90; dark gray bars, >60 and <90; light gray bars, >30 and ≤60; pale bars, >15 and ≤30; white bars, ≤15. **B.** Correlation of MITF-M protein levels with antigen expression. Members of the cell line panel from (**A**) are arranged in ascending order of MITF-M levels (*Y-axis*), with the corresponding antigen expression class for each cell line noted on the opposite axis (antigen positive, +; antigen negative, −; negative for Melan-A/MART-1 positive for gp100, M). Cell lines grouped to the left of the dashed line have MITF-M levels <1, whereas cells with MITF-M levels >1 are plotted to the right of the dashed line. Circled X-axis values correspond to anomalous lines K2 (antigen positive, low MITF-M) and CHOLLAR (antigen negative, relatively high MITF-M). **C.** Lack of correlation between MITF-M expression level and ERK activation in the cell line panel of (**A**). Each point represents one cell line, grouped in three categories (as shown) for low, intermediate, and high pERK levels. **D.** Scatter diagram for pERK levels from the cell line panel, grouped according to both *BRAF* and *NRAS* (Q61) mutational status. Horizontal dotted line shows relative pERK level of 0.1.

inhibitor had the opposite effect (e.g., SKMEL28, Mel-Juso, ML, and MALME-3M show negative indices for Melan-A/MART-1 augmentation with PD98059 but positive antigen augmentation with U0126). The majority of lines (19 of 22) responded to the MEK inhibitors with reproducible Melan-A/MART-1 indexes of >30; 8 of 22 showed antigen indexes of >80. U0126 seemed to be more effective overall than PD98059 for both antigens, with more responses characterized as strong (Fig. 3A). Relevant factors may be the inhibition of both MEK1 and MEK2 by U0126 (22, 23), whereas PD98059 has selectively greater activity towards MEK1 (24). However, in 7 of 22 cell lines, PD98059 elicited superior augmentation of both antigens (Fig. 3A). In almost all instances, the specific inhibitor giving the best responses for Melan-A/MART-1 also gave the best responses for gp100 in the same cell line. The 136.2 cell line was the only one of the 22 cell lines that was not concordant in this regard (Fig. 3A). The concordance of drug performance suggested that instances of improved

responsiveness with PD98059 are significant and based on currently undefined cell-specific properties. We assigned the cell line panel of Fig. 3A into High, Intermediate, and Low response categories based primarily on the effect of the MEK inhibitors on Melan-A/MART-1 levels within the experimental variables of our studies.

Role of MITF-M in Antigen Expression and MEK Inhibitor Antigen Augmentation

The transcription factor MITF-M is known to regulate many melanocytic genes (1, 3, 18). Given its corresponding relevance to melanoma antigen expression, Western blotting was used to assess relative levels of MITF-M (normalized to β-tubulin) in our cell line panel (Fig. 3A). When cell line expression levels of MITF-M are arranged in ascending order (Fig. 3B), a distinct correlation between levels of MITF-M and antigen expression status was observed. In general, antigen-positive cells were high for MITF-M, with relatively low levels seen for antigen negatives (including cells negative for Melan-A/MART-1 but showing gp100 expression). Antigen-positive cells had relative expression ratios >1.0 (shown to the right of the dashed line in Fig. 3B), and cells deficient in antigen expression had ratios <1.0 (shown to the left of the dashed line in Fig. 3B). Two exceptions were noted: one antigen-negative line (CHOLLAR) showed MITF-M levels as high as several positive lines, and one antigen-positive line (K2) had MITF-M levels lower than several negative lines (Fig. 3A and B). However, MITF-M expression showed no discernable correlation with ERK activation (Fig. 3A, pERK), shown as a scatter diagram in Fig. 3C with cells grouped into classes based on low, intermediate, and high pERK levels.

We then tested whether the MEK inhibitors affected MITF-M. Treatments with PD98059 or U0126 suppressed MITF-M mRNA levels, whereas Melan-A/MART-1 mRNAs were progressively augmented (Fig. 4A). These effects were seen in separate cell lines with both high initial MITF-M (MU89) and also low MITF-M levels detectable with sensitive PCR (MUX). In contrast, no such effects on the MITF-A microphthalmia isoform were observed (data not shown), using specific primers that exclude MITF-M (Table 1). The inhibitor-mediated ablation of MITF-M expression was also seen at the protein level. A Western blot is shown for representative antigen-positive K2 cells, which initially express detectable pERK and MITF-M protein (Fig. 4B; putative assignment of MITF isoforms is based on their reported differing molecular weights). MEK inhibitors ablated pERK and MITF-M proteins without comparable effects on β-tubulin or the putative MITF-A microphthalmia isoform, which is not restricted to the melanocytic lineage in the same manner as MITF-M (25).[5]

Effect of MEK Inhibitors at the Transcriptional Level

We then used reverse transcription-PCR (RT-PCR) to test whether the antigen enhancement effect correlated with changes in specific mRNA levels. When A375 (low antigen) melanoma cells were treated with U0126, the melanocytic antigens Melan-A/MART-1, tyrosinase, and gp100 all showed elevated levels of steady-state mRNA, without any observable change in β-actin levels (Fig. 5A). We also showed increased Melan-A/MART-1 mRNA levels by quantitative RT-PCR in additional antigen-positive and antigen-negative tumor lines (Fig. 5B). We noted

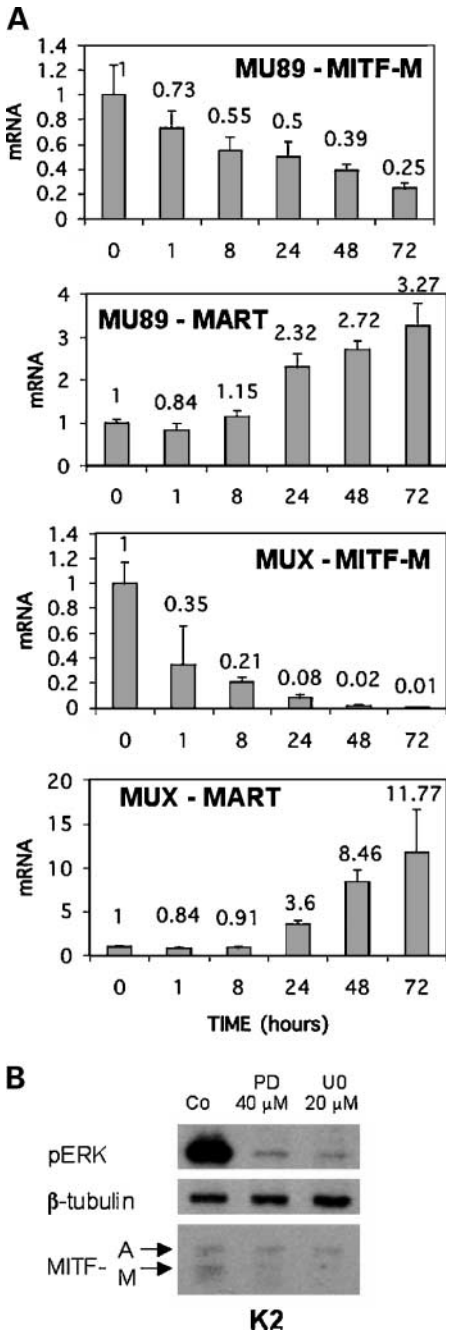

FIGURE 4. Effect of treatment with MEK inhibitor on expression of MITF-M. **A.** Time course of MITF-M and Melan-A/MART-1 mRNA expression following treatment with 10 μmol/L U0126. Top two, MU89; bottom two, MUX. mRNA levels are calculated relative to β-tubulin mRNA levels, and control untreated time point is normalized to 1.0. **B.** Effect of MEK inhibitors on MITF-M protein levels. Western blotting of antigen-positive K2 cells with antibodies to MITF-M, pERK, and β-tubulin. Cells were treated with the indicated concentrations of MEK inhibitors (*PD*, PD98059; *U0*, U0126; *Co*, control untreated cells) for 72 hours before harvesting for protein extracts. Bands were assigned as MITF-M or MITF-A isoforms based on molecular weight.

[5] Unpublished observations.

Table 1. Oligonucleotide Primers and Probes

Oligo name	Sequence
b-actin5′2.F	GAGATCACTGCCCTGGCACCCA
b-actin3′1.R	GCTCCAACCGACTGCTGTCACCTTCAC
B-actin-Q.F	ACCCAGCACAATGAAGATCAA
B-actin-Q.R	ATACTCCTGCTTGCTGATCCA
B-actin-Q.probe	HEX-CTCCTGAGCGCAAGTACTCCGTGT-BHQ1
BRAF m-RFLP.F	GGGCATGGATTACTTACACGCCA
BRAF g-RFLP.F	CTGTTTTCCTTTACTTACTACACCTCAGA
BRAF-RFLP.R	CTGTTCAAACTGATGGGACCCACTCCA
BRAF seq1	GTGTTGGAGAATGTTCCACTTACAACACA
BRAF seq2	GATACAAGCTGGAGCCCTCACACCA
BRAF seq3	CCTACACCTCAGCAGTTACAAGCCTTCA
BRAF seq4	ACAGGAAACGCACCATATCCCCCT
gp100-Q.F	TCTGGGCTGAGCATTGGG
gp100-Q.R	AGACAGTCACTTCCATGGTGTGTG
gp100-Q.probe	FAM-CAGGCAGGGCAATGCTGGGC-TAMRA
gp100.F	CTGATTGGTGCAAATGCCTCCTTCT
gp100.R	AGGAAGTGCTTGTTCCCTCCATCCA
MARTex2o.F	CAAGATGCCAAGAGAAGATGCTCACT
MARTex5o.R	GCTTGCATTTTTCCTACACCATTCCA
MART-Q.F	CCAATGCTCCACCTGCTTAT
MART-Q.R	AGGTGTCTCGCTGGCTCTTA
MART-Q.probe	CY5-TCTGCAGAACAGTCACCACCACCT-BHQ2
MITF-M-Q.F	GTAGAGGGAGGGATAGTCTACCG
MITF-M-Q.R	GGGTCTGCACCTGATAGTGATTA
MITF-M-Q.FAM	FAM-TCTCACTGGATTGGTGCCACCTAAA-BHQ1
MITF-A-Q.F	CATGAAGAGCCCAAAACCTATTAC
MITF-A-Q.R	ATGTCATACTGGAGGAGCTTATCG
MITF-A-Q.FAM	FAM-TCAACCGCTGAAGAGCAGCAGTTC-BHQ1
tyrosinase.F2	CAGCCCAGCATCATTCTTCTCCTCT
tyrosinase.R	GCAGTGAGGACGGCCCCTACCA
tyrosinase-Q.F	GGCTGTTTTGTACTGCCTGCT
tyrosinase-Q.R	AGGAGACACAGGCTCTAGGGAA
tyrosinase-Q.probe	FAM-AGTTTCCAGACCTCCGCTGGCCA-TAMRA
NRAS.mseq.F	AGAGGCAGTGGAGCTTGAGG
NRAS.mseq.R	TCAGGACCAGGGTGTCAGTG

NOTE: Probes for quantitative PCR indicated, all other oligos used as PCR or sequencing primers. All primers and probes for quantitative PCR designated with "Q" and showing 5′ and 3′ dye adducts. Target genes for each oligonucleotide indicated within name.
Abbreviations: F, forward PCR primers; R, reverse PCR primers.

no significant change in HLA-A mRNA levels following the same treatments with both compounds (data not shown). Increased mRNA levels correlated with augmentation of the Melan-A/MART-1 233-bp core promoter (26) by the same MEK inhibitors in reporter assays, without significant effect on the HLA-A promoter (Fig. 5C), suggesting that the enhancing effect was at the transcriptional level.

BRAF Expression Patterns in Melanoma Cell Lines

The striking effects of the MEK inhibitors suggested that a negative MAPK signal in melanoma cells was associated with increased antigen gene expression. MAPK activation would then be predicted to have the opposite effect. Because constitutively active mutant forms of BRAF (especially the V600E mutation) are common events in both benign and malignant melanocytic lesions (20, 21, 27-30), we screened a panel of melanoma cells for BRAF mutational status and compared this with their patterns of antigen expression. We used a PCR-RFLP system (Fig. 6) to rapidly detect the V600E mutation and distinguish wild-type and mutant alleles. No overall correlation between antigen expression and BRAF mutational status could be discerned (Fig. 3A), and there was likewise no convincing relationship between BRAF status of a cell line and its degree of responsiveness to MEK inhibitors.

We also checked for relative levels of pERK (normalized to β-tubulin levels). Cells bearing at least one mutant *BRAF* allele indeed showed ERK activation, but cells with homozygous wild-type *BRAF* fell into groups showing either very low or high pERK levels (Fig. 3A and D). Indeed, the highest pERK levels occurred in the homozygous wild-type *BRAF* lines MO and Roth (Fig. 3A). Because activating mutations in NRAS might be an alternate source of high steady-state pERK levels, we checked the cell lines for the Q61R/K/L mutations known to activate RAS proteins constitutively (20, 31-35). All cell lines bearing V600E mutant BRAF (whether homozygous or heterozygous) with significant pERK levels were wild type for NRAS (Fig. 3D), whereas cell lines wild type for both BRAF and NRAS showed low pERK (<0.1 relative level). Wild-type BRAF cells with high pERK levels (>0.4 relative level, Fig. 3D) each possessed activating NRAS mutations (Mel-Juso, Q61L; MO and Roth, Q61R). Only one cell line (H59-44T; Fig. 3A) with wild-type BRAF and a Q61K NRAS mutation failed to show elevated levels of pERK (Fig. 3D).

Levels of pERK showed no consistent link with the responsiveness of cells to MEK inhibitors (Fig. 3A). In addition, antigen-negative MM96L$^-$ cells, derived from MM96L$^+$ progenitors, acquired BRAF homozygosity but showed a reduction rather than an elevation in pERK levels. These data indicated that other factors must influence steady-state pERK turnover and showed that endogenous pERK levels alone do not predict levels of antigen expression (Fig. 3A). However, given the MEK inhibitor results, we asked whether exogenous application of a strong positive MAPK signal would override other variable regulatory controls and provide a better test for the effects of positive MAPK perturbation on antigen expression.

Transient Expression Effects of Wild-Type and Mutant BRAF in Melanoma Cells

We transiently transfected expression constructs for wild-type and V600E BRAF into melanoma cell lines and showed exogenous BRAF expression by Western blotting after 48 hours (Fig. 7A). In transient plasmid-based expression assays, it is important to focus on the subpopulation of cells that actively take up and express the transfected expression module. To test the concordance of expression between two independent cotransfected plasmids under our experimental conditions, we cotransfected green fluorescent protein (GFP) and Melan-A/MART-1 or gp100 expression plasmids into low-antigen cells and used two color fluorescence-activated cell sorting analysis: green fluorescence to assess GFP and phycoerythrin-conjugated antibody staining for the antigen expression (Materials and Methods). Concordance of expression between GFP and both of the antigen expression plasmids was high, with a low percentage (2.5-2.8%) of cells measurably expressing only one of the transfected markers (Fig. 7B).

We then tested the effects of transfected *BRAF* plasmids on levels of activated ERK (pERK) and endogenous melanoma antigens, using antigen-positive cells so that both increased and decreased antigen expression could be observed. In MU89 and MM96L$^+$ cells, transfection of V600E mutant BRAF

consistently resulted in elevation of pERK levels and suppression of endogenous Melan-A/MART-1 (Fig. 7C). In striking contrast, transfected wild-type BRAF caused suppression of pERK and elevation of antigen expression (Fig. 7C). This relationship between pERK and antigen expression paralleled the action of the MEK inhibitors (Fig. 1A-C).

Stable BRAF Transfectants

We attempted to derive cell lines stably expressing wild-type or mutant BRAF using the same antigen-positive cells (MU89 and MM96L$^+$). However, despite using a bicistronic vector (Materials and Methods) designed to force BRAF expression through coexpression of the selectable marker, the only transfectant isolated that expressed levels of exogenous BRAF >2-fold background was MU89 (with wild-type BRAF; Fig. 8A). Analysis of these transfectants showed reduced levels of pERK and increased antigen (Melan-A/MART-1 and gp100; Fig. 8B), in parallel with the transient transfection results. The difficulty of isolating cells with overexpressed mutant BRAF suggests a negative influence on cell viability when this activating mutation is present at high levels.

Effect of Antigen Elevation by MEK Inhibitors on Recognition by Cytotoxic T Cells

Because nominally antigen-negative cells were enhanced for Melan-A/MART-1 expression by MEK inhibitors (Fig. 2), we tested whether such treatments could also augment specific T cell–mediated cytotoxicity. A375 cells are poorly recognized by HLA-A-2/Melan-A/MART-1–specific cytotoxic T cells, but after culture with MEK inhibitor U0126, there is sufficient antigen induction to allow between 30% and 40% lysis at effector ratios between 20:1 and 2.5:1 (Fig. 9). Although even large numbers (20:1) of effector cells lyse less than half of the tumor targets, >70% lysis can be obtained against high antigen-expressing MU89 cells, using as few at 2.5 effectors per target (data not shown). These data indicate that enhanced antigen expression by MEK inhibitors in low Melan-A/MART-1 tumor cells results in enhanced killing in comparison to untreated cells, but the level of antigen expression is still suboptimal. The relative flatness of the lysis curves between 20:1 and 2.5:1 effector/target cells ratios results from the strong CTL activity by these cloned CTL, but only against tumor cells that have attained a threshold level of antigen expression. Significantly, the improvement in A375 lysis occurred despite its not being a strong responder to MEK inhibitors, even among antigen-negative cells (A375 lies at the lower end of "intermediate" responders; Fig. 3A).

Discussion

Previously, we showed reversible loss of antigen expression in melanomas (16, 17) mediated by soluble mediators, including the cytokine oncostatin M (17). Because antigen expression loss in melanomas does not indicate loss of functional antigen gene expression capability (16, 17), it is feasible to overcome the immunotherapeutic problem of low antigen expression by manipulating gene regulatory events. We, therefore, screened multiple agents to identify those able to modulate melanoma antigen expression. This resulted in the identification of the MEK inhibitors PD98059 and U0126 as up-regulators of multiple melanoma antigens (Figs. 1-3). Previously, Englaro et al. (36) showed enhancement of differentiation and tyrosinase levels in B16 murine melanoma cells by the MEK inhibitor PD98059, and more recently, inhibition of human MAPK signaling by anthrax lethal factor has been associated with melanogenesis and apoptosis (37).

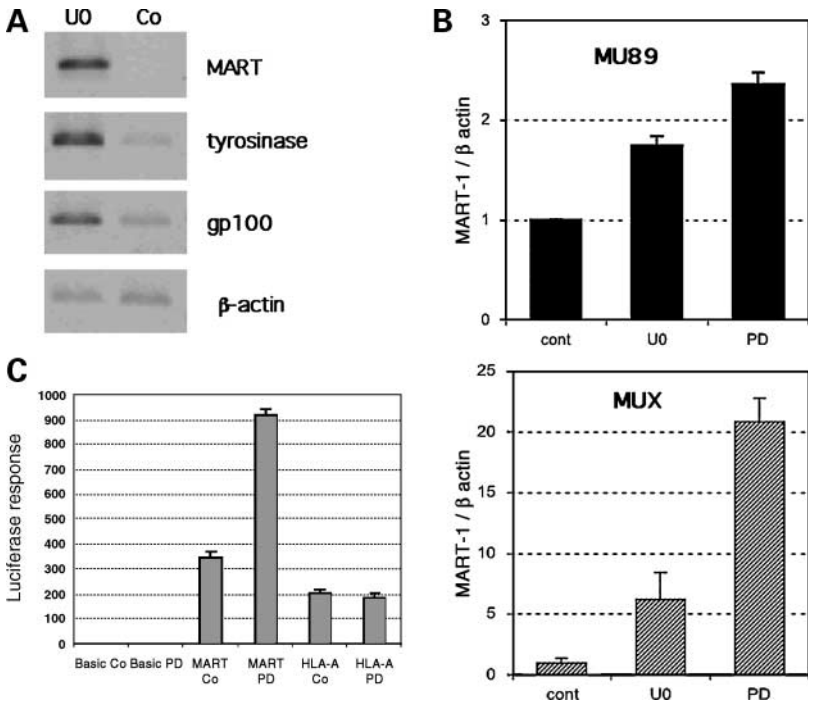

FIGURE 5. Effects of MEK inhibitors on melanoma antigen mRNA levels and promoter activity. **A**. Up-regulation of antigen expression in A375 cells by treatment with 40 μmol/L U0126 for 3 days (*U0*) compared with corresponding untreated control cells (*Co*), assayed by RT-PCR. **B.** Quantitative PCR analysis of Melan-A/MART-1 for melanoma cells treated with MEK inhibitors. Cells were treated with 20 μmol/L U0126 (*U0*) and 40 μmol/L PD98059 (*PD*) for 3 days. Columns, means of five replicates; bars, SD. **C.** Effect of MEK inhibitor on antigen promoter reporter activity, using the dual luciferase assay in MU89 cells, where firefly luciferase is used as the reporter and cotransfection of *Renilla* luciferase is used as a control. After transfection with pGL3-basic vector alone (*basic*), the minimal 233-bp Melan-A/MART-1 promoter in the same vector (26), or a 230-bp HLA-A promoter (63), medium was replaced at 24 hours with or without 20 μmol/L PD98059 (*PD*). After a further 48 hours, cells were harvested and assayed. Vertical axis shows ratio of firefly to *Renilla* luciferase activity times 1,000. Columns, means of four replicates; bars, SD.

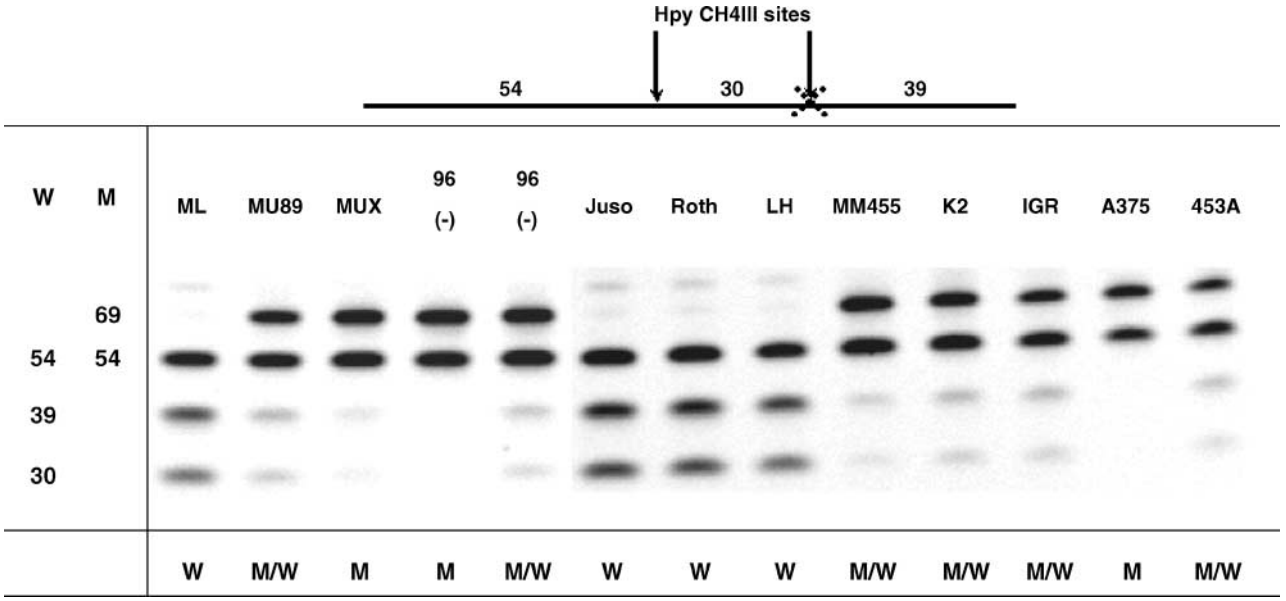

FIGURE 6. RFLP analysis to identify *BRAF* mutational status at V600E codon. PCR fragments from indicated melanoma cell genomic DNAs were amplified with primers BRAFg-RFLP.F and BRAF-RFLP.R (Table 1), digested with Hpy CH4III, and electrophoresed on a 3% low range ultra agarose gel. Top bar, pattern of sites within the PCR fragment. The site with cross through it is lost through the T1796A mutation, which alters the restriction fragment pattern after electrophoresis as shown to the left of gel. The RFLP analysis will yield bands of 69 and 54 bp for homozygous mutant; bands of 54, 39, and 30 bp for homozygous wild type; and heterozygotes will have the combined band patterns. Cell lines are as indicated except where abbreviated: 96(−), MM96L^{-}; 96(+), MM96L^{+}; Juso, Mel-Juso; IGR, IGR.39D. The mutational status of each cell line is indicated below each lane: W, homozygous wild type; M, homozygous mutant; M/W, heterozygous mutant and wild type. The specific nature of the mutations causing loss of the Hpy CH4III site was confirmed as T1796A by direct sequencing. Evaluation of mutational status of *BRAF* for the MUX cell line showed a faint 39-bp band, which suggested that MUX is not monoclonal for homozygous mutant *BRAF*. Indeed, single-cell PCR analysis shows that although true homozygous *BRAF* MUX cells are the major population, there are residual MUX cells that retain mutant/wild-type *BRAF*.

Dose-dependent apoptosis was clearly induced by MEK inhibitors in our melanoma cells. Melanoma cells may evade apoptosis by MAPK-mediated phosphorylation and inactivation of the apoptosis-promoting *bad* protein (38), with the result that MAPK inhibition is proapoptotic. In our experiments, the cells surviving MAPK inhibitor treatment show marked shape changes as well as increased antigen expression. Consistent with the observed morphologic alterations, direct effects of MAPK signaling on cytoskeletal proteins have been noted, including actin (39) and stathmin regulation of microtubules (40).

The known importance of MITF-M for melanocytic gene regulation (1, 3, 18) was entirely consistent with the fact that MITF-M protein levels in untreated tumor cells correlated highly with antigen expression (Fig. 3B). It has been recently reported (41) that mutational BRAF activation in melanocytes is associated with down-regulation and degradation of MITF-M, and that MITF-M is antiproliferative in melanoma. Although some cell lines in our panel indeed have BRAF V600E mutations accompanied by high levels of pERK and low MITF-M, this is not at all a consistent or necessary phenotype, as the steady-state concentrations of endogenous pERK showed no meaningful correlation with MITF-M (Fig. 3C). Cell lines within our panel (Fig. 3) with either very high MITF-M or low MITF-M are also not distinguishable based on their proliferation rates (data not shown). Others have in fact claimed that MITF-M is required for melanoma growth through regulation of cyclin-dependent kinase 2 (42). As a resolution to this seeming paradox, Wellbrock and Marais (41) proposed that low but definite amounts of MITF-M are required for melanoma survival and/or proliferation. This cannot be ruled out at present, but the exceedingly low MITF-M levels expressed by some cell lines (e.g., EW; Fig. 3A) are not supportive of this hypothesis.

MAPK phosphorylation has been reported to both directly activate MITF-M and to signal its degradation (43); thus, the net effect of MAPK inhibition on preformed MITF-M is not obvious. However, our data clearly show that MAPK inhibition by MEK inhibitors results in dramatic declines in both MITF-M mRNA and protein levels (Fig. 4). This effect seemed to be restricted to the MITF-M isoform, as no evidence for depletion of the MITF-A isoform was seen (Fig. 4).[6] The different MITF isoforms are transcribed from different promoters, with isoform-specific 5′ exons spliced onto common 3′ exons (25), and the respective isoforms are, therefore, subject to divergent transcriptional controls. Significant suppression of MITF-M expression by MEK inhibitors would seem unlikely to be correlated with increased MITF-M function. In addition, although MEK inhibitors may block MITF-M degradation, they would also be predicted to block its activation by the MAPK pathway (43). An alternate explanation is available in any case, from the proposal that MITF-M may not be necessary for melanocytic gene activation in some circumstances. Indeed, it has been shown that under conditions where the p38 stress MAPK pathway is activated, the USF1/2 transcription factors

[6] Unpublished data.

substitute for MITF-M in the transcriptional activation of tyrosinase (44, 45).

In this study, we used two chemically distinct MEK inhibitors (PD98059 and U0126), but others have been described and tested in phase I or II clinical trials (46). In a majority of cases in our study, cells responded positively with antigen augmentation for both inhibitors (14 of 22 for Melan-A/MART-1 and 17 of 22 for gp100; Fig. 3A). However, several observations indicated that the two drug treatments were not functionally equivalent. In some cases, cells responded poorly or even negatively to PD98059 yet showed significant antigen up-regulation with U0126 (Fig. 2). Apart from known differences in their respective selectivities towards MEK1 and MEK2 (22-24), these inhibitors may differ in other cell line–specific biological interactions. These results consequently show the need to evaluate the effect of any new MEK inhibitors on a sizable panel of cell lines if a realistic response pattern is to be obtained. Furthermore, the possibility of patient-specific therapy must be entertained.

Much evidence documents the importance of MAPK signaling for melanoma growth and progression (20, 33, 47-50). Activating mutations within the *BRAF* gene, especially that resulting in the V600E mutant protein, have been shown to be a major route for acquisition of constitutive MAPK activity by melanoma cells (20, 21, 27-30). Because results with MEK inhibitors showed that blocking of ERK activation was associated with antigen up-regulation, we compared BRAF mutational status, pERK levels, and antigen expression in our 22–cell line panel without finding any generalizable correlations (Fig. 3A). ERK activation also failed to correlate with MITF-M expression (Fig. 3C). Other studies (18, 51) also found no simple link between *BRAF* mutation and Melan-A/MART-1 or gp100 expression. Thus, antigen expression status of melanoma cells cannot be inferred from knowledge of either their *BRAF* mutational state or relative pERK levels. In particular, high endogenous pERK levels per se are not necessarily incompatible with significant antigen expression (Fig. 3A). A recent study (52) shows that the antiproliferative response to MEK inhibitors correlates with *BRAF* mutational status. Wild-type *BRAF* cells with RAS mutations were less sensitive to MEK inhibition, interpreted as arising from RAS signaling affecting alternate downstream pathways (52). However, no such dichotomy in the effects of MEK inhibitors on antigen expression of wild-type versus mutant BRAF cells is evident, irrespective of NRAS status (Fig. 3A and D). Thus, in circumstances where RAS signaling bypasses downstream components of the MAPK pathway, such alternative signaling is not important for regulating melanocytic antigen gene expression.

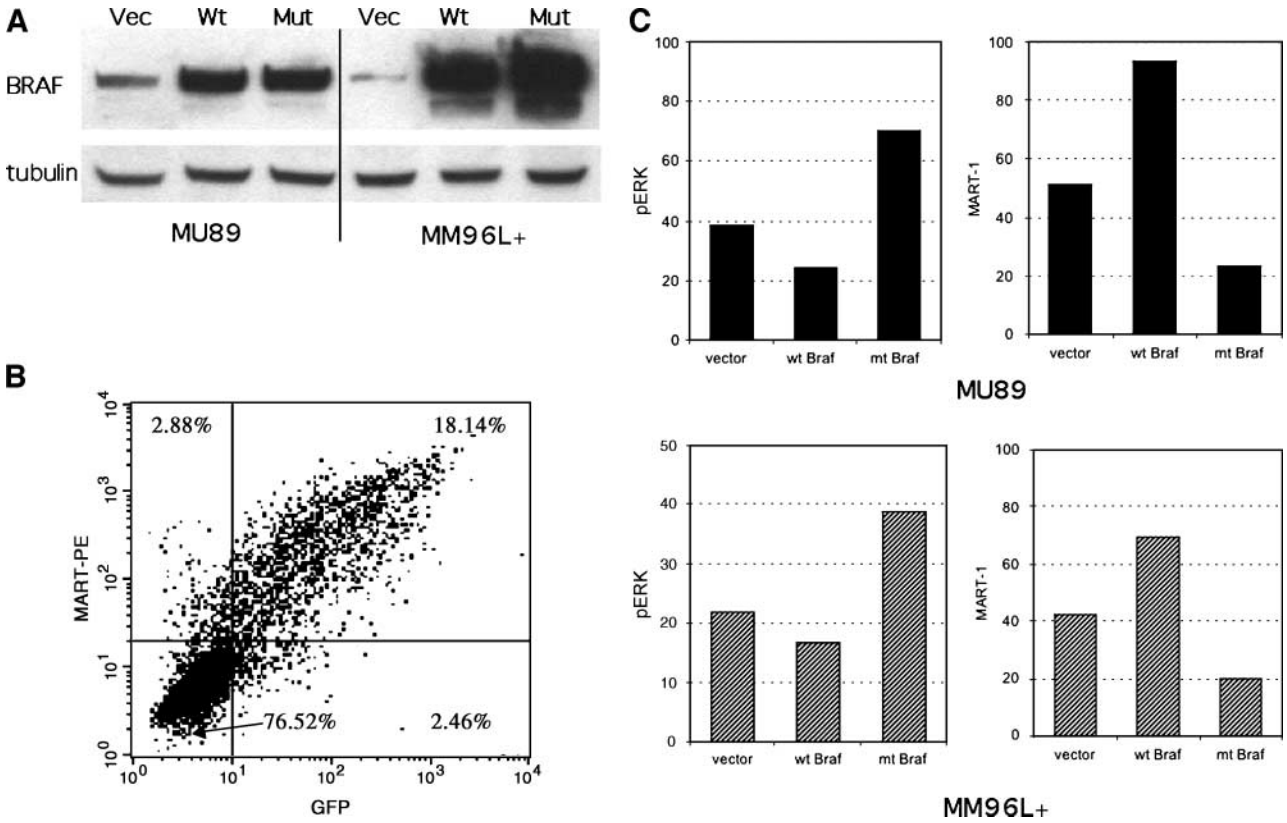

FIGURE 7. Regulation of melanoma antigen and pERK status by BRAF transient transfection. **A.** Western for BRAF 48 hours after transfection of MU89 and MM96L$^+$ cells with vector alone (*Vec*), wild-type BRAF (*Wt*), or V600E mutant BRAF (*Mut*). **B.** Concordance of cotransfected GFP and antigen gene expression assessed by flow cytometry. MUX cells were cotransfected with expression plasmids for GFP and Melan-A/MART-1 and stained for Melan-A/MART-1 with phycoerythrin-conjugated secondary antibody. Percentage of cells in each quadrant is indicated. **C.** Effect of transfection with expression constructs for wild-type and mutant-type BRAF on pERK and Melan-A/MART-1 levels. The levels of pERK and Melan-A/Mart-1 were determined by antibody staining of permeabilized cells followed by flow analysis. Cotransfection of GFP was used to gate for successfully BRAF-transfected cells (upper left quadrant in **B**), and the geometric mean fluorescence of these cells is graphed.

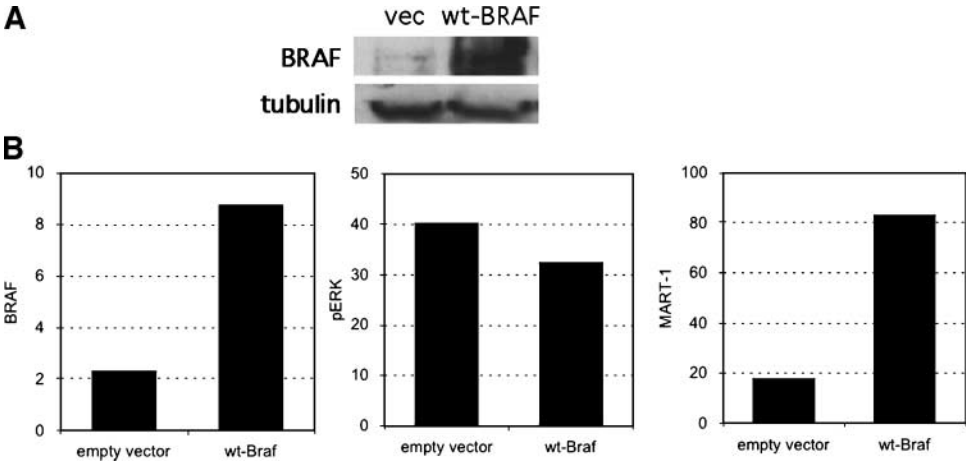

FIGURE 8. Regulation of melanoma antigen and activated pERK status by BRAF stable transfection. **A.** Western blotting for BRAF expression in MU89 stably transfected with empty pIRESneo3 vector alone (*Vec*) or wild-type BRAF (*wt-BRAF*). **B.** Flow cytometric analysis of BRAF expression, pERK level, and Melan-A/MART-1 expression in MU89 cells stably overexpressing exogenous wild-type BRAF.

Whereas endogenous levels of pERK do not correlate with antigen levels, artificial intervention that lowers the preexisting baseline of pERK (whether initially high or low; via MEK inhibition) has the effect of augmenting antigen expression. In turn, this implies that additional factors may determine the efficiency of pERK activity itself on its downstream substrates. A relatively low level of pERK in one cell type may be more functionally potent than much higher levels of pERK in a different cellular context. This supposition is consistent with data suggesting that, beyond a certain point, excessive ERK signaling is not compatible with cell proliferation, and that optimal conditions for tumor growth may involve a low but steady unregulated "trickle" of signaling (29). In our melanoma panel, levels of pERK show no correlation with the rate of cell growth of individual lines (data not shown).

Down-regulation of MAPK phosphatases is another means for achieving MAPK activation and may be associated with tumor promotion (53, 54). All these observations reinforce the intricacies of MAPK signaling control and indicate that a large panel of melanoma cells will inevitably show diversity in their specific perturbations of this signaling pathway. However, we considered that an exogenous strong positive MAPK signal might predominate over such variable influences and thus tested the effects of BRAF transfection on antigen expression. We used the same antigen-positive parental lines for transfection of wild-type or mutant BRAF expression constructs, as a way of directly testing the role of BRAF proteins on MAPK signaling and antigen levels. Exogenous expression of V600E mutant BRAF indeed increased ERK activation but lowered antigen expression in these cells. Notably, transient expression of wild-type BRAF had dramatically different effects than mutant BRAF because the wild-type protein suppressed pERK levels and augmented antigen expression (Fig. 7). Although not predicted, this effect on pERK and antigen levels paralleled that seen with the MEK inhibitors. Unlike V600E BRAF, wild-type BRAF requires phosphorylation at T599 and S602 for activation (55), and replacement of these residues with alanines (BRAF-AA) abrogates activity. Moreover, BRAF-AA acts as a dominant-negative mutant, potently blocking ERK activation (56). Given these observations, the effects of wild-type BRAF may be mediated through escape from activation by a significant fraction of the expressed protein, with a resulting dominant-negative effect on ERK activation. However, high-level expression of wild-type BRAF in certain melanoma cell lines has been implicated as another alternative route for MAPK activation (34); thus, overexpression of wild-type BRAF cannot be suppressive in all contexts. It is unlikely that the wild-type BRAF effect was a consequence of transient transfection in itself because we succeeded in isolating a stable transfectant of MU89 overexpressing wild-type BRAF, which showed similar characteristics to the transient expression system (Fig. 8A and B). Possibly, kinase activation of BRAF in MU89 (bearing both mutant and wild-type *BRAF* alleles) is inefficient or tempered by phosphatases such as DUSP6, which is known to be up-regulated in mutant BRAF cells (57). Such a phenotype in turn could lead to incomplete activation of wild-type BRAF

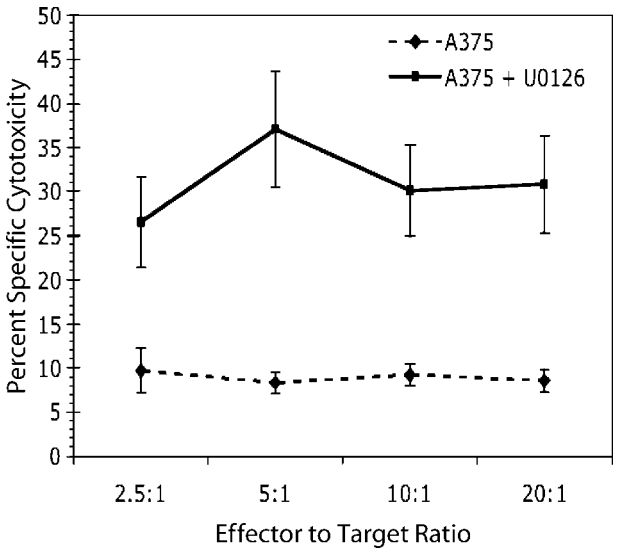

FIGURE 9. Enhancement of Melan-A/MART-1–specific cytotoxic T-cell responses. Points, % Melan-A/MART-1–specific cytotoxic T-cell killing of A375 tumor cells treated with 20 μmol/L U0126 for 6 days (*dotted line*) compared with untreated (*solid line*) A375 tumor cells; bars, SD. For this assay, each target/effector ratio was determined in triplicate, and spontaneous and maximum release was determined from the average of six wells each.

when exogenously expressed at high levels and is amenable to experimental testing.

Activating MAPK mutations in melanoma *NRAS* or *BRAF* genes seem to be mutually exclusive (20, 31-34), which has been interpreted as evidence for their influence on the same signaling pathway. Consistent with these reports, cells in our own melanoma panel with V600E mutant BRAF (whether homozygous or heterozygous) are also wild type for NRAS. However, V600E BRAF can be phosphorylated *in vitro* by mutant RAS with even higher BRAF kinase activation (20). It is then possible that beyond a threshold point, excessive BRAF kinase activity may become inhibitory, consistent with observations that very high ERK activity itself is detrimental to cell growth (29). We hypothesize that this may be a factor in our failure to obtain stable high-level expression with mutant BRAF in the specific cell lines used. Our data also indicate that NRAS-activating mutations may indeed explain the high levels of ERK activation observed in a subset of cells homozygous for wild-type BRAF (Fig. 3A and D). Conversely, wild-type BRAF cells with relatively low pERK levels were also wild type for NRAS, with one exception (H59-44T cells; Fig. 3A and D). The latter cells may have active ERK-mediated feedback mechanisms inhibiting wild-type BRAF activity (by analogy with RAF1; ref. 58), which are inactivated in the wild-type BRAF cells with both NRAS mutations and high pERK.

We have shown in this work that the augmented antigen expression mediated by MEK inhibitors is also associated with elevated specific T-cell cytotoxicity directed towards a known melanocytic antigen (Fig. 9). Although the U0126-treated antigen-negative cells (A375) were not killed as efficiently as constitutively strong antigen-positive tumor cells (MU89), these results show that the effects of the MEK inhibitors on antigen levels can be immunologically significant.

The critical role of MAPK activation in melanoma development has prompted attempts to exploit this pathway as a therapeutic target (59-61). In this context, it is noteworthy that MEK inhibitors induce both measurable apoptosis and immunologically relevant increased antigen expression. The biologically "available" MEK inhibitor CI-1040 was recently reported (46) to be insufficient for control of tumor growth in several tumor types, including melanoma. Although MEK inhibitors may have limited direct efficacy *in vivo*, our *in vitro* studies show that those cells surviving MEK inhibitor treatment show the highest levels of antigen expression. In turn, this indicates that a "double-barreled" strike against melanoma in the form of vaccines or adoptive immunotherapy to target tumor cells combined with treatment to enhance antigen expression by the tumor may greatly improve overall success rates of either therapy individually. This approach would allow the immune response to destroy antigen-enhanced cells that survive MEK inhibitor therapy.

Materials and Methods

Chemicals

MEK inhibitors PD98059 and U0126 were obtained from Cell Signaling Technologies (Beverly, MA). All other inhibitors were obtained from EMD Biosciences (San Diego, CA).

Cell Culture

The MU89 Melan-A/MART-1−, gp100-, and tyrosinase-positive cell line has been described (16). The low-antigen variant MUX was derived from MU89 cells (16). MM96L$^+$ and MM96L$^-$ are derived from MM96L (62), following the spontaneous divergence of the original cell line in culture into separate antigen-positive and low-antigen populations. These divergent cells were cloned by limiting dilution and termed MM96L$^+$ and MM96L$^-$ based on their melanocytic antigen levels. The common origin of these cells was confirmed by microsatellite analysis (data not shown). All cells were cultured in DMEM containing 10% fetal bovine serum at 37°C in 5% CO_2.

Apoptosis Assay for Melanoma Cells with MEK Inhibitors

Melanoma cells were treated with U0126 for various times and stained by Annexin V-FITC Apoptosis Detection kit (BioVision, Mountain View, CA). Each cell sample was then harvested with trypsin, stained with propidium iodide, Annexin V, and MART-1. Stained cells were then analyzed by flow cytometry. Percent apoptosis represents the percentage of intact cells in the Annexin V−positive and propidium iodide−negative gate.

Standard RT-PCR and Quantitative PCR

For assessments of each antigen by standard RT-PCR, 25 ng of reverse-transcribed total RNA was amplified. For β-actin assessment, 1-pg samples from the same reverse transcriptions were amplified using the same conditions (to avoid saturation owing to the relatively very high β-actin mRNA copy number per cell). RT-PCR products were electrophoresed on 1.4% agarose and confirmed for structure by sequencing. Primers used were MARTex2o.F/MARTex5o.R (Melan-A/MART-1), tyrosinase.F2/tyrosinase.R, gp100.F/gp100.R, and b-actin5′2. F/b-actin3′1.R (β-actin). (see Table 1 for primer sequences). For quantitative PCR, the total volume of each reaction was 25 μL, including 12.5 μL of Brilliant QPCR Master Mix (Stratagene, La Jolla, CA), optimized amount of primer and probe, and 25 ng (melanoma antigen-positive cells) or 100 ng (melanoma antigen-negative cells) of template. Real-time PCR (40 cycles at 95°C for 15 seconds and at 60°C for 1 minute; after initial heat activation at 95°C for 10 minutes) was done (five replicate reactions) with the Mx4000 Multiplex Quantitative PCR System (Stratagene). Data were analyzed with the Mx4000 Software Package (Stratagene). All quantitative PCR data are presented relative to the level of β-actin mRNA control. Data are normalized to 1.0 for untreated cells. Sequences for real-time PCR primers and probes are provided in Table 1.

PCR-RFLP Assay

Genomic DNA was amplified for PCR-RFLP analysis with BRAFg-RFLP.F and BRAF-RFLP.R primers. Restriction enzyme digestions of 5 μL of PCR products were carried out with 1 unit of Hpy CH4III (New England Bio Labs, Beverly, MA) at 37°C for 2 hours. After phenol extraction and ethanol precipitation, products were analyzed with electrophoresis with 4% to 20% gradient polyacrylamide gel and ethidium bromide

staining. Mutant fragments show loss of one of the two Hpy CH4III sites normally present within this segment. The specific nature of the mutations causing loss of the Hpy CH4III site was confirmed as T1796A by direct sequencing.

Flow Cytometric Analysis

Flow cytometric analyses of cytoplasmic Melan-A/MART-1 and gp100 expression were done as described previously (16). Flow cytometric analysis of pERK levels in melanoma cell lines was done according to the protocol of Cell Signaling Technologies using phospho-p44/42 MAPK monoclonal antibody (#9106; Cell Signaling Technologies). The secondary antibody was phycoerythrin-conjugated goat anti-mouse IgG antibody (BD Biosciences, San Jose, CA).

DNA and RNA Isolation and cDNA Synthesis

Genomic DNAs from melanoma cell lines were isolated by using QIAamp DNA kit (Qiagen, Valencia, CA), and corresponding total RNAs were extracted by using the RNeasy mini kit and RNase-Free DNase Set (Qiagen). cDNA synthesis was done with standard conditions using SuperScript III RNase H- reverse transcriptase (Invitrogen, Carlsbad, CA).

Analysis of BRAF and NRAS Mutations

BRAF gene mutation status at codon 600 was analyzed by PCR-RFLP (Fig. 6) and sequencing of amplified genomic DNA and cDNA. To screen for other mutations in *BRAF* cDNA in the region corresponding to exons 5 to 18, and to confirm the BRAF codon V600 mutational status assigned by RFLP, sequences of PCR fragments were obtained with the primer BRAF seq1-4. *NRAS* RT-PCR used primers NRAS.mseq.F/NRAS.mseq.R, and the products were sequenced directly to test for the presence of codon Q61 mutations. Primer sequences are shown in Table 1.

Expression Constructs

Melan-A/MART-1, wild-type, and mutant *BRAF* coding sequences were amplified by RT-PCR of appropriate melanoma mRNAs (antigen-positive and expressing wild-type or mutant *BRAF*). *BRAF* sequences were assembled from a series of shorter amplified segments. The G-C rich 5′ end of *BRAF* coding sequence required denaturants (0.5 mol/L betaine plus 2.5% DMSO) for successful amplification. The gp100 (Pmel17/SILVER) coding sequence was obtained as an IMAGE clone (3139788) from the American Type Culture Collection (Manassas, VA). All coding sequences were completely confirmed by sequencing, cloned in plasmids for transient expression under the control of the SV40 early promoter and SV40 3′ poly A signal. For stable transfections, *BRAF* coding sequences were appropriately inserted into the bicistronic vector pIRESneo3 (Clontech, Palo Alto, CA).

Transfections and Reporter Assays

Transient and stable transfections were done with the Fugene-6 reagent (Roche, Indianapolis, IN) under conditions optimized for the respective cell lines according to manufacturer's instructions. Transient cotransfections with an expression plasmid for GFP (using the E-GFP coding sequence; Clontech) were achieved with a ratio of 1:4, GFP/expression vector of interest. When the GFP expression plasmid was cotransfected along with an additional expression vector, staining for levels of endogenous antigens of interest was effected with phycoerythrin-conjugated secondary antibodies (BD Biosciences). Expression of GFP acts as an easily detectable marker for the positively transfected subpopulation of the total initial cells and allows selective fluorescence-activated cell sorting interpretation of the effects of the cotransfected plasmid within this positive subpopulation. Accordingly, in all transient tests of expression constructs, GFP-positive cells were gated and analyzed. Selection and maintenance of stable transfectants was achieved with G418 (Sigma, St. Louis, MO). For reporter assays, transfections (four replicates per determination, using 4×10^4 cells per 1 mL per 24-well plate) were done as above for Dual-luciferase assays (16) with cotransfected *Renilla* luciferase expressed from the SV40 early promoter. Cells were harvested and assayed for luciferase 48 to 72 hours after transfection.

Western Blotting

Melanoma cells were lysed in PhosphoSafe Extraction Buffer (Novagen, Madison, WI). Extracts were electrophoresed in reducing 10% SDS-polyacrylamide gels (20 μg per gel lane) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) saturated with StartingBlock Blocking Buffer (Pierce, Rockford, IL) containing 0.5% Tween 20. Blots were incubated with optimized dilutions of appropriate primary antibodies (BRAF: sc-166, Santa Cruz Biotechnology, Santa Cruz CA; pERK1/2: #9106; Cell Signaling Technologies; β-tubulin: Sigma; MITF: C5, Abcam, Cambridge, MA) and then further incubated with horseradish peroxidase–conjugated secondary antibody (Pierce). Bound antibodies were detected using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

Antigen-Specific Cytotoxicity Assays

A375 tumor cells were labeled with ^{51}Cr and cocultured for 4 hours with varying numbers of cloned CTLs specific for Melan-A/MART-1 as previously described (16). To assess the induction of antigen by MEK inhibitor, A375 tumor cells were cultured in the presence of 20 μmol/L U0126 for 6 days before labeling with ^{51}Cr in parallel with unstimulated A375 cells. As a control of antigen-specific killing, Melan-A/MART-1–expressing tumor cells, ^{51}Cr-labeled MU89, were tested in parallel with A375 cells.

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