Coexpression of NRAS^{Q61R} and BRAF^{V600E} in Human Melanoma Cells Activates Senescence and Increases Susceptibility to Cell-Mediated Cytotoxicity

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Abstract

Activating mutations in *BRAF* and *NRAS* oncogenes in human melanomas are mutually exclusive. This finding has suggested an epistatic relationship but is consistent even with synthetic lethality. To evaluate the latter possibility, a mutated NRAS^{Q61R} oncogene was expressed, under a constitutive or a doxycycline-regulated promoter, in a metastatic melanoma clone (clone 21) harboring an activated *BRAF*^{V600E} oncogene. After the first 10 to 12 *in vitro* passages, the constitutive NRAS^{Q61R} transfectant displayed progressive accumulation in G_0-G_1 phase of the cell cycle and stained for the senescence-associated β-galactosidase activity (SA-β-Gal). Inducible expression of NRAS^{Q61R}, by the Tet-Off system, in clone 21 cells (21NRAS^{61ON}) led to overactivation of the RAS/RAF/mitogen-activated protein kinase signaling pathway and, after the 10th *in vitro* passage, led to promotion of senescence. This was documented by reduced proliferation, flattened cell morphology, reduced growth in Matrigel, positive staining for SA-β-Gal, and expression of AMP-activated protein kinase and of the cell cycle inhibitor p21$^{waf1/Cip1}$. These effects were detected neither in 21 cells with silenced NRAS^{Q61R} (21NRAS^{61OFF}) nor in cells transfected with an inducible wild-type *NRAS* gene (21NRAS^{WTON}). In addition, when compared with parental 21 cells, or with 21NRAS^{61OFF}, 21NRAS^{61ON} and constitutive NRAS^{Q61R} transfectants cells showed increased susceptibility to cytotoxicity by both HLA class I antigen–restricted and nonspecific T cells and up-regulation of several MHC class I antigen processing machinery components. These results suggest a relationship of synthetic lethality between *NRAS* and *BRAF* oncogenes, leading to selection against "double-mutant" cells. (Cancer Res 2006; 66(13): 6503-11)

Introduction

Activating *RAS* mutations, mostly at codon 61 of *NRAS*, have been identified in 14% of melanoma lesions arising from soles or palms and in 20% of tumors arising from skin with chronic sun exposure but in only 3% to 5% of tumors arising from skin areas with intermittent sun exposure or from areas protected from the sun (1). In contrast, *BRAF* mutations, mostly at codon 600, have been found in 78% of the tumors from skin with intermittent UV exposure, the most common form of the disease (1). In spite of the overall high frequency of activated *NRAS* or *BRAF* in melanoma lesions, mutations of these two oncogenes have been found to be mutually exclusive in the same tumor (1–3). The lack of association between *KRAS* and *BRAF* mutations has also been found in colon, endometrial, and ovarian carcinoma lesions (4–6) and between *KRAS/NRAS/HRAS* and *BRAF* mutations in thyroid carcinoma lesions (7). However, human melanomas harboring both *NRAS* and *BRAF* mutations have been recently described (8–11). Nevertheless, analysis of clones isolated from one of such double-mutant tumors indicated that the two activating mutations are mutually exclusive at the single-cell level (10).

These findings have suggested an epistatic relationship between *RAS* and *BRAF* oncogenes (4). According to such a model, mutation of both *BRAF* and *RAS*, in cells where one or the other of these oncogenes is already activated, may not provide an additional significant growth/survival advantage. However, an alternative mechanism for mutually exclusive *NRAS* and *BRAF* mutations in melanoma cells is represented by "synthetic lethality." As recently reviewed by Kaelin, two genes are said to be synthetically lethal when mutations in either one is compatible with cell survival, whereas simultaneous mutations in both genes is not (12). Although synthetic lethality usually involves loss-of-function mutations, it could also result from gain-of-function changes, as in the instance of the activating *NRAS* and *BRAF* mutations found in melanoma cells.

To provide insights into the mechanisms that may explain mutual exclusion of activated *NRAS* and *BRAF* at the single-cell level, we developed a constitutive and a doxycycline-regulated system for expressing an oncogenic NRAS (NRAS^{Q61R}) in a metastatic melanoma clone (665/2/21) harboring wild-type *RAS* alleles (*HRAS, KRAS*, and *NRAS*) and a 1798 T/A *BRAF* mutation (*BRAF*^{V600E}) at one of the *BRAF* alleles. The results to be presented indicate that coexpression of activated *NRAS* and *BRAF* in the same melanoma cell activates a senescence program and boosts melanoma cell recognition by cytotoxic lymphocytes.

Materials and Methods

Melanoma cells. The melanoma clone 665/2/21 (thereafter named 21), harboring wild-type *RAS* genes, was obtained in our laboratory by cloning in soft agar the metastatic melanoma cell line 665/2 derived from patient 665 (HLA haplotype: A1,A9; B15,BW55; DR4,DRw6; DQw1, DQw3) as described (13). Clone 21 and the metastatic melanoma cell line Me 4473M (*NRAS*^{Q61R}; ref. 14) were routinely grown in RPMI 1640 (Bio Whittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (BD Biosciences, Clontech, San Jose, CA), 2 mmol/L L-glutamine (Bio Whittaker), 20 mmol/L HEPES buffer (Bio Whittaker), 200 units/mL penicillin (Pharmacia, Milan, Italy),

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and 40 μg/mL Gentalyn (Italfarmaco, Milan, Italy). Matrigel cultures were done by coating dishes with BD Matrigel Basement Membrane Matrix (BD Biosciences, Clontech) following the manufacturer's instructions.

Sequence analysis. Amplification of *BRAF* exon 15 and *NRAS* exon 3 was done with primers 1 to 4 (list of all primers in Supplementary Table S1) on genomic DNA isolated by standard methods from melanoma cells. Primers for pTRE-based plasmids, mapping on the response plasmid, were purchased by BD Biosciences, Clontech. Amplified products were gel purified (NucleoSpin Extract, Machinery-Nagel, Düren, Germany) and automatically sequenced using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, CA) with sequencing primers 5 to 10.

Construction of constitutive and inducible expression plasmids for NRAS^{WT} and NRAS^{Q61R}. Wild-type human *NRAS*, amplified with primers 11 and 12, was cloned by PCR into pcDNA3.1/V5/His plasmid using the Eukaryotic TOPO TA cloning Kit (Invitrogen, Carlsbad, CA) and sequenced to verify correspondence to the official *NRAS* cDNA sequence (RefSeq: NM_002524). The resulting constitutive expression vector (pcDNA3.1-Flag*NRAS*^{WT}), which contains also the Flag sequence (15), was then digested with *Bam*HI and *Not*I restriction enzymes, and the recovered fragment was cloned into *Bam*HI/*Not*I–digested response plasmid pTRE2hyg- (BD Biosciences, Clontech) to obtain pTRE2hyg-Flag*NRAS*^{WT}. pcDNA3.1-Flag*NRAS*^{WT} and pTRE2hyg-Flag*NRAS*^{WT} were then subjected to site-directed mutagenesis by the QuikChange II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) using primers 13 and 14. This yielded pcDNA3.1-Flag*NRAS*^{Q61R} and pTRE2hyg-Flag*NRAS*^{Q61R} plasmids containing the Q61R mutation, as result of an A/G substitution. The single nucleotide coding change A182G in both plasmids was confirmed by sequencing.

Generation of a melanoma clone constitutively expressing NRAS^{Q61R}. 21 cells were transfected with pcDNA3.1-Flag*NRAS*^{Q61R} plasmid and selected for 2 weeks in complete medium containing G418 (0.8 mg/mL; Sigma-Aldrich, St. Louis, MO). DNA was extracted from each of G418-resistant clones, with lysis buffer containing 50 mmol/L Tris (pH 8.5), 1 mmol/L EDTA, and 0.5% Tween 20 and amplified with GenomiPhi enzyme (Amersham Biosciences, Buckinghamshire, United Kingdom). Presence of Flag*NRAS*^{Q61R} insert was tested by PCR with primers 3 and 4, and Q61R mutation was detected by direct sequencing. A stable selected clone was named 21NRAS^{Q61R}.

Generation of melanoma clones with inducible expression of mutated or wild-type *NRAS*. The Tet-Off System (Clontech Laboratories, Palo Alto, CA) includes the tetracycline-controlled regulatory pTet-Off vector, the pTRE2hyg- response vector, and the pTRE2hyg-*Luc* control vector (see Supplementary Fig. S1*A* for details). Hygromycin B and doxycycline were both purchased from Clontech. FuGENE 6 (Roche Molecular Biochemicals, Mannheim, Germany) was used for all transfection experiments, according to the manufacturer's instructions. 21 cells were transfected with pTet-Off vector, and clones were isolated as described for the constitutive transfection. Each clone was split into two cultures in a 24-well plate and transfected with pTRE2hyg-*Luc* in presence or absence of doxycycline (1 μg/mL). Following a 48-hour incubation at 37°C, cells were harvested and lysed by Glo Lysis Buffer (Promega Corp., Madison, WI). Steady Reagent (Promega) was added to cell lysates and relative light units (RLU) were measured using a plate reader luminescence spectrophotometer (TECAN ULTRA 384, Gentronix, Manchester, United Kingdom). The RLU (−Dox)/ RLU (+Dox) ratio was calculated for each well. The clone displaying the highest RLU ratio, named 21Off, was selected as stable pTet-Off expressing recipient line for the second step of transfection. To this end, pTRE2hyg-Flag*NRAS*^{WT} or pTRE2hyg-Flag*NRAS*^{Q61R} plasmids were independently transfected into 21Off, and stable clones were selected with G418 (0.5 mg/mL) and hygromycin B (0.25 mg/mL) for 2 weeks. Antibiotic-resistant stable transfectants were propagated and maintained in the same selective medium. The resulting cell lines were tested for NRAS^{Q61R} mutation by PCR.

Immunoblotting. Protein lysates (30 μg), prepared from melanoma cells, were separated by SDS-PAGE under reducing conditions and transferred onto polyvinylidene difluoride membranes (Hybond-P, Amersham Biosciences). Membrane blocking in nonfat milk and incubation with primary antibodies were as described (16). Primary antibodies were anti-NRAS (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-actin (1:2,000), anti-extracellular signal-regulated kinase (anti-ERK; 1:5,000), and anti-activated mitogen-activated protein kinase (MAPK; Thr^{183} and Tyr^{185}; 1:10,000; Sigma-Aldrich); anti-phosphorylated AMP-activated protein kinase (anti-phospho-AMPK-α; Thr^{172}; 1:1,000), anti-Akt (1:1,000), anti-phospho-Akt (Ser^{473}; 1:1,000), anti-PTEN (1:1,000; Cell Signaling Technology, Danvers, MA); anti-p21 (BD Biosciences, PharMingen). Proteins were detected using the appropriate horseradish peroxidase–conjugated secondary antibody (goat anti-mouse IgG or goat anti-rabbit IgG) followed by development with the SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL) and autoradiography.

Ras activation assay. Activation state of NRAS was assessed by the RAS Activation Kit (Stressgen, San Diego, CA) according to the manufacturer's instruction. This assay uses a peptide corresponding to the RAS-binding domain of BRAF as a RAS-GTP–dependent probe to immunoprecipitate active NRAS from cell lysates (17). The immunoprecipitated active RAS was then detected by Western Blot analysis using the specific antibody included in the kit.

Proliferation assay. Cells were seeded in a 96-multiwell plate (Corning Inc., Corning, NY) in their own medium with or without doxycycline (1 μg/mL). A freshly prepared solution of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H* tetrazolium inner salt (MTS)/phenazine methosulfate (ratio 20:1) was added (20 μL) to each well every 24 hours, between 24 and 96 hours after beginning of the cell culture. Colorimetric evaluation of the conversion of MTS into a soluble formazan product was carried out by reading absorbance at 490 nm with a Bio-Rad 550 plate reader (Bio-Rad, Hercules, CA).

Senescence assay. Cells were cultured in their own medium with or without doxycycline (1 μg/mL) in a 12-well culture plate (Corning). Cells were fixed at 70% confluence and then incubated at 37°C overnight with the staining solution containing the X-gal substrate (Senescence Detection kit, MBL International Corp., Woburn, MA). Cells were then observed under a microscope for development of blue color.

Flow cytometry analysis. Cell cycle analysis was carried out in cultures at 50% to 60% confluency. To this end, cells were detached by a 3-minute trypsin (500 mg/L; Bio Whittaker) treatment, resuspended in cold ethanol 70%, and kept in ice for 20 minutes. After washing in Vindelov Solution (3.4 mmol/L sodium citrate, 0.01% NP40, 1.5 mmol/L Spermina Tetrahydrochloride, 0.5 mmol/L Tris, 10 μg/mL RNase), cells were stained with propidium iodide (Sigma-Aldrich). Cell cycle analysis was carried out by a FACSCalibur flow cytometer (BD Biosciences), and phases of the cell cycle were evaluated by the ModFit Software (BD Biosciences). Expression of cell surface or intracellular antigens (in saponin-permeabilized cells) was carried out as previously described (18). Monoclonal antibodies (mAb) to Delta (SY-5), MB1 (SJJ-3), LMP2 (SY-1), LMP7 (SY-3), LMP10 (TO-7), Tapasin (TO-3), Calnexin (TO-5), Calreticulin (TO-11), ERp57 (TO-2), MICB (SJJ-5), and β2-microglobulin (L368) have been described elsewhere (19–21). The TAP1-specific mAb NOB-1 and the TAP2-specific mAb NOB-2 are secreted by hybridomas derived from the fusion of murine myeloma cells P3-X63-Ag8.653 with splenocytes from BALB/c mice immunized with partial length TAP1 recombinant protein (434-735) and a keyhole limpet hemocyanin–conjugated TAP1 peptide (717-735) and with partial length TAP2 recombinant protein (316-703), respectively, by the strategy described elsewhere (20). Staining for cell surface antigens was done with mAbs directed to HLA class I (W6/32; ref. 22), HLA-DR, HLA-DQ, HLA-DP (all from BD Biosciences), integrin β_1, β_4 (BD Biosciences, PharMingen), integrin α_3, $\alpha_v\beta_3$, VLA-2, VLA-4, VLA-5, VLA-6, intercellular adhesion molecule-1 (ICAM-1), and LFA-3 (all from Chemicon International, Temecula, CA). Cells were then stained with a fluorescein-conjugated affinity purified goat anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA) and analyzed with a FACSCalibur cytofluorimeter (BD Biosciences).

Cell-mediated cytotoxicity and limiting dilution analysis of cytotoxic effector frequency. Nonspecific lymphokine-activated killer (LAK) cells were generated by culturing peripheral blood lymphocytes (PBL) of healthy donors in RPMI 1640-10% pooled human serum with 1%

phytohemagglutinin (Murex Biotech Ltd., Temple Hill, DA) for 3 days and then with interleukin-2 (IL-2) at 3,000 IU/mL (Chiron, Emeryville, CA) for 2 weeks. Lysis of targets by LAK cells was evaluated by a 4-hour ^{51}Cr release assay at effector/target cell ratios from 100:1 to 1.5:1, as described previously (23). A T-cell line was generated by culturing PBL from a HLA-mismatched healthy donor for 3 weeks in mixed lymphocytes tumor culture (MLTC) with irradiated (30,000 cGy) melanoma transfectants bearing a silenced NRAS^{Q61R}, in RPMI 1640 supplemented with 300 IU/mL IL-2 and 10% pooled human serum. The culture was restimulated weekly. The resulting T-cell line was used in a split-well limiting dilution assay, as described (23). The HLA class I antigen restriction of the lysis was assessed by measuring its inhibition by the HLA class I antigen–specific mAb W6/32, as described (23). The threshold of significant lysis, criteria to score a well containing HLA class I antigen–restricted T cells and nonrestricted cytotoxic effectors, and data analysis for frequency determination have been described elsewhere (23).

Results

Constitutive or doxycycline-regulated expression of mutated NRAS^{Q61R} in a BRAF^{V600E} melanoma clone. Melanoma clone 21 expresses wild-type *HRAS, KRAS, NRAS* genes (13) but harbors a heterozygous BRAF^{V600E} mutation (Supplementary Fig. S1*B*) and bears a homozygous inactivating Tyr > His mutation at codon 236 in exon 7 of *p53* gene (24) and a homozygous deletion of exon 3 of the *CDKN2A* locus that prevents p16^{INK4A} and p14^{ARF} expression (data not shown). This clone was used for generating cells with constitutive or inducible expression of an exogenous NRAS^{Q61R}. Constitutive NRAS^{Q61R} expression was achieved by transfecting with pcDNA3.1-Flag*NRAS*^{Q61R}. Out of 100 clones initially isolated, 54 grew in selective medium, and 18 of the latter maintained the mutated insert. However, only 1 of the 18 (thereafter named 21NRAS^{Q61R}) expressed the exogenous mutated NRAS at the protein level (Supplementary Fig. S2*A*). The mutated NRAS could be distinguished from the endogenous NRAS thanks to the reduced electrophoretic mobility caused by the addition of the Flag peptide to the *NRAS* transgene (Fig. 1*A*). To generate cells with inducible NRAS^{Q61R} expression, clone 21 cells were initially transfected with the regulatory pTet-Off vector. Clones expressing a functional regulator tTA protein were identified by a luciferase assay detecting doxycycline-regulated light emission 48 hours after transient transfection with the pTRE2hyg-*Luc* reporter plasmid. One of the 22 responsive clones, named 21Off, was selected for subsequent experiments because it showed doxycycline dose-dependent RLU reduction and, at 1 μg/mL doxycycline, reached RLU values similar to those displayed by nontransfected cells (Fig. 1*B*). 21Off cells were then transfected with plasmids encoding mutated (pTRE2hyg-Flag*NRAS*^{Q61R}) or wild-type (pTRE2hyg-Flag*NRAS*^{WT}) NRAS. Eight of 48 hygromycin-resistant clones, transfected with pTRE2hyg-Flag*NRAS*^{Q61R}, grew in selective medium; however, only two of them expressed the oncogenic NRAS (Supplementary Fig. S2*B*). The presence of mutated *NRAS* and *BRAF* in these two clones was confirmed by sequence analysis (Supplementary Fig. S1*C*). In contrast, 30 of 48 clones transfected with pTRE2hyg-Flag*NRAS*^{WT} kept growing and expressed the wild-type inserted *NRAS* (Supplementary Fig. S2*B*). Expression and doxycycline-dependent regulation of the transgenic mutant or wild-type NRAS was evaluated by Western blotting analysis (Fig. 1*C*). In the absence of doxycycline, oncogenic NRAS as well as the wild-type endogenous NRAS were detected using NRAS-specific mAb. Addition of doxycycline (1 μg/mL) to the culture medium inactivated the transcription of the exogenous NRAS in a time-dependent fashion. The gene was completely silenced following a 48-hour incubation (Fig. 1*C*). In contrast, no effect was detected on the transcription of the endogenous gene. A clone with inducible NRAS^{Q61R} expression (thereafter named 21NRAS^{61ON} or 21NRAS^{61OFF}, depending on expression or not of the exogenous mutant NRAS) and a clone with inducible wild-type NRAS expression (thereafter named 21NRAS^{WTON} or 21NRAS^{WTOFF}, depending on expression or not of the exogenous wild-type NRAS) were selected for further experiments.

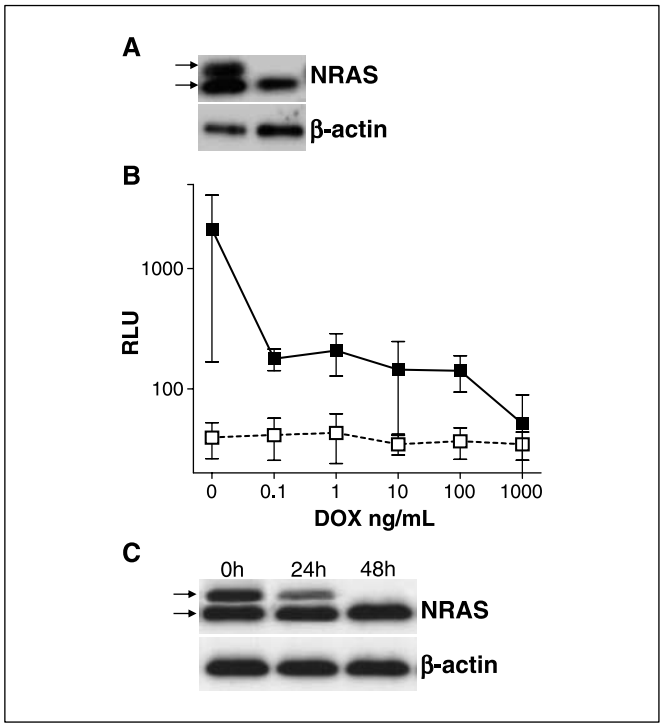

Figure 1. Constitutive and doxycycline-regulated expression of mutated NRAS^{Q61R} in BRAF^{V600E} melanoma clone 21. *A,* expression of Flag-tagged NRAS^{Q61R} in 21NRAS^{Q61R} cells by Western blot analysis using NRAS-specific mAb (*top arrow,* exogenous Flag-tagged NRAS^{Q61R}; *bottom arrow,* endogenous wild-type NRAS). *B,* luciferase activity of 21Off cells either untransfected (□) or transiently transfected with pTRE2hyg-*Luc* (■) and cultured for 48 hours at 37°C in the absence or in presence of increasing doxycycline (*DOX*) concentrations. *Points,* mean RLU of triplicate measurements; *bars,* SD. *C,* time course silencing of NRAS^{Q61R} transgene. 21NRAS^{61ON} cells were cultured in the absence or with doxycycline (1 μg/mL) for up to 48 hours. Cell lysates were then tested by Western blot analysis with an NRAS-specific mAb (*top arrows,* exogenous Flag-tagged NRAS^{Q61R}; *bottom arrows,* endogenous wild-type NRAS). β-Actin mAb was used as loading control.

NRAS activation, ERK, and Akt phosphorylation in "double-mutant" 21NRAS^{61ON} cells. To assess the function of the exogenous activated *NRAS* oncogene, we used the RAS-binding domain of BRAF as a RAS-GTP–dependent probe (17) to immunoprecipitate active NRAS from cell lysates. As shown in Fig. 2, more activated NRAS protein was immunoprecipitated from 21NRAS^{61ON} than from 21NRAS^{61OFF} cells. Doxycycline-regulated expression of the mutated NRAS^{Q61R} led to levels of active NRAS protein similar to those found in the human melanoma cell line Me 4473M harboring a mutated *NRAS*^{Q61R} allele (Fig. 2). In addition, active NRAS levels were similar in 21NRAS^{61OFF} and in the parental clone 21 cells (Fig. 2). Because NRAS mutations can result in dual activation of MAPK and phosphatidylinositol 3-kinase (PI3K) pathways (1, 11), we then evaluated levels of total and phosphorylated ERK and Akt in 21 cells with expressed or silenced NRAS^{Q61R}. Total endogenous NRAS and total amount of ERK

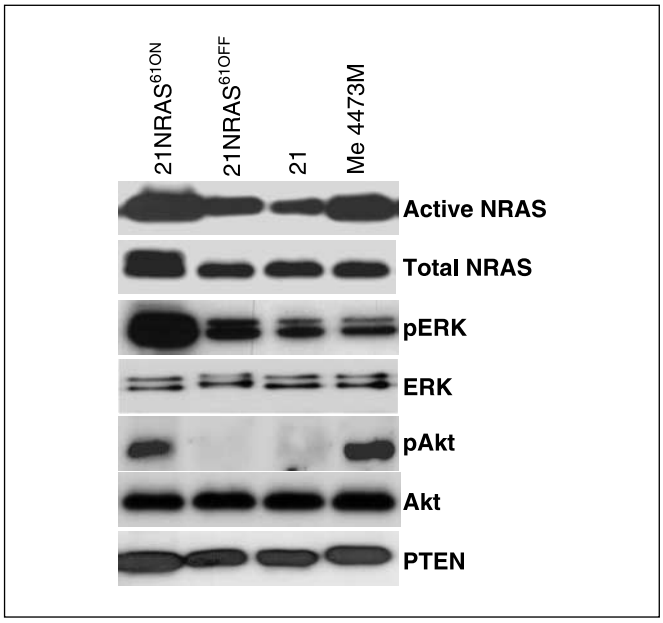

Figure 2. NRAS activation, ERK, and Akt phosphorylation in 21NRAS^{61ON} cells. Western blot analysis of active NRAS immunoprecipitated from 21NRAS^{61ON}, 21NRAS^{61OFF}, clone 21 (BRAF^{V600E}), and melanoma Me 4473M (NRAS^{Q61R}), and of total NRAS, phosphorylated ERK (*pERK*), ERK, phosphorylated Akt (*pAkt*), Akt, and PTEN from cell lysates of the same samples.

proteins were similar in all cell lines (Fig. 2). In contrast, levels of phosphorylated ERK were much higher in 21NRAS^{61ON} cells compared not only with 21NRAS^{61OFF} cells but even with the parental clone 21 expressing only BRAF^{V600E} and with melanoma Me 4473M expressing only NRAS^{Q61R}. Moreover, level of total Akt was similar in all samples, whereas phosphorylated Akt was found only in cells bearing an activated in NRAS^{Q61R} (21NRAS^{61ON} cells and Me 4473M). PTEN protein expression was found in all the samples (Fig. 2). Similar results were obtained by comparing the constitutive 21NRAS^{Q61R} transfectant with the parental clone 21 cells (data not shown). These results indicate that coexpression of activated *NRAS* and *BRAF* oncogenes in the same melanoma cells is associated with overactivation of components of the RAS effector cascade.

Senescent phenotype develops in BRAF^{V600E} melanoma cells after constitutive or inducible expression of NRAS^{Q61R}. *In vitro* growth properties of the 21NRAS^{Q61R} constitutive transfectant were similar to those of the parental clone 21 in the early *in vitro* passages. However, after the 12th *in vitro* passage, 21NRAS^{Q61R} cells began to lengthen their doubling time (data not shown). Cell cycle analysis between the 12th and the 17th *in vitro* passage indicated a progressive accumulation of 21NRAS^{Q61R} cells in the G_0-G_1 phase of the cell cycle (25.75-51.90%; Fig. 3*A*) and a corresponding decrease in the fraction of cells in S phase (64.12-29.38%). No such changes were seen in the parental clone 21, nor in a clone that underwent the same selection procedure as 21NRAS^{Q61R}, but did not express mutated NRAS (21NRAS^{WT} in Fig. 3*A*). Furthermore, by a colorimetric assay, using X-gal as a substrate at pH 6, 21NRAS^{Q61R} cells at the 12th *in vitro* passage began to stain positive for the senescence-associated β-galactosidase marker (SA-β-Gal; Fig. 3*B*), consistent with activation of cellular senescence (25), whereas both 21NRAS^{WT} and clone 21 cells remained negative.

Growth of 21NRAS^{61ON} cells in comparison with 21NRAS^{61OFF} also slowed down after the 10th *in vitro* passage. Proliferation was therefore quantified using an MTS assay. As shown in Fig. 4, $A_{490\ nm}$ values were similar in 21 cells cultured in presence or absence of doxycycline, in 21NRAS^{WTON} and 21NRAS^{WTOFF} (at different *in vitro* passages), in 21NRAS^{61ON} (9th *in vitro* passage), and 21NRAS^{61OFF} (both at 9th and 15th *in vitro* passages). In contrast, absorbance values were significantly lower in 21NRAS^{61ON} cells, at the 15th *in vitro* passage, compared with all other samples. A reduced growth of 21NRAS^{61ON} cells at the 15th *in vitro* passage was also observed following 1 week culture in Matrigel (Fig. 4*B*), an ECM protein gel produced by the EHS tumor and known to be highly effective in supporting cell proliferation (26, 27). After seeding into Matrigel, cells expressing NRAS^{Q61R} either did not divide or formed much smaller colonies than 21NRAS^{61OFF} cells (Fig. 4*B*), whereas 21NRAS^{WTON} and 21NRAS^{WTOFF} could grow to a similar extent (Supplementary Fig. S3). In addition, beyond the 10th *in vitro* passage, 21NRAS^{61ON} cells became larger and flatter than 21NRAS^{61OFF} cells (Fig. 4*C*), a cell morphology usually associated with a senescent phenotype (28, 29) and not observed in either 21NRAS^{WTON} or 21NRAS^{WTOFF} (Supplementary Fig. S3). At this stage, 21NRAS^{61ON} cells, like the constitutive 21NRAS^{Q61R}, began to stain positive for the senescence-associated marker SA-β-Gal activity, whereas 21NRAS^{61OFF} as well as 21NRAS^{WTON} and 21NRAS^{WTOFF} did not (Fig. 4*C*; Supplementary Fig. S3). 21NRAS^{61ON} and 21NRAS^{61OFF} cells were then evaluated for the expression of additional markers of senescence. Phospho-AMPK, a serine/threonine protein kinase recently shown to be up-regulated in senescent cells (30–32), was detected in the inducible 21NRAS^{61ON} and in the constitutive 21NRAS^{Q61R} transfectants but only at late *in vitro* passages (15th and 14th, respectively; Fig. 4*D*), whereas it was not expressed in all other cells, including presenescent 21NRAS^{61ON} (9th *in vitro* passage) and 21NRAS^{Q61R} (8th *in vitro* passage). Cyclin-dependent kinase inhibitor p21$^{waf1/Cip1}$ (p21), a mediator of growth arrest (33), could not be detected in nonsenescent 21NRAS^{61OFF}, 21NRAS^{WTON}, and 21NRAS^{WTOFF} (all passages tested) but was expressed in 21NRAS^{61ON} and 21NRAS^{Q61R}, at the senescent and the presenescent stages. Both p21 and phospho-AMPK were not detected in melanoma cells expressing only BRAF^{V600E} (clone 21) or only NRAS^{Q61R} (Me 4473M). Expression of several molecules involved in the p53 and p16/pRB pathways, like p53 itself, proliferating cell nuclear antigen, MDM2, and phosphorylated and underphosphorylated Rb did not change when activated NRAS and BRAF oncogenes were expressed both in the same cell (data not shown). Taken together, these results suggest that coexpression of activated NRAS and BRAF in the same melanoma cells can activate a senescence program that induces cell growth arrest.

Coexpression of activated NRAS and BRAF affects immune recognition and antigen processing machinery phenotype of melanoma cells. Additional mechanisms may contribute to selection against "double-mutant" tumor cells bearing activated *NRAS* and activated *BRAF* oncogenes. To provide evidence in support of this possibility, we evaluated susceptibility of constitutive and inducible NRAS^{Q61R} transfectants to cell-mediated cytotoxicity, as the immune system is the main extrinsic suppressor of tumor growth (34). The constitutive transfectant 21NRAS^{Q61R} was significantly (P = 0.0078) more susceptible, in terms of lytic units, to cell-mediated lysis, by IL-2-activated nonspecific LAK effectors generated from six different donors compared with parental clone 21 cells (Fig. 5*A*). A limiting dilution assay for

the determination of cytotoxic T-cell effector frequency (35) was then used to compare 21NRAS^{61ON} and 21NRAS^{61OFF} cells for HLA-restricted and nonrestricted cytotoxic T cell–mediated lysis. To this end, an alloreactive T-cell line, established from PBL of an HLA-mismatched donor by repeated MLTC stimulation with 21NRAS^{61OFF}, was used. Frequency of all lytic effectors (i.e., independently from inhibition of lysis by anti-HLA class I mAb) and of HLA class I–restricted alloreactive cytotoxic T-cell effectors cells was much higher on 21NRAS^{61ON} than on 21NRAS^{61OFF} targets, in spite of the fact that the effectors had been selected on the latter targets (Fig. 5*B*). Lysis of 21NRAS^{61ON} was significantly higher than lysis of 21NRAS^{61OFF} (P = 0.0189; Fig. 5*C*) even by the autologous HLA class I–restricted CTL clone 8B3 that was previously isolated from tumor-infiltrating lymphocytes (TIL) of patient 665 and was shown to be able to specifically lyse melanoma 665/2 cells (13).

To determine whether the increased susceptibility to cell-mediated lysis of 21NRAS^{61ON} cells reflected changes in the expression of molecules involved in target-cytotoxic cell interactions, 21NRAS^{61ON} and 21NRAS^{61OFF} cells were then compared for the expression of a large number of cell surface and intracellular molecules, including HLA class I antigen processing machinery (APM) components. These molecules play a major role in the generation of HLA class I-peptide complexes, which are recognized by CD8$^+$ CTL. Flow cytometry analysis of cells intracellularly stained with APM component-specific mAb showed a higher expression of constitutive proteasome subunits delta and MB1; of immunoproteasome subunits LMP2, LMP7, and LMP10; and of chaperones calnexin and calreticulin and ERp57 in 21NRAS^{61ON} cells than in 21NRAS^{61OFF} cells (Fig. 6*A*). No differences in expression were detected between the two cell types in the expression of the transporter associated with antigen processing (TAP) subunits TAP1 and TAP2 and of the chaperone tapasin. Furthermore, 21NRAS^{61ON} cells and 21NRAS^{61OFF} cells did not differ in the cell surface expression of HLA class I antigens; HLA-DR, HLA-DQ, HLA-DP antigens; $\alpha_v\beta_3$ integrin; β_1, β_4, α_2, α_3, α_4, α_5, and α_6 integrin subunits; and adhesion molecules ICAM-1 and LFA-3 (Fig. 6*A*). In addition, all APM molecules found differentially expressed in 21NRAS^{61ON} cells compared with 21NRAS^{61OFF} cells showed a similar expression pattern in 21Off cells treated or not with doxycycline (Fig. 6*B, top*). Moreover, 21 cells transfected with an inducible wild-type NRAS (21NRAS$^{WTON/OFF}$) did not show differential expression of the APM components when treated or not with doxycycline (Fig. 6*B, bottom*). Lastly, the death receptors Fas/CD95, TRAIL-R1 TRAIL-R2,

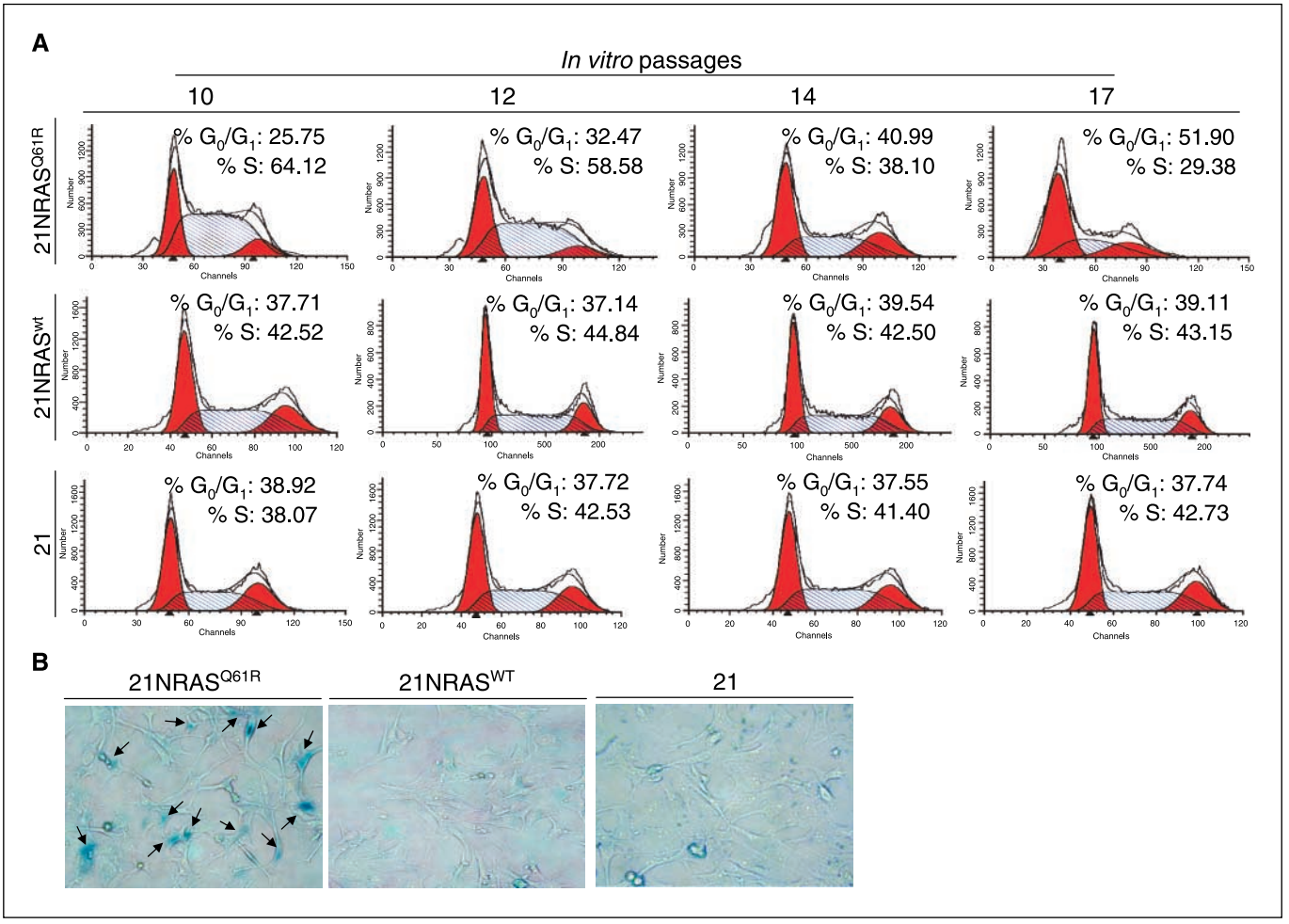

Figure 3. Accumulation in the G_0-G_1 phase of the cell cycle and expression of a senescence-associated marker in BRAF^{V600E} melanoma cells with constitutive NRAS^{Q61R} expression. *A,* cell cycle analysis of 21NRAS^{Q61R} cells, compared with 21NRAS^{WT} and with parental 21 cells between the 10th and the 17th *in vitro* passage. *B,* SA-β-Gal staining of the parental 21 line, of 21NRAS^{Q61R} cells, and 21NRAS^{WT} (both at the 14th *in vitro* passage).

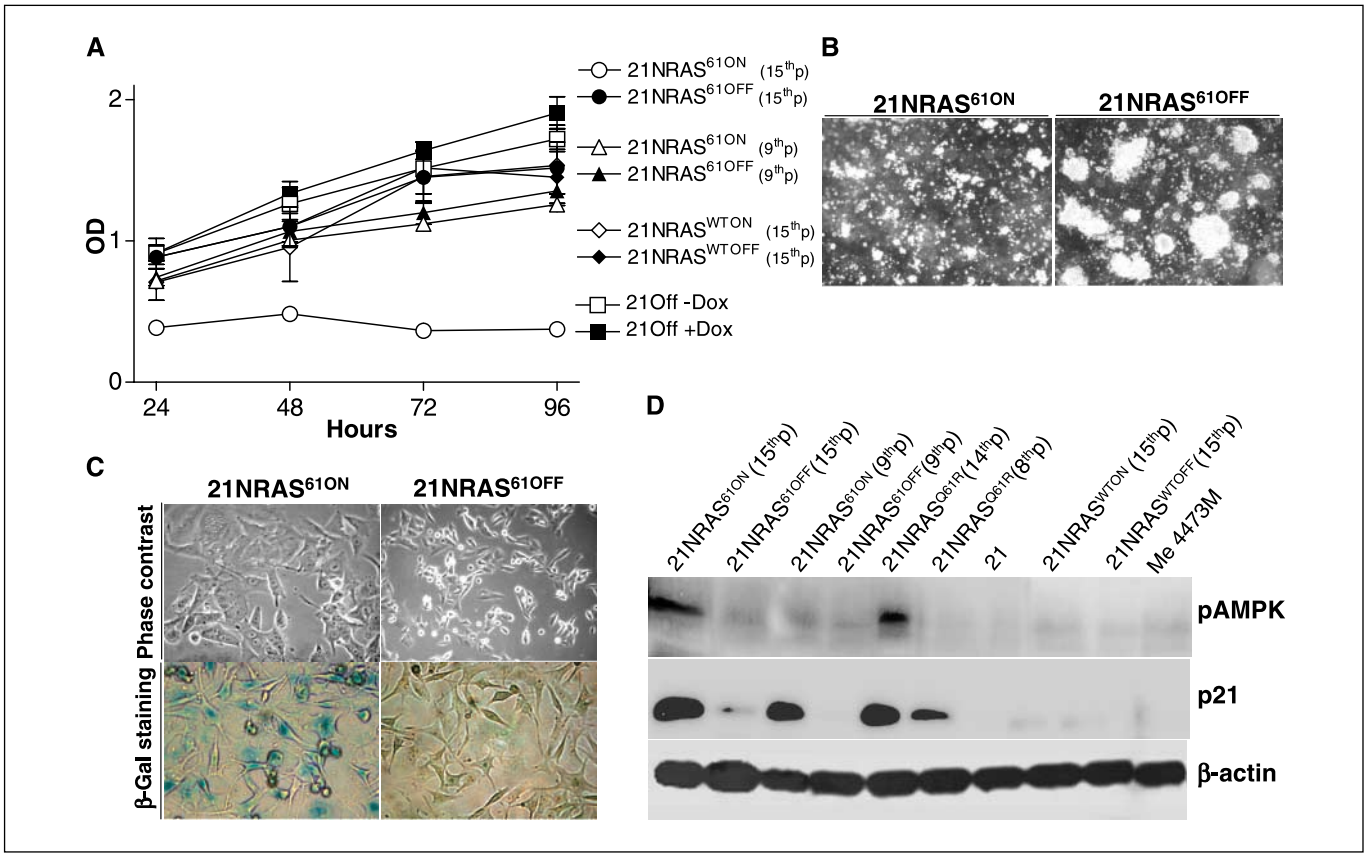

Figure 4. Differential growth characteristics and expression of senescence-associated markers in 21NRAS^{61ON} and 21NRAS^{61OFF} cells. *A,* proliferation, evaluated by MTS assay, of 21NRAS^{61ON} and 21NRAS^{61OFF} (at both the 9th and at the 15th *in vitro* passage), of 21NRAS^{WTON} and 21NRAS^{WTOFF} (at the 15th *in vitro* passage), and of 21Off cells cultured with/without doxycycline (*Dox*; 1 μg/mL). *Points,* mean $A_{490\ nm}$ (*OD*) of triplicates of one of three experiments; *bars,* SD. $A_{490\ nm}$ values of 21NRAS^{61ON} at the 15th *in vitro* passage were significantly different at all time points from those of the other samples (ANOVA followed by SNK test, $0.001 < P < 0.05$). *B,* growth in Matrigel for 1 week of 21NRAS^{61ON} and 21NRAS^{61OFF} cells at the 15th *in vitro* passage. Magnification, ×40. *C,* morphology and senescence-associated β-Gal activity of 21NRAS^{61ON} compared with 21NRAS^{61OFF} cells at the 15th *in vitro* passage. *D,* Western blot analysis of 21NRAS^{61ON} and 21NRAS^{61OFF} at both the senescent (15th) and nonsenescent (9th) *in vitro* passage, of constitutive transfectant 21NRAS^{Q61R} at both senescent (14th) and nonsenescent (8th) *in vitro* passage, of clone 21 (BRAF^{V600E}), of 21NRAS^{WTON} and 21NRAS^{WTOFF} at the 15th *in vitro* passage, and melanoma Me 4473M (NRAS^{Q61R}) for expression of phospho-AMPK (*pAMPK*), p21, and β-actin (as loading control).

TRAIL-R3, and TRAIL-R4 were not differentially expressed in 21NRAS^{61ON} and 21NRAS^{61OFF} cells (data not shown).

Taken together, these data indicate that coexpression of activating NRAS and BRAF mutations in the same melanoma cells may affect recognition by both nonspecific and HLA-restricted cytotoxic effectors and expression of several APM molecules involved in the generation of HLA class I–peptide complexes recognized by T cells.

Discussion

Early evidence on mutual exclusion of activating *KRAS* and *BRAF* mutations in colorectal cancer suggested that *RAS* and *BRAF* oncogenes may exert equivalent effects in tumorigenesis, due to the common MAP/ERK kinase (MEK)-ERK effector cascade they regulate (4). In agreement with this possibility, in melanoma, concomitant mutation of BRAF and loss of PTEN have been described (11, 36). Such condition could induce biochemical effects similar to an NRAS mutation, as activated NRAS can result in dual activation of MAPK and PI3K pathways (1, 11). In agreement, in this study, both these pathways were activated in melanoma clone 21 after expression of a mutated NRAS oncogene. However, other studies have identified NRAS or BRAF mutation-specific gene expression profiles in thyroid carcinoma and in melanoma (7, 37) and a selective susceptibility to MEK inhibition in BRAF mutant tumors but not in NRAS mutant tumors (38). These data suggest that activated *NRAS* and *BRAF* oncogenes may not necessarily drive equivalent phenotypic effects. In addition, analysis of clones isolated from a "double-mutant" melanoma indicated that NRAS and BRAF activating mutations do not coexist in the same cells (10), suggesting that the two mutations may be incompatible with cell survival. This possibility is in agreement with the concept of synthetic lethality recently reviewed by Kaelin (12). Genes having a synthetic lethal relationship may be identified among those that behave as "double-edged" swords for neoplastic cells, as able to deliver signals that may promote or inhibit cell proliferation and/or survival (12). Interestingly, both activated RAS and RAF proteins can play such contrasting roles depending on cell context and on the function/deficiency of other pathways, including PI3K, Rb, and the p53 pathways. Thus, activated NRAS contributes to neoplastic transformation of human melanocytes and to development of invasive melanomas, when the Rb and p53 pathways are inhibited (39). Similarly, activated BRAF is a transforming oncogene in immortalized melanocytes (40). On the other hand, several recent articles have shown that premalignant cells from different tissues can activate a senescence program in response to oncogenic RAS

(29), and that activated BRAF triggers senescence in melanocytes from nevi (41).

In agreement with the hypothesis that oncogenic NRAS and BRAF may be synthetically lethal, this study shows that after the first 9 to 10 *in vitro* passages, the constitutive and the doxycycline-regulated transfectants expressing both NRAS^{Q61R} and BRAF^{V600E} had reduced *in vitro* growth compared with control cells, progressive accumulation in G_0-G_1 phase of the cell cycle, and positive staining for the acidic SA-β-Gal. Furthermore, analysis of the doxycycline-regulated transfectants provided additional evidence for senescence, as indicated by increased levels of phospho-AMPK and of the cyclin-dependent kinase inhibitor p21. Activation of apoptosis as an alternative mechanism leading to elimination of the cells expressing NRAS^{Q61R} and BRAF^{V600E} cannot be ruled out. However, no evidence consistent with programmed cell death, such as cell shrinkage, membrane blebbing, and formation of apoptotic bodies (42), was observed in the cultures. Taken together, these findings suggest that constitutive or conditional expression of a mutant NRAS oncogene in a metastatic melanoma naturally expressing a BRAF^{V600E} oncogene is sufficient, in the absence cell cycle regulators, like p16/pRB and p14^{ARF}/p53 (28, 29), to activate senescence.

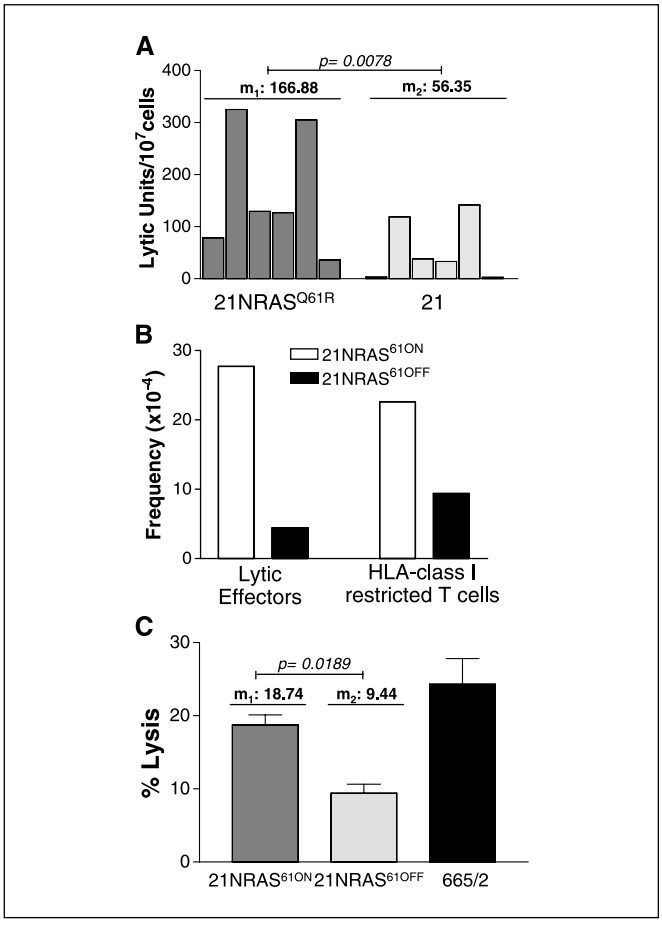

Figure 5. Increased susceptibility to cell mediated lysis of melanoma cells expressing constitutive or inducible NRAS^{Q61R}. *A,* lysis of 21NRAS^{Q61R} compared with clone 21 by LAK cells generated from six donors. Results expressed as lytic units (lytic units/10^7 lymphocytes, based on 30% target cell lysis). m_1 and m_2: means of the six lytic unit values corresponding to 21NRAS^{Q61R} and 21 cells, respectively; *p*: *P* obtained from a two-tailed *t* test of the two data sets. *B,* frequency of cytotoxic cells (lytic effectors) and of HLA class I–restricted cytotoxic T-cell effectors recognizing 21NRAS^{61ON} and 21NRAS^{61OFF} as evaluated by LDA with an alloreactive T-cell line generated by MLTC against 21NRAS^{61OFF} cells. Frequency of cytotoxic effectors expressed as cytotoxic cells/10^4 lymphocytes. *C,* lysis of 21NRAS^{61ON}, 21NRAS^{61OFF}, and parental 665/2 tumor by the autologous HLA class I–restricted CTL clone 8B3. m_1 and m_2: means of the % lysis relative to 21NRAS^{61ON} and 21NRAS^{61OFF}, respectively; *p*: *P* obtained by a two-tailed *t* test on the two data sets.

Cellular senescence is a genetic program leading to irreversible cell cycle arrest and can be mediated by several genes, including some oncogenes (28, 29). In fact, in primary murine and human cells activated components of the RAS/RAF/MAPK signaling cascade are known to trigger senescence responses that prevent the unlimited expansion of these cell types (43–45). The selective association of senescence in 21NRAS^{61ON} cells with p21 up-regulation is in agreement with the described requirement for this cell cycle inhibitor in senescence associated with high levels of oncogenic RAS activity (33, 45). Although p21 is the main target of p53, several p53-independent mechanisms for its induction have also been reported. They may underlie p21 induction in double-mutant 21NRAS^{61ON} cells, where p53 is functionally deficient (data not shown). For example, in the p53-negative U937 myeloid cell line, NRAS-induced senescence was dependent on p21 up-regulation mediated by IFN regulatory factor-1 (46). In human diploid fibroblasts undergoing replicative senescence, enzymatic components of the cyclic guanosine 3′,5′-monophosphate (cGMP) pathway were inhibited and exposure of colorectal cancer cells to a pharmacologic inhibitor of cGMP synthesis induced activation of p21 and senescence (47). In melanoma cells and melanocytes, senescence seems to be regulated by the basic helix-loop-helix leucine zipper microphthalmia-associated transcription factor (MITF, ref. 48). MITF is able to induce a G_1 cell cycle arrest that is dependent on p21 activation through modulation of its direct transcriptional repressor Tbx2 (49, 50). BRAF^{V600E} signaling seems to down-regulate MITF at a level compatible with cell proliferation because high MITF levels have antiproliferative effects (51). Low MITF levels are linked to reduced survival rates and increased metastases in patients with intermediate thickness melanoma (52). However, induction of p21 in 21NRAS^{61ON} cells is independent from MITF up-regulation because both senescent 21NRAS^{61ON} and nonsenescent 21NRAS^{61OFF} cells had similar levels of MITF protein expression (data not shown). It cannot, however, be ruled out that differential MITF transcriptional activity as a result of increased MAPK signaling could play some role in senescence induction (53). In addition to induction of p21 expression, senescent 21NRAS^{61ON} cells displayed up-regulation of AMPK. This metabolic enzyme is a key regulator of response to low ATP levels and is induced to prevent ATP-consuming processes under conditions of low cellular energy (30). Recently, AMPK activity was found to be increased in cells undergoing senescence, whereas its overactivation promoted senescence in primary human fibroblasts (31, 32).

Activation of senescence, by coexpression of oncogenic NRAS and BRAF in the same melanoma cells, may not be the only mechanism leading to selection against "double-mutant" cells. Because the immune system is the main extrinsic tumor suppressor factor, we evaluated whether cells with expressed or silenced NRAS^{Q61R} had significantly different susceptibility to cell-mediated cytotoxicity by measuring frequencies of both HLA class I–restricted and nonrestricted cytotoxic effectors directed against 21NRAS^{61ON} or 21NRAS^{61OFF}. Due to the lack of autologous lymphocytes, these effectors were generated by

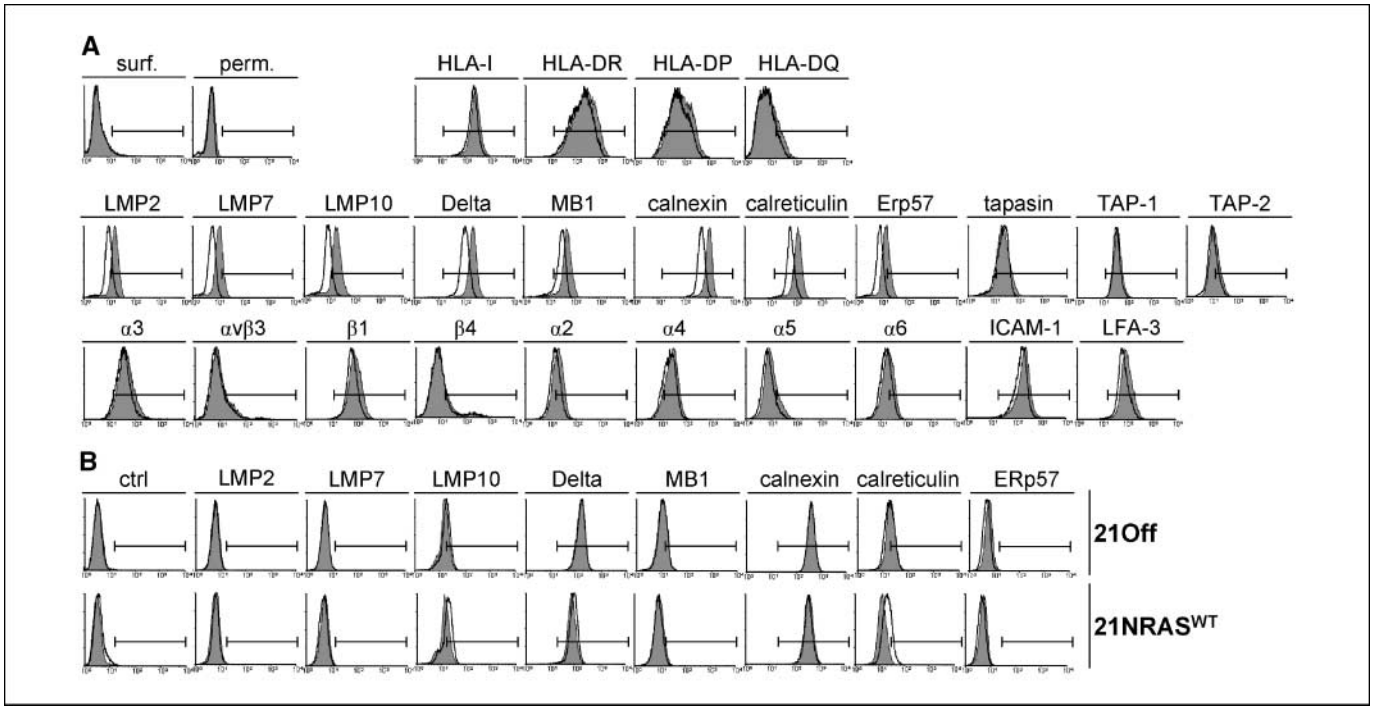

Figure 6. APM component up-regulation in BRAF^{V600E} melanoma cells expressing inducible NRAS^{Q61R}. *A,* flow cytometry analysis for cell surface molecules (HLA class I and II antigens; $\alpha_v\beta_3$ integrin; β_1, β_4, α_2, α_3, α_4, α_5, and α_6 integrin subunits; ICAM-1 and LFA-3 adhesion molecules) and for intracellular APM components (delta, MB1, LMP2, LMP7, LMP10, TAP-1, TAP-2, calnexin, calreticulin, ERp57, tapasin) in 21NRAS^{61ON} cells (*gray histograms*) and 21NRAS^{61OFF} (*empty histograms*). *surf.,* nonpermeabilized cells stained with secondary antibody only; *perm.,* permeabilized cells stained with secondary antibody only. *B, top,* flow cytometry analysis in permeabilized cells for selected APM components in 21Off cells without doxycycline (*gray histograms*) or with 1 μg/mL doxycycline (*empty histograms*). *Bottom row,* flow cytometry analysis in permeabilized cells for selected APM components in 21NRAS^{WTON} (*gray histograms*) or 21NRAS^{WTOFF} (*empty histograms*). *ctrl,* cells stained with secondary antibody only.

repeated restimulation of lymphocytes from an HLA-mismatched donor with 21NRAS^{61OFF} cells in MLTC. The results indicated that the frequency of cytotoxic effectors was markedly higher on 21NRAS^{61ON} than on 21NRAS^{61OFF} either when the cytotoxicity assay was done in the presence or absence of W6/32 mAb to inhibit the HLA class I antigen–restricted component of the lysis. Differential lysis of 21NRAS^{61ON} and 21NRAS^{61OFF} cells was observed even by using an autologous HLA class I–restricted CTL clone isolated from TILs and previously characterized (13). Interestingly, increased lysability of 21NRAS^{61ON} was associated with up-regulation of a specific set of components of the HLA APM that contribute to generation of MHC-peptide complexes recognized by T cells (54). These data, together with those indicating activation of senescence, are consistent with the possibility that double-mutant cells, harboring both activated *NRAS* and *BRAF* oncogenes, may be selected against by cell autonomous and extrinsic mechanisms.

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