Mutation of B-Raf in Human Choroidal Melanoma Cells Mediates Cell Proliferation and Transformation through the MEK/ERK Pathway* 

Received for publication, August 6, 2003
Published, JBC Papers in Press, August 12, 2003, DOI 10.1074/jbc.M308709200

Armelle Calipel†‡, Gaëlle Lefevre†‡, Celio Pouponnot†, Frédéric Mouriaux‡, Alain Eychène‡, and Frédéric Mascarelli‡¶

From the †Institut Biomédical des Cordeliers, INSERM U450, 75006 Paris, France and ‡Unité Mixte de Recherche-146 CNRS, Institut Curie, 91406 Orsay, France

The BRAF gene, encoding a mitogen-activated protein kinase kinase kinase, is mutated in several human cancers, with the highest incidence occurring in cutaneous melanoma. The activating V599E mutation accounted for 80% of all mutations detected in cutaneous melanoma cell lines. Reconstitution experiments have shown that this mutation increases ectopically expressed B-Raf kinase activity and induces NIH3T3 cell transformation. Here we used tumor-derived cell lines to characterize the activity of endogenous mutated B-Raf protein and assess its specific role in transformation. We show that three cell lines (OCM-1, MKT-BR, and SP-6.5) derived from human choroidal melanoma, the most frequent primary ocular neoplasm in humans, express B-Raf containing the V599E mutation. These melanoma cells showed a 10-fold increase in endogenous B-RafV599E kinase activity and a constitutive activation of the MEK/ERK pathway that is independent of Ras. This, as well as melanoma cell proliferation, was strongly diminished by siRNA-mediated depletion of the mutant B-Raf protein. Moreover, blocking B-RafV599E-induced ERK activation by different experimental approaches significantly reduced cell proliferation and anchorage-independent growth of melanoma cells. Finally, quantitative immunoblot analysis allowed us to identify signaling and cell cycle proteins that are differentially expressed between normal melanocytes and melanoma cells. Although the expression of signaling molecules was not sensitive to U0126 in melanoma cells, the expression of a cluster of cell cycle proteins remained regulated by the B-RafV599E/MEK/ERK pathway. Our results pinpoint this pathway as an important component in choroidal melanoma cell lines.

One of the main hallmarks of cancer is the acquisition by transformed cells of self-sufficiency in growth signals. In contrast, normal cells require mitogenic growth signals transmitted by transmembrane receptors to enter the cell cycle (1). The Raf/MEK1 (MAPK or ERK kinase)/ERK (extracellular signal-regulated kinase) module of the mitogen-activated protein kinase cascade is a major intracellular mediator of mitogenic signaling that regulates numerous biological processes (2, 3). Activation of this cascade downstream of membrane-bound receptors occurs through the interaction of Raf proteins with the small GTPase Ras upon its conversion to the GTP loaded, activated form. Mutations in genes encoding components of the Ras/Raf/MEK/ERK signaling pathway contribute to the development of many human cancers. Point mutations in HRAS, KRAS, and NRAS that lead to the constitutive activation of their protein products are found in ~30% of all human cancers (4), suggesting that the deregulation of downstream effectors of Ras is involved in oncogenesis. Although constitutive activation of ERK1/2 was detected in 36% of 50 studied human tumor cell lines (5), no mutations have been found in either ERK1/2 or MEK1/2 in human tumors. However, mutations in the BRAF gene were detected recently in ~20% of 530 human tumoral cell lines (6). B-Raf, the major MEK activator, has a higher affinity for and is a more efficient activator of MEK1 and MEK2 than Raf-1 (7–11). Two classes of BRAF gene mutation have been detected in the kinase domain of B-Raf; both of them lead to a constitutive increase in its kinase activity (6). One consists of single amino acid substitutions within the glycine-rich loop located in exon 11 of BRAF. These mutations are frequently associated with a RAS gene mutation. The other consists of single amino acid substitutions within the activation loop (exon 15 of BRAF). This class is never associated with a RAS gene mutation (6). B-Raf is more strongly activated by the second class of mutations, which may explain why the first class is always found associated with a RAS mutation. The highest incidence of BRAF mutations was found in cutaneous melanomas. Sixty-six percent of malignant cutaneous melanomas contain somatic missense mutations in BRAF, and the V599E single substitution in exon 15 of BRAF accounts for ~80% of all mutations in this cancer (6).

In contrast to cutaneous melanomas, which have been extensively studied, little is known about the molecular pathogenesis of choroidal melanomas. Choroidal melanoma is the most frequent primary intraocular neoplasm in adult humans in western countries. Cutaneous and choroidal melanomas share a common embryological origin, the neural crest, and similar...
histological features, but they differ in epidemiological and cytogenetic aspects. No RAS mutations have been detected in choroidal melanoma (12, 13), and no data concerning BRAF mutations in choroidal melanoma were reported by Davies et al. (6), leaving open the question of the involvement of the Ras/Raf/MEK/ERK signaling pathway in this cancer. In addition, despite the identification of mutations in the BRAF gene, the specific role of endogenous mutated B-Raf in tumor progression has not been documented. For instance, the kinase activity of endogenous mutated B-Raf protein in cancer cells has not been investigated thus far. Likewise, the contribution of B-Raf to the MEK/ERK pathway in the establishment of the transformed phenotype has yet to be directly assessed in tumor-derived cell lines.

In this study, we report the presence of the V599E B-Raf mutation in three short-term choroidal melanoma cell cultures. We have investigated the consequences of this mutation on the basal kinase activity of endogenous B-Raf protein and the downstream activation of the MEK/ERK module relative to normal human choroidal melanocytes. Using small interfering RNA (siRNA)-mediated inhibition of B-Raf expression in melanoma cells, we demonstrate that both constitutive activation of the MEK/ERK module and cell proliferation are linked directly to the presence of endogenous mutated B-Raf protein. We also show that one hallmark of transformation, anchorage-independent growth in soft agar, is strongly dependent on the B-Raf/MEK/ERK pathway in the choroidal melanoma cell lines. Finally, we identify several signaling molecules and cell cycle proteins that are differentially expressed between normal melanocytes and melanoma cells. Strikingly, only the expression of a subset of cell cycle proteins proved to be regulated by the B-RafV599E/MEK/ERK pathway in melanoma cells.

MATERIALS AND METHODS

Cell Cultures and Treatments—Normal choroidal melanocytes were isolated from post-mortem human enucleated eyes (generously provided by Dr. C.-A. Maurage, CHRU Lille, France, and Prof. P. Gain, EA 3063, Saint-Etienne, France) as described previously (14). They were cultured in F12 medium supplemented with 20% fetal calf serum (FCS), 2.5 μg/ml fungizone/amphotericin B, 50 μg/ml gentamycin, 2 mM t-glutamine, 10 ng/ml cholaer toxin, 0.1% isobutyl methylxanthine, and 10 ng/ml FGF2 (obtained from Dr. H. Prats (IN-SERM U339, Toulouse, France) (14)). OCM-1 (ocular choroidal melanoma-1), MTK-Br (kindly provided by Dr. M. Jager, University of Leiden, The Netherlands) and SP6.5 cells (kindly provided by Dr. F. Malecaze, Ophthalmology Dept., CHU Toulouse, France) were grown in RPMI 1640 medium supplemented with 5% FCS, 2.5 μg/ml fungizone/amphotericin B, 50 μg/ml gentamycin, and 2 mM t-glutamine (complete medium) (15). Normal melanocytes and melanoma cells were cultured at 37 °C in a humidified air/CO2 (19:1) atmosphere.

Stock solutions of pharmacological inhibitors were made by dilutions in dimethyl sulfoxide (Me2SO) such that the final Me2SO concentration in the culture media did not exceed 0.1%. Cells were seeded in triplicate in 24-well plates at a density of 5 × 104 cells/well for normal melanocytes and 2.5 × 104 cells/well for melanoma cells. The plates were incubated for 3 days at 37 °C in a humidified air/CO2 (19:1) atmosphere. Inhibitors of Ras (FPT III inhibitor and farnesylthiosalicylic acid) and MEK1/2 (U0126) (VWR International) were added 2 h before induction of cell proliferation, on the first day of stimulation and on day 3 of the cell proliferation assay. Cell proliferation was assessed daily by two methods: 1) counting the cells remaining in the culture dish after staining with trypan blue and 2) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method (16). The number of trypan blue-positive cells never exceeded 3% of the total cell population. Cells were examined by phase-contrast microscopy before being treated and before the MTT assay, to assess the degree of cell death. Cell cycle analysis of growth inhibition was calculated by comparison with control Me2SO-treated cells.

Plasmid Construction and Cell Transfection—The Ca5 domain of murine Ksr-1 (amino acid sequence from Gly-450 to the stop codon) was cloned from pGB79/Ksr-CA5 (17) into the pEGFP-C1 vector (Clontech–La Jolla) such that the KSR (kinase suppressor of Ras) domain was fused to enhanced green fluorescent protein (EGFP).

Inhibition of B-RafV599E Expression Using Small Interfering RNA (siRNA)19 nucleotides of siRNA for B-RafV599E (forward, 5′-GAG-AUG-AUG-AGU-UAG-UGU-A-adTdT-3′; and reverse, 5′-CAC-UCC-AUC-GAG-AUU-UCC-C-dTdT-3′) targeting 1795 nucleotides downstream of the start codon were synthesized (Eurogentec). As a control, a nonspecific siRNA duplex containing the same nucleotides but in irregular sequence (scrambled) was used (forward, 5′-AAA-UUG-GUG-GAG-CU-UUG-adTdT-3′; and reverse, 5′-UCA-AUG-GCU-CAC-AUU-U-
dUTP-3’). Transfection of siRNA duplex (300 ng) was performed using LipofectAMINE 2000 reagent as recommended by the manufacturer (Invitrogen) for 5 h. Then, the transfection medium was replaced by the culture medium.

Genomic DNA Purification and Mutation Screening—Genomic DNA from normal melanocytes and melanoma cell lines was prepared according to standard procedures, checked in an 0.8% agarose gel, and quantified by spectrophotometry. Polymerase chain reaction (PCR) primers were designed to amplify exons 11 and 15 of the human BRAF gene. The following primers were used: Exon 11, sense, 5′-AA-ACA-CTT-GGT-A- GA-CGG-GAC-3′, and antisense, 5′-GTC-TAC-AAG-GGA-TGG-TGC-GAT-G-3′; exon 15, sense, CTT-CAT-GAA-GAC-CTC-ACA-GT and antisense, 5′-CCT-GGA-TCC-ATT-TTG-ATG-GAC-3′. PCR was performed with 250 ng of genomic DNA using the Expand High Fidelity PCR kit (Roche Molecular Biochemicals). PCR products were precipitated and separated in 3% agarose gels. The 117-bp (exon 11) and 111-bp (exon 15) PCR fragments were purified and sequenced (MWG Biotech).

Transformation Assay—Cell transformation was analyzed in a clonogenic assay by the ability of cells to form colonies in soft agar under anchorage-independent conditions. Melanoma cells were suspended in 0.3% agar in complete medium and plated on a layer of 0.7% agar in complete medium in 6-well culture plates (3 × 10^5 cells/well). U0126 was added 2 h before plating cells and then every 3 days during the culture period.

Statistics—Two-tailed Student’s t tests (normal distributions with equal variances) and Mann-Whitney tests (nonparametric tests) were used for statistical analysis.

RESULTS

Constitutive Activation of the MEK/ERK Module in Choroidal Melanoma Cells—When cultured in the presence of 20% FCS, 10 ng/ml FGF2, 10 ng/ml cholera toxin, and 0.1 mM isobutyl methylxanthine, normal human choroidal melanocytes grew slowly, with a doubling time of ~72 h (Fig. 1A). In contrast, the human choroidal melanoma cell lines OCM-1, MKT-BR, and SP-6.5 grew rapidly in medium supplemented only with 5% FCS, with a doubling time of 18–24 h (Fig. 1A). These three cell lines also proliferated in the presence of 0.5% FCS, unlike normal melanocytes despite the addition of FGF2, isobutyl methylxanthine, and cholera toxin (Fig. 1B). This suggested that a major mitogenic signaling pathway is constitutively activated in melanoma cells. We concentrated our attention on the Ras/Raf/MEK/ERK signaling cascade, a major mediator of proliferation in many cell types.

Initially, we compared the activation levels of ERK1/2 following a 24-h period of serum stimulation in the three melanoma cell lines versus normal melanocytes. In the absence of serum, high basal levels of ERK1/2 phosphorylation/activation were observed in the three melanoma cell lines only (Fig. 2A). 24 h of serum stimulation slightly increased ERK1/2 phosphorylation in normal melanocytes; however, the level of phosphorylated ERK1/2 never reached that observed in the three melanoma cell lines (Fig. 2A). Kinetic analysis at different times of serum stimulation confirmed these observations (Fig. 2B). Consistent with this finding, the phosphorylation/activation of MEK1/2, the immediate upstream activators of ERK1/2, was strong and sustained in all three melanoma cell lines relative to normal cells (Fig. 2C). Interestingly, the level of MEK1/2 expression is lower in normal melanocytes than in melanoma cells (Fig. 2C; see below and Fig. 8). These data clearly show that the basal level of MEK/ERK module activation is much higher in melanoma cells than in normal choroidal melanocytes.

Constitutive Activation of the MEK/ERK Module Is Ras-independent in Choroidal Melanoma Cells—The small GTPase Ras is the major upstream activator of the MEK/ERK signaling pathway in response to proliferative signals. Although no Ras mutations have been detected in choroidal melanoma (12, 13), we nevertheless investigated whether Ras is involved in the constitutive activation of the MEK/ERK module and proliferation in choroidal melanoma cell lines. Farnesylation, a post-translational modification required for Ras membrane anchoring and activation (19), is prevented by treatment with FPT III inhibitor (20). Thus, we used this pharmacological compound to investigate the role of Ras in melanoma cell proliferation. Treatment of OCM-1, MKT-BR, and SP-6.5 cells with 25 μM FPT III inhibitor reduced their proliferation by 35, 38, and 29%, respectively, after 6 days of culture (Fig. 3A). A similar inhibitory effect (41, 39, and 32% in OCM-1, MKT-BR, and SP-6.5, respectively) was observed in melanoma cells treated with 50 μM farnesylthiosalicylic acid, a Ras antagonist that dislodges Ras from its membrane anchoring sites (Fig. 3A). To confirm the role of Ras in melanoma cell proliferation, we transfected cells with an expression vector for a dominant negative mutant of Ras, RasN17. RasN17 overexpression reduced cell proliferation in OCM-1, MKT-BR, and SP-6.5 by 38, 34, and 61%, respectively, 6 days after cell transfection (Fig. 3A). These data suggested that Ras plays a significant role in choroidal melanoma cell proliferation. Therefore, we investigated whether the inhibition of Ras, either through treatment with FPT III inhibitor or overexpression of RasN17, affected the activation of the MEK/ERK module in this context. Interestingly, ERK1/2 phosphorylation was not diminished by either inhibitory strategy in any of the three melanoma cell lines (Fig. 3B). FPT III inhibitor, and C, Ras N17). These data suggested that the role of Ras on melanoma cell proliferation is not mediated through the activation of the MEK/ERK module and that constitutive activation of this pathway is independent of Ras activity.
A Somatic Mutation in the BRAF Gene Induces High Basal Kinase Activity in Choroidal Melanoma Cells—The results presented above strongly suggested that constitutive activation of the MEK/ERK pathway in choroidal melanoma cells occurs downstream of Ras. While this work was underway, mutations in exons 11 and 15 of the BRAF gene were reported to occur at a high frequency in human cutaneous melanoma (6). Cell proliferation could be inhibited by blocking ERK1/2 phosphorylation, suggesting that mutated B-Raf signals through the ERK1/2 signaling pathway to promote cell growth in cutaneous melanoma cells (6). Moreover, the mutations in exon 15 of BRAF rendered the activation of ERK1/2 independent of Ras in cutaneous melanoma cells, leading us to hypothesize that such mutations could also occur in choroidal melanoma cells. Western blot analysis revealed that

![Diagram of cell proliferation and ERK activity](image)

**Fig. 2.** High levels of MEK and ERK activation in choroidal melanoma cell lines. A, NCM and melanoma cells (OCM-1, MKT-BR, and SP-6.5 cells) were cultured as described in the legend for Fig. 1. After overnight serum starvation, melanoma cells and normal melanocytes were stimulated with FIC medium and RPMI supplemented with 5% FCS, respectively, and lysed before (−) or 24 h after serum stimulation (+). Equal amounts of protein extracts were separated on SDS-PAGE and analyzed by Western blotting with anti-phospho-ERK1/2 (P-ERK1 and P-ERK2) and anti-ERK2 antibodies. B, cells were treated as described in A, and a kinetic analysis of ERK1/2 phosphorylation was performed during a 24-h culture period. C, cultures were treated as described in B, and MEK1/2 phosphorylation was analyzed by Western blotting with and anti-phospho-MEK1/2 and anti-MEK1/2 antibodies. The data presented are representative of four independent experiments.

**Fig. 3.** Ras inhibition affects cell proliferation but not ERK activation in choroidal melanoma cells. A, effects of Ras inhibition on melanoma cell proliferation. OCM-1, MKT-BR, and SP-6.5 cells were cultured for 3 days and then treated or not with the Ras inhibitor compounds, FPT III inhibitor (25 μM) and farnesylthiosalicylic acid (FTS) (50 μM), or transiently transfected with either HA1-tagged RasN17 dominant negative mutant or the empty vector. The MTT colorimetric assay was used to assess melanoma cell proliferation after a 6-day culture period, and the percentage of inhibition was obtained by comparing treated and control cultures. B, Western blot analysis of ERK1/2 phosphorylation (P-ERK1 and P-ERK2) in FPT III inhibitor-treated and untreated cultures over a 48-h culture period. C, Western blot analysis of ectopic Ras expression and ERK1/2 phosphorylation in cultures transfected with either HA1-tagged RasN17 (+) or the empty vector (−) for 6 days. HA1-tagged RasN17 expression was detected using a monoclonal anti-HA1 antibody. Similar results were obtained in three independent experiments. Note that the strongest inhibitory effect of RasN17 expression on cell proliferation was observed in SP6.5 cells (A), in which the highest level of RasN17 expression was observed (C).
the B-Raf protein is expressed in choroidal OCM-1 melanoma cells at a level comparable with that found in normal choroidal melanocytes (Fig. 4A), ruling out the possibility that the constitutive activation of ERK1/2 was because of increased B-Raf expression in melanoma cells. Therefore, we screened for mutations in exons 11 and 15 of the \textit{BRAF} gene in the three choroidal melanoma cell lines. \textit{BRAF} exons 11 and 15 were amplified by PCR from genomic DNA and sequenced. No mutations were detected in exon 11 in any of the melanoma cell lines or in normal melanocytes (data not shown). In contrast, a single-base substitution was detected in exon 15 in the three melanoma cell lines relative to normal choroidal melanocytes (Fig. 4B). This T1796A substitution leads to the replacement of a valine by glutamic acid at position 599 (V599E).

Transient overexpression of the V599E mutated form of B-Raf results in a 10.7-fold increase in B-Raf basal kinase activity in COS cells (6), however the basal kinase activity of endogenous B-Raf has never been compared between normal cells and tumor-derived cells containing the V599E mutation. To characterize the biological effects of this mutation, we measured the endogenous basal B-Raf kinase activity in OCM-1 cells and in normal choroidal melanocyte cultures after overnight serum starvation. Following B-Raf immunoprecipitation, its kinase activity was measured in a kinase cascade assay using GST-MEK and GST-ERK (kinase dead) as sequential substrates. The background activity of GST-MEK was determined by a control immunoprecipitation in the absence of protein extract (—). Sample were separated on SDS-PAGE and transferred to Immobilon-P membranes. GST-ERK phosphorylation levels ($^{32}$P) were visualized and quantified using a PhosphorImager (Amersham Biosciences). The amount of immunoprecipitated B-Raf was then verified by Western blotting (WB) by probing the membrane with the B-Raf antibody. The relative kinase activity was calculated as described under “Materials and Methods.”

To establish that the activating V599E B-Raf mutation is responsible for the constitutive activation of the MEK/ERK module in choroidal melanoma cells, we used an siRNA-based approach to inhibit B-Raf$^{V599E}$ protein expression (Fig. 4D). Transfection of OCM-1 cells with control scrambled siRNA had no effect on B-Raf expression, ERK phosphorylation, or cell proliferation. In contrast, transfection with B-Raf-specific siRNA down-regulated B-Raf protein expression substantially, leading to a decrease in both ERK activation and cell proliferation. This demonstrated the direct role of endogenous, mutated B-Raf in the control of both processes.
phosphorylation was analyzed during a 48-h culture period. Microscopy observation (data not shown). Similar results were obtained when the GFP-KSR-CA5 fusion protein was verified by direct fluorescent microscopy at 6 days post-transfection over a 48-h culture period. Expression of ERK1/2 phosphorylation was analyzed by Western blotting using anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. ERK1/2 phosphorylation was analyzed by Western blotting with anti-phospho-ERK1/2 and anti-ERK2 antibodies (P-ERK1 and P-ERK2). B, cells were treated as described in A, and ERK1/2 phosphorylation was analyzed during a 48-h culture period. C, OCM-1 cells were transfected with the pEGFP/KSR-CA5 plasmid (+) or the pEGFP-C1 empty vector (-). ERK1/2 phosphorylation was analyzed by Western blotting using anti-phospho-ERK1/2 and anti-ERK1/2 antibodies at 6 days post-transfection over a 48-h culture period. Expression of the GFP-KSR-CA5 fusion protein was verified by direct fluorescent microscopy observation (data not shown). Similar results were obtained in four independent experiments.

**Constitutive Activation of B-Raf/MEK/ERK Signaling Is Responsible for the Proliferation of Choroidal Melanoma Cells**—We wanted to investigate further the contribution of the B-Raf/MEK/ERK signaling pathway to melanoma cell proliferation and transformation. To confirm that constitutive activation of ERK was controlled at the level of MEK, we treated normal melanocytes and the three melanoma cell lines with U0126, a pharmacological inhibitor of MEK1/2 (21) and thereby of ERK1/2 phosphorylation (Fig. 5). The inhibitor strongly blocked ERK1/2 phosphorylation/activation in the three melanoma cells after a 24-h culture period (Fig. 5A). Kinetic analysis showed that U0126 treatment inhibited ERK activation within 10 min, an effect that lasted over a 48-h culture period in the three melanoma cell lines (Fig. 5B). We used another experimental approach to confirm these results. KSR is a scaffold protein that regulates the Raf/MEK/ERK signaling cascade by interacting with the three components of the module (2, 17, 22). Overexpression of the KSR domain (CA5) strongly inhibits the Ras/MEK/ERK signaling pathway through the formation of a stable complex with MEK (23, 24). In OCM-1 cells, transfection of KSR-CA5 induced a strong and sustained inhibition of ERK1/2 phosphorylation, confirming the role of MEK1/2 in the activation of ERK1/2 in melanoma cells.

To determine the role of the constitutively active B-RafV599E/MEK/ERK module in melanoma cell growth, we analyzed the effects of its inhibition on the proliferation of normal melanocytes and melanoma cells (Fig. 6). In normal melanocytes, MEK1/2 inhibition with U0126 did not significantly inhibit proliferation, which was reduced by 11% relative to untreated cells after a 6-day culture period (Fig. 6A). In contrast, proliferation of the three melanoma cell lines was sensitive to U0126 treatment. At low concentration (2 μM), U0126 reduced the proliferation of OCM-1, MKT-BR, and SP-6.5 melanoma cells by 90, 95, and 46%, respectively, after a 6-day culture period (Fig. 6A). A higher concentration of U0126 (20 μM) completely abolished the proliferation of OCM-1 and MKT-BR cells and reduced the proliferation of SP6.5 cells by 67% after a 6-day culture period (Fig. 6A). To confirm these results, we analyzed the effects of KSR-CA5 overexpression on melanoma cell growth. KSR-CA5 reduced the proliferation of OCM-1, MKT-BR, and SP-6.5 melanoma cells by 62, 72, and 76%, respectively, after a 6-day culture period following cell transfection (Fig. 6B). These data demonstrate that the B-RafV599E/MEK/ERK module plays a key role in the signaling system that promotes choroidal melanoma cell proliferation, whereas its wild-type counterpart is only a minor component in the proliferation of normal choroidal melanocytes.

To analyze the role of B-RafV599E/MEK/ERK signaling in cell transformation, we examined the ability of choroidal melanoma cells to grow under anchorage-independent conditions. Cell transformation was assessed by the ability of cells to form colonies in soft agar, a property closely associated with the malignant phenotype. The three melanoma cell lines formed numerous and large colonies in soft agar, revealing a strongly transformed phenotype (Fig. 7). Inhibition of MEK1/2 with 20 μM U0126 markedly inhibited the formation of colonies (Fig. 7). Taken together, these data demonstrate that the B-Raf-mediated constitutive activation of the MEK/ERK signaling pathway is responsible not only for proliferation but also for transformation in OCM-1, MKT-BR, and SP-6.5 cells.

**Evidence for a Direct Link between the B-Raf/MEK/ERK Signaling Pathway and the Cell Cycle Machinery in the Control of Choroidal Melanoma Cell Proliferation**—The molecular events downstream from the MEK/ERK signaling pathway required to promote cell cycle entry are not completely defined and may be cell type- and cell stage-specific. The MEK/ERK signaling pathway controls cell proliferation in part by modulating the transcription of genes encoding proteins involved in regulation of the cell cycle, such as cyclin D1 (25). Therefore, we first investigated the effects of inhibiting the B-RafV599E/MEK/ERK module on the level of cyclin D1 protein expression in the OCM-1, MKT-BR, and SP-6.5 melanoma cell lines. Western blot analysis indicated that cyclin D1 is expressed in the three cell lines (Fig. 8A). MEK inhibition with U0126 (20 μM) substantially reduced cyclin D1 levels in the three cell lines, with the strongest effect observed in OCM-1 cells (Fig. 8A). These results suggest that the B-RafV599E/MEK/ERK pathway controls cell proliferation in part by driving cyclin D1 expression in melanoma cells.
We next wanted to characterize the molecular events occurring during conversion of a normal melanocyte into a transformed melanoma cell, as well as the contribution of the B-Raf/MEK/ERK pathway to this process. To this end, we looked for changes in the expression level of proteins involved in cell cycle regulation, signal transduction, and cell differentiation. A quantitative immunoblot analysis with a panel of ~120 monoclonal antibodies revealed that a cluster of proteins were altered in OCM-1 melanoma cells in comparison with normal choroidal melanocytes (Fig. 8B). Small G proteins of the Ras and Rho families, such as H-Ras, Rap1, Rap2, Rho and Rac1, were down-regulated in OCM-1 melanoma cells (Fig. 8B). On the other hand, expression of PLC-γ1, PKCδ, P90RSK, and MEK1 was up-regulated in melanoma cells. Notably, expression of Raf (26–28), a chaperone for multiple protein kinases including Raf, was also found overexpressed in choroidal melanoma cells. Among the proteins of the cell cycle, expression of MEK/ERK pathway to this process. To this end, we looked for changes in the expression level of proteins involved in cell cycle regulation, signal transduction, and cell differentiation. A quantitative immunoblot analysis with a panel of ~120 monoclonal antibodies revealed that a cluster of proteins were altered in OCM-1 melanoma cells in comparison with normal choroidal melanocytes (Fig. 8B). Small G proteins of the Ras and Rho families, such as H-Ras, Rap1, Rap2, Rho and Rac1, were down-regulated in OCM-1 melanoma cells (Fig. 8B). On the other hand, expression of PLC-γ1, PKCδ, P90RSK, and MEK1 was up-regulated in melanoma cells. Notably, expression of Raf (26–28), a chaperone for multiple protein kinases including Raf, was also found overexpressed in choroidal melanoma cells. Among the proteins of the cell cycle, expression of the cyclin-dependent kinases (Cdk) inhibitor, p27Kip1, was decreased in melanoma cells, in agreement with a recently published study (29). Conversely, several positive regulators of the cell cycle, including cyclin A, Cdk1/Cdc2, and Cdk4, as well as the checkpoint proteins such as proliferating cell nuclear antigen and CHK1 (checkpoint kinase 1), were increased in melanoma cells (Fig. 8B). Other cell cycle proteins, such as cyclin D3, cyclin B, p21Waf1/Cip1, p19Arf, and Rb (retinoblastoma), remained unchanged (data not shown). To evaluate the contribution of the B-RafV599E/MEK/ERK pathway to these modifications in protein expression, a similar comparative study was performed for 24 h using untreated OCM-1 melanoma cells versus those treated with 20 μM U0126. Interestingly, among proteins in which expression was modified in OCM-1 cells relative to normal melanocytes (Fig. 8B), a subgroup containing only cell cycle regulators appears to be controlled by the MEK/ERK pathway (Fig. 8C). More specifically, expression of proliferating cell nuclear antigen, cyclin A, and Cdk1/Cdc2 was down-regulated upon U0126 treatment, whereas p27Kip1 expression was up-regulated (Fig. 8C). In contrast, MEK inhibition did not affect the expression level of proteins belonging to the signal transduction category. Interestingly, inhibition of the B-Raf/MEK/ERK pathway resulted in an increase in the expression of TRP-1, a differentiation marker specific for the melanocytic lineage (30).

In conclusion, these data strongly suggest that the V599E mutation in the B-Raf gene, which increased B-Raf basal kinase activity, induced sustained activation of the MEK/ERK module, leading to cell cycle regulation and transformation.

**DISCUSSION**

**BRAF** was first identified as an oncogene by its ability to induce proliferation of avian primary cell cultures upon retroviral transduction and to transform NIH3T3 cells upon transfection with Ewing sarcoma DNA (31, 32). Although its role as a major activator of the MEK/ERK pathway has been demonstrated in a large number of physiological processes (2, 8–11, 18), its role in cell transformation has been poorly documented until 2002, when activating mutations were identified in the BRAF gene in human cancers, with the highest frequencies found in cutaneous melanomas (6) and papillary thyroid carcinoma (33, 34). The V599E substitution in the activating loop within exon 15 of the BRAF gene accounted for ~80% of the total BRAF mutations in cutaneous melanoma cell lines and for 66% in primary cutaneous melanomas (6). However, this had not been linked directly to the kinase activity of endogenous mutated B-Raf protein. In addition, the contribution of endogenous B-RafV599E-mediated constitutive activation of the MEK/
ERK pathway in cell transformation has not been directly assessed in tumor-derived cell lines.

In the present study, we report the presence of the V599E B-Raf mutation in three short-term cell cultures derived from choroidal melanoma. Furthermore, we have investigated the consequences of this mutation on the basal kinase activity of endogenous B-Raf and the downstream activation of the MEK/ERK module in comparison with normal human choroidal melanocytes. Thus, for the first time, the role of this endogenous pathway in cell cycle regulation and cell transformation has been investigated directly in melanoma cell lines. The similar levels of B-Raf in both normal human choroidal melanocytes and choroidal melanoma cells allowed us to investigate the basal kinase activity of endogenous B-Raf protein carrying this mutation in a physiological context. We observed a 10-fold increase in endogenous B-Raf\textsuperscript{V599E} activity from melanoma cells in comparison with endogenous wild-type B-Raf in normal melanocytes. This finding provides a physiological basis for the previous study reporting a similar 10-fold increase in ectopically expressed Myc epitope-tagged B-Raf\textsuperscript{V599E} (6). Our study also provides the first demonstration that constitutive activation of the MEK/ERK module in melanoma cells is directly linked to the presence of endogenous mutated B-Raf protein. Indeed, we show that siRNA-mediated inhibition of B-Raf expression in OCM-1 cells significantly decreased both ERK activation and cell proliferation. This prompted us to further investigate the role of B-Raf downstream signaling pathway in melanoma cell proliferation and transformation. Using several experimental approaches, we found that inhibition of the MEK/ERK module strongly decreased the proliferation rate of three choroidal melanoma cell lines (OCM-1, MKT-BR, and SP-6.5) as it did in cutaneous melanoma cell lines carrying the V599E mutation (6). Furthermore, we showed that choroidal melanoma cells not only display a high growth rate but also a marked transformed phenotype. U0126 treatment prevented the ability of melanoma cells to form colonies in soft agar, thereby demonstrating the requirement of B-Raf\textsuperscript{V599E}-mediated MEK/ERK activation for melanoma cell transformation. Again, our data support, in a more physiological context, previous studies reporting that the ectopic expression of epitope-tagged B-Raf V599E induced transformation in established rodent fibroblasts (6).

To gain further insight into the molecular mechanisms of tumoral progression in melanoma cells, we looked for changes in the expression levels of proteins between normal melanocytes and melanoma cells, as well as in melanoma cells treated with U0126. A quantitative Western blotting analysis was performed with a panel of 120 monoclonal antibodies raised against proteins involved in cell cycle regulation, signal transduction, and cell differentiation. This approach was validated by the observation that two proteins found differentially expressed in this analysis, namely MEK1 and p27\textsuperscript{KIP1}, had been identified as such otherwise (this study and in Ref. 29, respectively). Global analysis of the results identified two main clusters of proteins differentially expressed between normal melanocytes and melanoma cells: signaling molecules and cell cycle regulators. Strikingly, only the latter proved to be regulated by the B-Raf\textsuperscript{V599E}/MEK/ERK pathway in melanoma cells.

Although the expression levels of several signaling molecules are either up- or down-regulated in melanoma cells, none appears to be under the direct control of the MEK/ERK pathway. Notably, several GTPases of the Ras superfamily were decreased in melanoma cells. This correlates with the observation that proliferation of normal melanocytes and melanoma cells does not require the same signaling pathways. Indeed, we showed that melanoma cell proliferation depends strongly on the B-Raf\textsuperscript{V599E}/MEK/ERK pathway, whereas that of normal melanocytes does not. This might reflect the lower dependence of melanoma cells carrying the B-Raf mutation on the signaling pathways regulated by these GTPases. Conversely, normal melanocyte proliferation is not sensitive to U0126 treatment, suggesting that these cells use other effectors, distinct from the MEK/ERK pathway, downstream of these GTPases.

In contrast, the key role of B-Raf mutation in melanoma cell proliferation and in the acquisition of the transformed phenotype is strengthened by the following two observations. First, MEK1, p90RSK (an ERK substrate), and Cdc37 were increased
in a MEK/ERK-independent manner in melanoma cells. All three proteins directly participate in the Raf pathway. The Cdc37 co-chaperone was shown, in concert with Hsp90, to be required for Raf proteins function (26–28). Genetically selected overexpression of these three molecules may well enhance the strong transformed phenotype associated with the V599E B-Raf mutation in melanoma cells. Second, the expression of a cluster of cell cycle proteins remains under the control of the B-Raf/V599E/MEK/ERK pathway in melanoma cells. Positive regulators, namely cyclin A and cyclin D1, Cdc2/Cdk1, and proliferating cell nuclear antigen, are increased, whereas the p27Kip1 inhibitor is decreased. Alterations in the expression of regulation of these molecules leads to abnormal cancer cell division (35, 36). One striking example is p27Kip1, for which down-regulation is necessary for G1/S phase transition and is associated with tumor progression. Our data on p27Kip1 are consistent with its up-regulation upon U0126 treatment in cutaneous melanoma cell lines (37) and its previously reported degradation by the MEK/ERK pathway in OCM-1 cells (29). These observations are certainly relevant to melanoma biology, because we also found an inverse correlation between the number of mitotic figures and p27Kip1 expression in primary choroidal melanomas (38).

With respect to positive regulators of the cell cycle, we have shown that cyclin D1 expression can be strongly reduced upon U0126 treatment in the three melanoma cell lines. High levels of cyclin D1 have been detected in dysplastic cutaneous nevi (39), and the presence of cyclin D1 is associated with a more aggressive course and a histologically unfavorable prognosis in choroidal melanomas (40). Thus, the B-Raf/MEK/ERK pathway controls the expression of at least two major regulators of the G1-to-S phase, cyclin D1 and p27Kip1, in choroidal melanoma cells. Likewise, positive regulators of the S- and the G2-M phases, namely cyclin A and Cdc2/Cdk1, appear to be also controlled by the B-Raf/V599E/MEK/ERK pathway. Although the implication of these molecules in tumoral progression has been less well documented, several examples of their deregulation in cancer cells have been reported (35, 36, 41, 42). Taken together these observations stress the importance of constitutive activation of the B-Raf/V599E/MEK/ERK pathway in the control of cell cycle progression in melanoma cells.

Interestingly, our proteomic analysis revealed another contribution of B-Raf2MEK/ERK pathway in the establishment of the transformed phenotype through the repression of a differentiation program in melanoma cells. Indeed, treatment of OCM-1 cells with U0126 resulted in an increase in the expression of TRP-1 (tyrosine-related protein 1), a differentiation marker specific for the melanocytic lineage (30).

Although we have firmly established the role of B-Raf/V599E in choroidal melanoma cells proliferation, we have also shown that inhibition of endogenous Ras significantly decreased the cell proliferation rate, without affecting MEK/ERK activation. In contrast, it was reported that proliferation of cutaneous melanoma cells containing this mutation was not dependent on Ras, as shown by the lack of S-phase inhibition with a Ras-neutralizing antibody (6). These results disclose a major difference between cutaneous and choroidal melanoma cells, both containing the B-Raf V599E mutation, and suggest the existence of a parallel signaling pathway downstream of Ras that does not involve B-Raf in choroidal melanoma cells. Recent reports have demonstrated that other Ras downstream signaling pathways involving phosphatidylinositol 3-kinase and Rac exchange factors cooperate for Ras-induced cell proliferation in different cell systems (24, 43, 44). Taken together, these findings indicate that although the B-Raf/V599E/MEK/ERK pathway is required for choroidal melanoma cell proliferation, it is not sufficient and might cooperate with other endogenous signaling pathways. These results suggest that, in contrast to cutaneous melanoma, BRAF mutation is probably not an early event in choroidal melanoma progression. They could explain why we and others have failed to detect the V599E BRAF mutation in primary choroidal melanomas thus far (45, 46). This could be because of the low number of cells containing the mutation within one choroidal melanoma samples, in which only cell culture conditions could allow their selection/amplification. Thus, another genetic event, which remains to be characterized, is probably required in addition to the V599E BRAF mutation for choroidal melanoma progression.

The BRAF V599E mutation is the first genetic alteration identified in choroidal melanoma cells that is common to cutaneous melanomas. Additional mutations in other genes must occur for the progression to malignancy in cutaneous melanomas (47). For instance, mutations in both BRAF and CDKN2A (encoding p16INK4A) have been detected in cutaneous melanomas (48, 49), suggesting that association of these two events is important for the progression to malignancy in this cancer. p16INK4A inhibits Cdk4 activity by binding to cyclin D-Cdk4 complexes and displaces p27Kip1 from cyclin D-Cdk4 to cyclin E-Cdk2 complexes, thereby inhibiting Cdk2 activity (36). Interestingly, we found that both cyclin D1 and Cdk4 are overexpressed in OCM-1 melanoma cells, whereas p27Kip1 is down-regulated. These findings suggest that, as in cutaneous melanoma, concomitant alterations in both the B-Raf/MEK/ERK and the p16INK4A-cyclin D/Cdk4 pathways occur in choroidal melanoma. Although CDKN2A is rarely mutated in choroidal versus cutaneous melanoma (50–54), our data indicate that alteration of the pathway could otherwise occur through the deregulation of its components.

In conclusion, we have demonstrated that the B-Raf/MEK/ERK pathway is specifically involved in the proliferation and transformation of choroidal melanoma cells carrying the V599E mutation. These data, which further explain the complex relationship among B-Raf activation, cell cycle machinery, and malignant transformation, may have important implications for the design of specific therapeutic strategies for the control of melanoma progression. In this respect, our demonstration that mutated B-Raf can be targeted by an siRNA approach in melanoma cells is certainly promising.

Acknowledgment—We are grateful to Dr. Robert Hipskind for critical reading of the manuscript.

REFERENCES
1. Hanahan, D., and Weinberg, R. A. (2000) Cell 100, 57–70
2. Peyssonnaux, C., and Eyaché, A. (2001) Biol. Cell 93, 53–62
3. Pearson, G., Robinson, F., Beers Gibson, T., Xu, B-E., Karandikar, M., Berman, K., and Cobb, M. H. (2001) Endocr. Rev. 22, 153–183
4. Bos, J. L. (1989) Cancer Res. 49, 4682–4689
5. Hoshino, R., Chatani, Y., Yamori, T., Tsuuro, T., Oka, H., Yoshida, O., Shimada, Y., Arii, S., Wada, H., Fujimoto, J., and Hohno, M. (1999) Oncogene 18, 815–822
6. Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clug, S., Teague, J., Wooffenden, H., Garnett, M. J., Bottomley, W., Davis, N., Dicks, E., Eccles, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mouod, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gustran, B. A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Mahtani, N., Chenevix-Trench, G., Riggins, J. G., Bignier, D. D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J., Leung, S. Y., Yuen, S. T., Weber, B. L., Seigler, H. F., Darrow, T. L., Paterson, H., Marais, R., Marshall, C. J., Wooster, R., Stratton, M. R., and Futreal, P. A. (2002) Nature 417, 949–954
7. Catling, A. D., Reuter, C. W., Cox, M. E., Parson, S. J., and Weber, M. J. (1994) J. Biol. Chem. 269, 36034–36041
8. Pritchard, C. A., Samuels, M., L., Bosh, E., and McMahon, M. (1995) Mol. Cell. Biol. 15, 6430–6442
9. Marais, R., Light, Y., Paterson, H. F., Mason, C. S., and Marshall, C. J. (1997) J. Biol. Chem. 272, 4738–4743

2 A. Calipiel, G. Lefevre, C. Pouponnet, F. Mouriaux, A. Eyaché, and F. Mascarelli, unpublished results.
B-Raf Mutation and ERK Activation in Choroidal Melanoma Cells

10. Papin, C., Denouel, A., Calothy, G., and Eyche, A. (1996) Oncogene 12, 2213–2221

11. Papin, C., Denouel-Galy, A., Laugier, D., Calothy, G., and Eyche, A. (1998) J. Biol. Chem. 273, 24938–24947

12. Moyer, C. M., Van der Helm, M. J., Van der Kwaast, T. H., De Jong, P. T., Ruiter, D. J., and Zwarthoff, E. C. (1991) Br. J. Cancer 64, 411–413

13. Soparker, C. N., Hao, N., and Liggett, P. E. (1989) Invest. Ophthalmol. Vis. Sci. 30, 2210–2219

14. Hu, D. N., McCormick, S. A., Ritch, R., and Pelton-Henrion, K. (1993) J. Natl. Cancer Inst. 85, 321–329

15. Kan-Mitchell, J., Mitchell, M. S., Rao, N., and Liggett, P. E. (1993) Invest. Ophthalmol. Vis. Sci. 34, 2210–2219

16. Mosmann, T. (1983) J. Immun. Methods 65, 55–63

17. Denouel-Galy, A., Douville, E. M., Warne, P. H., Papin, C., Laugier, D., Calothy, G., Downward, J., and Eyche, A. (1998) Curr. Biol. 8, 46–55

18. Buscic, R., Abbe, P., Mantoux, F., Aberdam, E., Peyssonnaux, C., Eyche, A., Ortonne, J. P., and Baillet, R. (2000) EMBO J. 19, 2900–2910

19. Adjer, A. (1998) J. Natl. Cancer Inst. 90, 1062–1074

20. Wang, D., Xu, Y., and Brecher, P. (1998) J. Biol. Chem. 273, 33027–33034

21. Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Freese, W. S., Van Dyk, D. E., Pitts, W. J., Karl, R. A., Hobbs, P., Copeland, R. A., Magolda, R. E., Scherle, P. A., and Trzaskos, J. M. (1998) J. Biol. Chem. 273, 18625–18632

22. Roy, F., Laberge, G., Douziech, M., Ferland-McCollough, D., and Therrien, M. (2002) Genes Dev. 16, 427–438

23. Cacace, A. M., Michaud, N. R., Therrien, M., Mathies, K., Copeland, T., Robin, G. M., and Morrison, D. K. (1999) Mol. Cell. Biol. 19, 229–240

24. Peyssonnaux, C., Provot, S., Felder-Schmittbuhl, M. P., Calothy, G., and Eyche, A. (2000) Mol. Cell. Biol. 20, 7068–7079

25. Wilkinson, M. G., and Millar, J. B. (2000) FASEB J. 14, 2147–2157

26. Cutforth, T., and Robin, G. M. (1994) Cell 77, 1027–1036

27. Jaiswal, R. K, Weissinger, E., Kolch, W., and Landreth, G. E. (1996) J. Biol. Chem. 271, 20626–20629

28. Grammatikakis, N., Lin, J. H., Grammatikakis, A., Tsichlis, P. N., and Cochran, B. H. (1999) Mol. Cell. Biol. 19, 1661–1672

29. Delmas, C., Aragou, N., Poussard, S., Cottin, P., Darbon, J. M., and Manenti, S. (2003) J. Biol. Chem. 278, 12443–12451

30. Cacace, A. M., Michaud, N. R., Therrien, M., Mathies, K., Copeland, T., Robin, G. M., and Morrison, D. K. (1999) Mol. Cell. Biol. 19, 229–240

31. Godin, C. R., and Fisher, D. E. (1997) Cell Growth & Differ. 8, 935–940

32. Marx, M., Eyche, A., Laugier, D., Bechade, C., Crisanti, P., Dezelée, P., Pessac, B., and Calothy, G. (1998) EMBO J. 7, 3269–3273

33. Ikawa, S, Fukui, M, Ueyama, T, Tamaoki, N, Yamamoto, T, and Toyoshima, K (1988) Mol. Cell. Biol. 8, 2651–2654

34. Cohen, Y, Xing, M, Mambo, K, Guo, Z, Wu, G, Trank, R, Beller, U, Westra, W, H, Ladenson, P, W, and Sidransky, D (2003) J. Natl Cancer Inst. 95, 625–627

35. Kimura, E, Nikiforova, M, Zhu, Z, Knauf, J, A, Nikiforov, Y, E, and Fagin, J, A (2003) Cancer Res. 63, 1454–1457

36. O’Connor, D, S, Wall, N, R, Porter, A, C, and Altieri, D, C (2002) Cancer Cell 2, 43–44

37. Sherr, C, J, and Roberts, J, M (1999) Genes Dev. 13, 1501–1512

38. Kerteszewski, M, Heinrich, P, C, Kauffmann, M, E, Bohm, M, MacKiewicz, A, and Behrmann, I (2000) Biochem. J. 357, 297–303

39. Mouriaux, F, Chabaud, F, Maurage, C, A, Malecave, F, and Labalette, P (2001) Invest. Ophthalmol. Vis. Sci. 41, 2837–2843

40. Ewanowicz, C, Brynes, R, K, Medeiros, L, McCourtay, A, and Lai, R (2001) Arch. Pathol. Lab. Med. 125, 208–210

41. Keyomarsi, K, and Pardee, A, B (1993) Proc. Natl. Acad. Sci. 90, 1112–1116

42. Ho, Y, Takeda, T, Sakon, M, Monden, M, Tsujimoto, M, and Matsumura, S (2000) Oncology 59, 68–74

43. Gilie, H, and Downward, J (1999) J. Biol. Chem. 274, 22033–22040

44. Treinies, I, Paterson, H, E, Hooper, S, Wilson, R, and Marshall, C, J (1999) Mol. Cell. Biol. 19, 321–329

45. Edmunds, S, C, Cree, I, A, Di Nicolantonio, F, Hungerford, J, L, Hurten, J, S, and Kelsell, D, P (2003) Br. J. Cancer 88, 1403–1405

46. Cohen, Y, Goldenberg-Cohen, N, Parrella, P, Chowers, I, Merbs, S, L, Pe’er, J, and Sidransky, D (2003) Invest. Ophthalmol. Vis. Sci. 44, 2876–2878

47. Walker, G, J, and Hayward, N, K (2002) J. Investig. Dermatol. 119, 783–792

48. Lee, J, Y, Dong, S, M, Shin, M, S, Kim, S, Y, Lee, S, H, Kang, S, J, Lee, J, D, Kim, C, S, Kim, S, H, and Yoo, N, J (1997) Biochem. Biophys. Res. Commun. 237, 667–672

49. Papp, T, Fensel, H, Zimmermann, R, Bastrop, R, Weiss, D, G, and Schiiffmann, D (1999) J. Med. Genet. 3, 610–614

50. Ohta, M, Nagai, H, and Shimizu, M (1994) Cancer Res. 54, 5269–5272

51. Singh, A, D, Wang, M, X, Donoso, L, A, Shields, C, L, De Potter, P, and Shields, J, A (1996) Semin. Ocul. 6, 768–772

52. Wang, X, Egan, K, M, Gragoudas, E, S, and Kelsey, K, T (1996) Melanoma Res. 6, 405–410

53. Merbs, S, L, and Sidransky, D (1999) Invest. Ophthalmol. Vis. Sci. 40, 779–783

54. Soulier, N, Bressac-de Paillerets, B, Desjardins, L, Levy, C, Bomblrd, J, Gorin, I, Schlenger, P, and Stoppa-Lyonnet, D (2000) Br. J. Cancer 82, 818–824
Mutation of B-Raf in Human Choroidal Melanoma Cells Mediates Cell Proliferation and Transformation through the MEK/ERK Pathway
Armelle Calipel, Gaelle Lefevre, Celio Pouponnot, Frédéric Mouriaux, Alain Eychène and Frédéric Mascarelli

J. Biol. Chem. 2003, 278:42409-42418.
doi: 10.1074/jbc.M308709200 originally published online August 12, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308709200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 54 references, 26 of which can be accessed free at
http://www.jbc.org/content/278/43/42409.full.html#ref-list-1