Ras-Associated Protein-1 Regulates Extracellular Signal-Regulated Kinase Activation and Migration in Melanoma Cells: Two Processes Important to Melanoma Tumorigenesis and Metastasis

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Abstract

Melanoma is one of the most devastating malignances with a rising incidence and lack of effective treatments for advanced disease. Constitutive activation of the mitogen-activated protein kinase (MAPK) pathway and altered expression of α5β3 integrin are critical for melanoma development and progression. Ras-associated protein-1 (Rap1), a Ras family member of the small GTPases, has emerged as a key mediator in these two important processes. In this study, we have shown Rap1 activation in cells derived from two human metastatic melanomas and also in three of seven cutaneous metastatic melanoma tissues. We found increased extracellular signal-regulated kinase (ERK) activity in the tumors with detected Rap1 activity that interestingly harbored neither BRAF nor N-Ras mutation, suggesting a role for Rap1 in ERK activation in vivo. We also showed Rap1 and ERK activation by both hepatocyte growth factor (HGF) and 8CPT-2Me-cAMP (an activator of Epac, a Rap1 guanine nucleotide exchange factor) in two human melanoma cell lines. In addition, the activation of ERK by HGF was reduced, at least in part, by small interfering RNAs against Rap1 and a dominant-negative Rap1. Finally, a functional role for Rap1 activation was shown by Rap1-induced α5β3 integrin activation and consequent increased melanoma cell migration in vitro. Taken together, these results show that Rap1 is involved in the activation of MAPK pathway and integrin activation in human melanoma and suggest a potential role for Rap1 in melanoma tumorigenesis and metastasis. (Cancer Res 2006; 66(16): 7880-8)

Introduction

Melanoma derives from the malignant transformation of cutaneous melanocytes, the pigment-producing cells that reside in the basal layer of the epidermis in human skin. The incidence of melanoma has been rising steadily over recent decades and intensive, episodic UV exposure is one of the major etiologic factors. Successful treatment depends on early detection followed by definitive surgical excision using wide margins. Once metastasis develops, however, melanoma is relatively resistant to current therapeutic regimens (1). Success in identifying novel therapeutic targets in melanoma lies in our further understanding of the molecular mechanisms involved in melanocyte malignant transformation, tumor growth, invasion, and host response. As in many other tumors, dysregulated cell growth/proliferation and survival/apoptosis are known to play critical roles in melanoma pathogenesis.

The mitogen-activated protein kinase (MAPK) pathway has emerged recently as the central growth-stimulatory pathway in melanoma. Activating mutations in Ras, particularly N-Ras and BRAF, and hyperactivation of their downstream effector kinase, extracellular signal-regulated kinase (ERK), have been reported in a large percentage of human melanomas and melanoma cell lines (2, 3). The signature BRAF mutations in melanoma represent a T to A change resulting in a valine to glutamic acid change at residue 599 (V599E) within the activation segment of BRAF. It has been shown that BRAF (V599E) hotspot mutation results in higher kinase activity, stimulates endogenous ERK phosphorylation, and induces transformation of NIH3T3 cells (2). Recent studies propose that distinct subgroups of melanomas expressing varying copy numbers and mutation frequencies of BRAF exist, where the upstream factors responsible for increased ERK activity are not straightforward (4, 5). More specifically, a recent study has shown enhanced ERK activity despite a low frequency of BRAF and N-Ras mutations in acral melanoma (6), suggesting that factors other than BRAF and N-Ras mutations contribute to constitutive activation of MAPK/ERK pathway.

Ras-associated protein-1 (Rap1), a close member of Ras in the small GTPase family, regulates two important cellular processes: Ras/BRAF/ERK activation and integrin-mediated cell adhesion/migration (7, 8). Like Ras, Rap1 functions as a molecular switch, cycling between an inactive and an active form under the regulation of guanine nucleotide exchange factors (GEF) and GTPase-activating proteins (GAP). GEFs activate Rap1 by facilitating the replacement of GDP with GTP, whereas GTPs inhibit Rap1 by enhancing the intrinsic GTPase activity of Rap1 to hydrolyze the bound GTP to GDP. Rap1 was originally identified as an antagonist of Ras-induced transformation. Studies have shown subsequently, however, that Rap1 activation induces either activation or inhibition of ERK and such effects are cell type specific (9, 10). Compelling evidence suggests that Rap1 is crucial for the control of cell adhesion and migration through the inside-out activation of integrins (i.e., transient overexpression of activated Rap1 stimulates α5β3 integrin-dependent adhesion in human T and B cells). In addition, inhibition of CD31-induced T-cell adhesion has been shown using a dominant-negative form of Rap1 (11). In vivo, recent evidence shows another consequence of dysregulation of Rap1 activation, the development of chronic myelogenous leukemia in a murine model (12), suggesting a role for Rap1 in malignancy.

It is well established that the α5β3 integrin plays a critical role in melanoma cell invasion (13). In some melanoma models,
perturbation of αvβ3 function inhibits melanoma growth and invasion in vitro as well as metastasis in vivo (14). Conversely, ectopic expression of either αv or β3 subunits promotes melanoma growth and induces conversion from radial to vertical growth phase in primary human melanoma (15, 16), suggesting that αvβ3 integrin expression and function are associated with a more invasive phenotype in melanoma. Despite these findings, however, the exact mechanism of how αvβ3 integrin is regulated in melanomas has not yet been determined.

Such findings about Rap1, together with the well-recognized function of αvβ3 integrin and the Ras/BRAF/ERK signaling pathway in melanoma, prompted us to investigate whether Rap1 was involved in the regulation of melanoma tumorigenesis and invasion. Here, we show that Rap1 activation is detected in three of seven human cutaneous metastatic melanomas compared with normal human melanocytes; the activation of Rap1 by the specific Epac activator 8CPT-2Me-cAMP and hepatocyte growth factor (HGF) and consequent induction of ERK activity in two melanoma cell lines that is partially blocked by a dominant-negative Rap1A and Rap1 small interfering RNA (siRNA). Finally, we show that Rap1 activation enhances melanoma cell migration and regulates αvβ3 integrin activity in A375 and MeWo melanoma cells. Together, these findings suggest that Rap1 plays a role in melanoma tumorigenesis and metastasis.

Materials and Methods

Cells and melanoma tissues. Human metastatic melanomas were obtained in accordance with an approved protocol (LAC and Tissue Procurement Core, Siteman Cancer Center, Washington University School of Medicine, St. Louis, MO). Primary human melanocytes were purchased and maintained in medium 254 with supplemented growth factors from Cascade Biologies (Portland, OR). Melanoma cell lines A375, MeWo, A2058, SK-MEL-2, C32, and 501-mel were purchased from American Type Culture Collection (Manassas, VA).

Reagents. Anti-phosphorylated ERK (9101 and 4376), phosphorylated Src, β-actin, and total ERK antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-BRAF antibody (sc-166) and goat anti-mouse and goat anti-rabbit horseradish peroxidase–conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Epac activator 8-(4-chloro-phenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate (8CPT-2Me-cAMP) and protein kinase A (PKA) inhibitor H-89 were purchased from BIOMOL (Plymouth Meeting, PA). Anti-BRAF antibody (sc-166) and goat anti-rabbit horseradish peroxidase–conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Beverly, MA). Anti-BRAF antibody (sc-166) and goat anti-rabbit horseradish peroxidase–conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Beverly, MA). Anti-BRAF antibody (sc-166) and goat anti-rabbit horseradish peroxidase–conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Beverly, MA). Anti-BRAF antibody (sc-166) and goat anti-rabbit horseradish peroxidase–conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Beverly, MA). Anti-BRAF antibody (sc-166) and goat anti-rabbit horseradish peroxidase–conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Beverly, MA). Anti-BRAF antibody (sc-166) and goat anti-rabbit horseradish peroxidase–conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Beverly, MA).

Reverse transcription-PCR (RT-PCR). Reverse transcription-PCR (RT-PCR) was done with specific primers for β3 integrin, HGF, and c-Met. Real-time PCR primers for Rap1A, Rap1B, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems (Foster City, CA) and reactions were carried out in triplicate on an ABI PRISM 7700 Sequence Detector System (Applied Biosystems) as described previously (17). Specific primers for direct sequencing covering exons 11 and 15 of the BRAF and exons 2, 3, 4, and 5 of N-Ras were described previously (2).

Rap1 activation assay and Western blot. Cells were serum deprived for 24 hours before challenging with 8CPT-2Me-cAMP (100 μM) for 30 minutes and total cell lysates were prepared. Two 50-μm frozen sections were homogenized in cold lysis buffer. The lysates were then cleared by centrifugation at 10,000 × g for 15 minutes. In vitro Rap1 activation was assayed by using human RafGDS-RRD fused with glutathione S-transferase to affinity precipitate cellular Rap1-GTP (EZ-Detect Rap1 Activation kit, Pierce, Rockford, IL) according to the manufacturer’s instructions. Total cell lysates were subjected to Western blot analysis using specific antibodies.

Immunohistochemistry. Five-micron fresh frozen melanoma tumor tissue sections were stained with phosphorylated ERK antibody (Cell Signaling Technology; 4376) according to the manufacturer’s instructions. Sections incubated with either primary or secondary antibody only served as negative controls.

Immunofluorescence and laser confocal scanning microscopy. Cells were challenged with either vehicle or 8CPT-2Me-cAMP (100 μM/L) for 30 minutes. For BRAF and Rap1 staining, cells were fixed in 3% paraformaldehyde and cold methanol, respectively. Cells were blocked with 0.2% bovine serum albumin (BSA) for 30 minutes before incubation with either anti-BRAF (1:10) antibody in 0.5% saponin in PBS or monoclonal anti-Rap1 (1:10) antibody in 0.2% BSA for 1 hour at room temperature followed by incubation with fluorescent dye–conjugated secondary antibodies (1:50 Jackson Immunoresearch Laboratories, Bar Harbor, ME) for 45 minutes. Cells incubated with either primary or secondary antibody alone served as controls. Cells were mounted in 80% glycerol in PBS and subjected to laser confocal microscopic analysis (LSM510, Zeiss, Jena, Germany).

Analysis of αvβ3 integrin expression by flow cytometry. Cells were stimulated with 8CPT-2Me-cAMP for 16 hours and lifted with 1× trypsin-EDTA (Life Technologies, Carlsbad, CA). Cells were incubated on ice for 30 minutes with either an anti-β3 antibody IA4 (a generous gift from Dr. Steven Teitelbaum, Washington University, St. Louis, MO) or a specific antibody against activated αvβ3 integrin, WOW-1 (a generous gift from Dr. Sanford Shattil, University of California, San Diego, CA; ref. 18). After washing, cells were incubated for another 20 minutes with fluorescein–conjugated donkey anti-mouse IgG (Jackson Immunoresearch) on ice in the dark, washed again, and analyzed on a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA). Cells incubated solely with primary or secondary antibody served as controls.

Migration assay. A375 and MeWo cells (5 × 104) were plated onto the inserts (pore size, 8 μm) of a 24-Transwell system (Corning Costar, Cambridge, MA) and serum starved overnight following incubation with 8CPT-2Me-cAMP (100 μM/L) for 6 and 24 hours, respectively. Cells that migrated to the bottom well were fixed in 3% paraformaldehyde for 15 minutes. Nuclear staining with Hoechst 33258 (Sigma, St. Louis, MO) was then done for 5 minutes at a dilution of 1:400 and cells were counted using fluorescent microscopy. Migration was similarly evaluated in cells transfected with Rap1 siRNA.

Transient transfections of plasmids and siRNAs. Human dominant-negative Rap1A (myc-Rap1A-17N; University of Missouri-Rolla cDNA Resource Center, Rolla, MO) was cut and ligated into pcDNA 3.1+ (Invitrogen, Carlsbad, CA). MeWo cells were transfected with Eugene 6 (Roche, Indianapolis, IN) with Rap1A-17N cDNA in DMEM according to the manufacturer’s instructions. Medium was changed 20 hours after transfection. Transient transfected cells were selected with G418 at 1 mg/mL (Life Technologies) for 48 hours after transfection for 7 consecutive days. siRNAs were designed based on RNA interference sequence guidelines. siRNA sequence was generated with a point mutation at nucleotide 19 in the sense strand, which creates a single nucleotide mismatch, making siRNA duplex function asymmetrically (19). The siRNA sequences (synthesized by Ambion, Austin, TX) were as follows: Rap1A sense 5′-gcaggagacagaacauuaucuc and antisense 5′-uaaauugacucguccuag and Rap1B sense 5′-acagacagacacuauacuc and antisense 5′-uaaauugacacacucguc. A random scramble siRNA that did not match any known sequence after National Center for Biotechnology Information Blast search was used as a control sense 5′-uucuccacggcaauccug and antisense 5′-ccgagacguaggagc. siRNAs against Rap1A and Rap1B, together with pMX-GFP-IREs-puro (a plasmid expressing both green fluorescent protein and puromycin resistance) at a ratio of 4:1, were introduced into A375 cells via nucleofection with an Amaza nucleofector (Koeln, Germany) using solution R/program K-017 according to the manufacturer’s instructions. Twenty hours after nucleofection, cells were selected with puromycin at 3 μg/mL for another 24 hours. Total cell lysates were used to confirm the efficacy of blocking Rap1 expression in transfected cells.

Statistical analysis. Group mean values were compared by two-tailed Student’s t test.
**Results**

**ERK and Rap1 activation is detected in human metastatic melanoma.** We first determined Rap1 activity in cells derived from human metastatic melanomas. Single-cell suspensions were prepared from two fresh metastatic melanomas and kept in DMEM with 20% FCS. Active Rap1-GTP was immunoprecipitated with human RaIgDS-RBD-GST fusion protein. Rap1 activation was evident in melanoma cells in culture compared with normal human melanocytes (Fig. 1A). We then wanted to determine Rap1 activity in fresh melanoma tissues. Cell lysates from seven cutaneous metastatic melanomas were prepared (see Material and Methods). Equal amounts of protein from each sample were analyzed for Rap1 activity. As shown in Fig. 1A, Rap1 activity was detected in three of seven samples (M5092, M1697, and M5123) compared with normal human melanocytes. We further examined the Rap1 expression in these tissue samples by real-time PCR. Interestingly, higher Rap1A expression levels were found in the three samples (M5092, M1697, and M5123) that had shown Rap1 activity (Fig. 1B), suggesting a correlation between increased Rap1 expression and activity. We then determined ERK activity (phosphorylated ERK) in these tumors. Total cell lysates were used and ERK activity was detected by Western blot using specific anti-phosphorylated ERK antibody. As quantitated by densitometry, varying degrees of increased ERK activity were detected in all seven samples compared with normal human melanocytes (Fig. 1C). Because increased ERK activity has been associated with activating mutations of *BRAF* or *N-Ras* in melanoma, we conducted mutational analysis of *BRAF* and *N-Ras* in these samples. (Due to difficulties with PCR amplification, sample M10617 could not be further analyzed.) As shown in Fig. 1C, three samples harbored an activating mutation in *BRAF* (M954, M1697, and M12807) and only sample M12592 contained an *N-Ras* mutation. Interestingly, two samples (M5092 and M5123) carrying wild-type (WT) *BRAF* and *N-Ras* also showed an increase in ERK activity of 5- and 3-fold, respectively (Fig. 1C). To further validate our observation in samples M5092 and M5123, ERK activity was examined in vivo by immunohistochemistry using an antibody specific for phosphorylated ERK. Samples M12592 and M1697 were also examined as positive controls. Normal skin served as negative control (data not shown). As shown in Fig. 1D, enhanced ERK activity was detected in melanoma cells in samples M5092 and M5123 as well as in samples M12592 and M1697. Together, these findings suggest that Rap1 may be involved in ERK activation in tumors M5092 and M5123. To further characterize the role of Rap1 activation in melanoma tumorigenesis, we then used human melanoma cell lines.

**Rap1 is expressed in human melanoma cell lines.** There are two isoforms of Rap1 in mammalian cells, Rap1A and Rap1B, which
HGF has been implicated as an autocrine growth factor involved in melanoma tumorigenesis and progression (20). We then examined the expression of HGF and its receptor c-Met. By RT-PCR, we found that both Rap1A and Rap1B were expressed in all six cell lines, and Rap1A was the predominant isoform in A375, MeWo, SK-MEL-2, and 501-mel cells (Fig. 2A). To further confirm Rap1 expression on the protein level, total cell lysates were prepared and Rap1 protein expression was detected by Western blot analysis using anti-Rap1 antibody (Fig. 2B). Direct sequencing revealed that A375, A2058, and C32 cells had signature BRAF mutations, whereas SK-MEL-2 and 501-mel cells contained N-Ras mutations. However, MeWo cells carried neither BRAF nor N-Ras mutations (Fig. 2B).

Rap1 is involved in HGF-induced ERK activation in human melanoma cell lines. The activation of receptor tyrosine kinase (RTK) HGF/c-Met signaling pathway is associated with the development and progression of many tumors, including melanoma (20). In melanoma, the contribution of HGF to tumor development has been shown in a murine melanoma model, where HGF transgenic neonatal mice develop melanoma following a single erythema dose of UV irradiation (21). Rap1 is activated through stimulation of various transmembrane receptors, including RTKs, heterotrimeric G protein–coupled receptors, and cytokine receptors (7, 11). We then determined whether HGF regulated Rap1 activation in human melanoma cells. A375 and MeWo cells were serum starved for 24 hours and stimulated with 100 ng/mL HGF (R&D Systems, Minneapolis, MN) for the times indicated. Consistent with previous reports (22), HGF rapidly stimulated ERK phosphorylation in both cell lines (Fig. 3A). A375 cells, harboring the signature BRAF mutation, had a higher basal level of ERK activation compared with MeWo cells, WT for BRAF and N-Ras. Active GTP-bound Rap1 was also detected using a Rap1 activation assay. HGF induced an increase of Rap1-GTP as early as 5 minutes following stimulation in both cell lines (Fig. 3A). The stimulation of both ERK and Rap1 by HGF suggested that Rap1 could mediate HGF-induced ERK activation in human melanoma cells. However, because many other pathways, including Ras, may be activated by this growth factor, we wanted to determine whether Rap1 was directly involved in HGF-induced ERK activation in our cells. Therefore, we first transiently expressed a dominant-negative form of the predominately expressed Rap1A (Rap1A-17N). Transiently transfected MeWo cells were then stimulated with HGF (100 ng/mL) for 5 and 30 minutes, respectively, and Rap1 and ERK activation was determined. As shown in Fig. 3B, the Rap1A-17N-transfected cells had a 2.4- and 2.2-fold increase of ERK activation compared with 3.8- and 4.2-fold increase in empty vector–transfected cells, respectively. To further confirm the role of Rap1 in HGF-induced ERK activation in melanoma cells, we then introduced Rap1 siRNAs into A375 cells. Figure 3C shows diminished ERK activity on introduction of Rap1 siRNAs. Furthermore, blocked expression of Rap1 by Rap1 siRNAs also blunted ERK activation following HGF stimulation (Fig. 3C). These findings indicate that, in addition to Ras, Rap1 is at least partially involved in the HGF-induced activation of MAPK/ERK pathway in melanoma cells.

Figure 2. Rap1 is expressed in human melanoma cell lines. A, Rap1A and Rap1B are expressed in all six melanoma cell lines. Real-time PCR was done for Rap1A and Rap1B in human melanoma cell lines. Rap1A is the predominantly expressed Rap1 isoform in A375, MeWo, SK-MEL-2, and 501-mel melanoma cell lines. B, Rap1 protein is detected in all six melanoma cell lines with reciprocal BRAF and N-Ras mutations. Total cell lysates from six melanoma cell lines were subjected to Western blot and total Rap1 was detected using Rap1 antibody. β-Actin was used as a loading control. All cell lines expressed Rap1 protein. Direct BRAF and N-Ras sequencing of these cell lines showed reciprocal BRAF and N-Ras mutations in five of the six cell lines examined, whereas melanoma cell line MeWo was WT for both BRAF and N-Ras. C, HGF/c-Met and human β3 integrin are expressed in human melanoma cell lines. By RT-PCR, all six melanoma cell lines expressed β3 integrin and c-Met. All cell lines expressed HGF, GAPDH served as internal control.
Because PKA is required for cAMP-induced ERK and cAMP can directly regulate Epac, we wanted to determine if PKA was involved in 8CPT-2Me-cAMP-induced ERK activation in melanoma cells. A375 cells were pretreated with the PKA inhibitor H-89 (10 μM) for 30 minutes followed by stimulation with 8CPT-2Me-cAMP (100 μM) for 15 minutes. Activation of both Rap1 and ERK was not affected by the PKA inhibitor H-89 (Fig. 3D), confirming that 8CPT-2Me-cAMP-stimulated Rap1-induced ERK activation was PKA independent. Taken together, these findings suggest that a module of Rap1/BRAF/ERK, independent of PKA, exists in certain human melanoma cells.

**Translocation of Rap1 and BRAF to cell membranes on Rap1 activation.** As described earlier, increased BRAF is also detected in the same cell lysate fractions of activated Rap1 (Rap1-GTP) in melanoma cells (Fig. 3D), which is in line with other studies, showing that the activation of Rap1 is capable of recruiting BRAF to the cell membrane (24). We then wanted to determine the localization of Rap1 and BRAF on Rap1 activation. Melanoma cells A375 and MeWo were stimulated with 8CPT-2Me-cAMP (100 μM) for 30 minutes following an overnight serum starvation. Cells were fixed and stained with specific antibodies against Rap1 and BRAF, respectively (Materials and Methods). We found that both Rap1 and BRAF were translocated to the cytoplasm of untreated cells. However, both Rap1 and BRAF were translocated to the membranes in the treated cells, particularly in the protrusions of cell membranes undergoing spreading following Rap1 activation (Fig. 4). Our data are consistent with recent findings (25) and implicate activation of Rap1 in melanoma cell cytoskeletal organization and cell spreading.

**Induction of αvβ3 integrin activation and enhanced melanoma cell migration on Rap1 activation.** A new paradigm in Rap1-regulated cellular events is the finding that it regulates integrin activity (8, 26). Rap1 has been shown to regulate integrins associated with the actin cytoskeleton, including β1, β2, and β3 via a process termed inside-out signaling. We postulated that Rap1 may regulate αvβ3 integrin activation in human melanoma cell lines. A375 and MeWo cells were serum starved and incubated with 8CPT-2Me-cAMP (100 μM) for 16 hours. Activated αvβ3 integrin was detected by flow cytometry using a specific antibody, WOW-1, that recognizes the activated conformation of the αvβ3 integrin.
αβ3 integrin. As shown in Fig. 5A, a 37% and a 24% increase in activated αβ3 integrin was found in A375 and MeWo cells, respectively, following Rap1 activation. Total β3 integrin was also detected using IA2 antibody directed against the β3 subunit. We found no change in the total surface level of β3 integrin following 8CPT-2Me-cAMP stimulation in either cell line (data not shown), suggesting increased activated, but not total, expression of β3 integrin on Rap1 activation.

Src is a non-RTK that becomes autophosphorylated on engagement of αβ3 integrin to its ligand, vitronectin. Phosphorylation of Src is a key mediator of integrin “outside-in” signaling (27). Thus, Src phosphorylation level can be used as a surrogate marker for increased activation of αβ3 integrin. To further substantiate αβ3 integrin activation following Rap1 activation, we determined Src phosphorylation level. A375 and MeWo melanoma cells were treated with 8CPT-2Me-cAMP (100 μmol/L) for 30 minutes followed by either seeding on tissue culture plates precoated with vitronectin (5 μg/mL) or exposed to vitronectin in suspension. After 30 minutes, total cell lysates were prepared and phosphorylated Src expression was determined by Western blotting. Increased Src phosphorylation following Rap1 activation was found in both melanoma cell lines (Fig. 5B), consistent with increased αβ3 integrin ligand-binding capacity and subsequent integrin activation.

Finally, we asked whether increased αβ3 integrin activity would enhance melanoma cell migration. Melanoma cells were seeded in the upper chamber of an 8-μm Transwell insert coated with vitronectin and stimulated with 8CPT-2Me-cAMP (100 μmol/L) for either 6 (A375) or 24 (MeWo) hours. The cells that migrated to the lower chamber were fixed and nuclei stained with Hoechst 33258 and counted. In both cell lines, 8CPT-2Me-cAMP induced a 3- to 4-fold increase in melanoma cell migration in response to Rap1 activation compared with untreated cells (P < 0.005; Fig. 5C), indicating that Rap1 induced αβ3 integrin activation and consequent enhanced melanoma cell migration. This was further substantiated by using A375 cells transfected with Rap1 siRNA, where migration was attenuated in Rap1 siRNA-transfected cells compared with controls following 8CPT-2Me-cAMP stimulation (Fig. 5D).

Discussion

It has been shown that Rap1 is rapidly activated following engagement of growth factors and their receptors (7); however, the ability of Rap1 to regulate ERK is less straightforward. In several cell types, Rap1 attenuates growth factor–induced, Ras-mediated ERK activation by sequestration of RAF-1 and other Ras effectors in an inactive complex (28). On the other hand, accumulating evidence also indicates that Rap1 can activate ERK through the direct binding and activation of BRAF in certain, but not all, BRAF-expressing cells (10, 29). Finally, it has been shown that extracellular signal-regulated activation of Rap1 fails to interfere with Ras signaling toward ERK in certain cell types (30). These seemingly contradictory findings show that the mechanisms underlying the regulation of ERK by Rap1 are complex, highly cell type specific, and with the response most likely dictated by the conditions of stimulation in the context of cell expression of specific isoforms of the MAPK kinase kinase RAFs (ARAF, BRAF, and RAF-1; ref. 7). In our study, we show the involvement of Rap1 in the activation of the MAPK/ERK pathway in melanoma cell lines, where both HGF and 8CPT-2Me-cAMP activate Rap1 and ERK. The activation of ERK by HGF can be reduced, at least in part, by the transient expression of a dominant-negative form of Rap1 and by Rap1 siRNA, thus supporting a role for Rap1 in HGF-induced ERK activation. Interestingly, previous work in melanocytes and B16 murine melanoma cells has shown that Ras, and not Rap1, mediates the cAMP-dependent activation of ERK (31). Along these lines, a separate study in melanoma cells using the histone deacetylase inhibitor FK228 to induce Rap1 expression showed a late (6- and 24-hour) decrease in ERK activity (25). Although this is in contrast to our findings of Rap1-induced ERK activation, we examined more immediate (5- and 30-minute) responses of Rap1 and ERK activity using the melanoma growth factor HGF. As described previously, these findings must be viewed in cell type– and stimulator-specific context.

Recently, genetic studies in Drosophila support the notion that Rap1 binds to and directly activates D-Raf, leading to ERK activation following extracellular stimulation (32). In these studies, targeted disruption of D-Rap1 expression decreased RTK-dependent ERK activation. Interestingly, mammalian BRAF shares sequence similarities with DRAF but not RAF-1. We have, in fact, shown the correlation of Rap1 activation and increased ERK activity in melanomas both in vivo and in vitro, and our data supports a similar Rap1/BRAF/ERK module in human melanoma cells. These results are further substantiated by findings in native
kidney cells that show an essential role for Epac in coupling cAMP signaling to Rap1, BRAF, and ERK (33).

Activation of the MAPK pathway is common in cutaneous melanoma and signaling molecules along this pathway have become attractive therapeutic targets (34). However, the dependence of increased ERK activity on activating mutations in BRAF or N-Ras has been challenged recently. One study shows that, in acral melanoma, increased ERK activity is detected despite a low incidence of BRAF or N-Ras mutations in these tumors (6). By immunohistochemistry, Curtin et al. (4) found that melanomas without BRAF or N-Ras mutations showed less frequent ERK activity and concluded that the MAPK pathway is not activated upstream of ERK in these tumors. However, a low sensitivity for detection of ERK activity using immunohistochemistry in formalin-fixed tissues has been reported previously (6). In our study, using fresh frozen human melanoma tumor samples, all seven samples showed increased ERK activity by Western blot, with only four samples harboring either BRAF or N-Ras mutations. Sample M1697 that had the highest level of Rap1 activation also carried the activated BRAF mutation (V599R) and showed a concomitant 26-fold increase in ERK activity. Likewise, sample M12807, carrying the signature BRAF mutation (V599E), showed the highest (27-fold) increase in ERK activity. In studies using NIH3T3 cells, it has been reported that the BRAF V599E mutation results in a 2-fold increase in kinase activity compared with BRAF V599R mutation (3) and consequent similar relative levels of ERK phosphorylation (2). If these findings translate to the in vivo situation, the 26-fold increase in ERK activity in M1697 cannot be simply explained by the V599R BRAF mutation alone. Most interestingly, two samples that were WT for both BRAF and N-Ras had increased Rap1 and ERK activation (M5092 and M5123), possibly suggesting a role for an activated Rap1-induced increase in ERK activity in these tumors. In fact, the synergistic effect of Rap1 activation in the context of an activating mutation in BRAF may suggest the existence of an active feedback loop of Rap1/BRAF/ERK activation. This potential effect is also supported by our in vitro studies, where A375 melanoma cells that carry the signature V599E BRAF mutation have higher levels of Rap1 activation than MeWo cells that are WT for both BRAF and N-Ras. Similar findings have been reported by other investigators (25).

Several independent observations show a role for Rap1 involvement in inside-out signaling to integrin in certain cells.

**Figure 5.** Rap1 induces $\alpha_{\text{v}}\beta_{3}$ integrin activation and enhances melanoma cell migration. A, A375 and MeWo melanoma cells were serum starved for 24 hours followed by stimulation with 8CPT-2Me-cAMP (100 $\mu$mol/L). After 16 hours, cells were lifted and stained with antibody specifically directed against activated $\alpha_{\text{v}}\beta_{3}$ integrin (WOW-1) or antibody against $\beta_{3}$ integrin subunit (1A2). Cells were then analyzed by fluorescence-activated cell sorting. In 8CPT-2Me-cAMP-treated cells A375 and MeWo, there was a 37% and 24% increase in activated $\alpha_{\text{v}}\beta_{3}$ integrin (bold lines), respectively, compared with control cells (lighter lines). B, A375 and MeWo melanoma cells were suspended in serum-free medium supplemented with 8CPT-2Me-cAMP (100 $\mu$mol/L) for 30 minutes. Cells ($2 \times 10^6$) were then seeded onto vitronectin-precoated plates or left in suspension. After 30 minutes, cells were lysed and phosphorylated Src expression was determined by Western blotting using a phosphorylated Src-specific antibody. Increased phosphorylation of Src was observed in cells plated on vitronectin. C, cells ($5 \times 10^4$) were plated in the inserts of a Transwell and stimulated with 8CPT-2Me-cAMP. After specified times (6 hours for A375 and 24 hours for MeWo cells), cells that had migrated to the bottom wells were fixed and nuclei stained with Hoechst 33258. Increased melanoma cell migration was evident in cells treated with 8CPT-2Me-cAMP ($P < 0.05$). By quantification, there was a 3- to 4-fold increase in migration compared with untreated cells. D, blocked cell migration by Rap1 siRNAs in A375 cells. Cells with Rap1 siRNA or scramble RNA were plated in the inserts of a Transwell and stimulated with 8CPT-2Me-cAMP. After 6 hours, cells migrated to the bottom wells were fixed, stained, and counted. Decreased cell migration was evident in A375 cells with Rap1 siRNAs compared with A375 cells with scramble RNA.
Rap1 in Melanoma

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