Exchange Protein Directly Activated by Cyclic AMP Increases Melanoma Cell Migration by a Ca^{2+}-Dependent Mechanism

Erdene Baljinnyam, Mariana S. De Lorenzo, Lai-Hua Xie, Mizuka Iwatsubo, Suzie Chen, James S. Goydos, Martha C. Nowycky, and Kousaku Iwatsubo

Abstract

Melanoma has a poor prognosis due to its strong metastatic ability. Although Ca^{2+} plays a major role in cell migration, little is known about the role of Ca^{2+} in melanoma cell migration. We recently found that the exchange protein directly activated by cyclic AMP (Epac) increases melanoma cell migration via a heparan sulfate–related mechanism. In addition to this mechanism, we also found that Epac regulates melanoma cell migration by a Ca^{2+}-dependent mechanism. An Epac agonist increased Ca^{2+} in several different melanoma cell lines but not in melanocytes. Ablation of Epac1 with short hairpin RNA inhibited the Epac agonist–induced Ca^{2+} elevation, suggesting the critical role of Epac1 in Ca^{2+} homeostasis in melanoma cells. Epac-induced Ca^{2+} elevation was negated by the inhibition of phospholipase C (PLC) and inositol triphosphate (IP3) receptor. Furthermore, Epac-induced cell migration was reduced by the inhibition of PLC or IP3 receptor. These data suggest that Epac activates Ca^{2+} release from the endoplasmic reticulum via the PLC/IP3 receptor pathway, and this Ca^{2+} elevation is involved in Epac-induced cell migration. Actin assembly was increased by Epac-induced Ca^{2+}, suggesting the involvement of actin in Epac-induced cell migration. In human melanoma specimens, mRNA expression of Epac1 was higher in metastatic melanoma than in primary melanoma, suggesting a role for Epac1 in melanoma metastasis. In conclusion, our findings reveal that Epac is a potential target for the suppression of melanoma cell migration, and, thus, the development of metastasis.

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Introduction

Melanoma is a major form of cancer and is prevalent worldwide. The median life span of patients with advanced stage melanoma is less than a year because there are no effective therapies once the tumor has spread to vital organs (1). The process of tumor cell metastasis is conventionally understood as the migration of individual cells that detach from the primary tumor, enter lymphatic vessels or the bloodstream, attach to endothelial cells and undergo transendothelial extravasation, and proliferate in organs (2). Despite numerous efforts in the research field, the understanding and controlling of melanoma cell migration/metastasis has been unsuccessful.

In addition to the conventional cyclic AMP (cAMP) signaling pathway through protein kinase A, a new protein kinase A–independent signaling pathway has been identified with the discovery of the exchange protein directly activated by cAMP (Epac), a guanine nucleotide exchange factor (3). Two isoforms of Epac, Epac1 and Epac2, were shown to mediate cAMP signaling through activation of a small-molecular weight G protein, Rap1 (4). In cancer cells, reports show that Epac mediates cell adhesion in Ovcar3 cells (5), apoptosis (6), and growth arrest (7) in B lymphoma cells, formation of embryonic vasculogenic networks in melanoma cells (8), and proliferation of prostate carcinoma cells (9). We have previously reported that Epac increases melanoma cell migration by a heparan sulfate–related mechanism (9). Additionally, we found that Epac could also increase melanoma cell migration without significantly increasing heparan sulfate production, suggesting the existence of a heparan sulfate–independent mechanism in Epac-induced melanoma cell migration.

Ca^{2+} is one of the major second messengers, and is known to regulate cell migration (10, 11). However, little attention has been paid to the role of the Ca^{2+} signal in cancer cell migration. In other cell types, there are a few reports demonstrating the role of Epac on the Ca^{2+} signal. In HEK293 cells, Ca^{2+} elevation was shown by overexpression of Epac1 (12). This report also suggested the involvement of phospholipase C (PLC) in the Epac-induced Ca^{2+} signal. Another report showed that Epac increases Ca^{2+} via ryanodine receptors in cardiac myocytes (13). In the current study, we studied the role of Epac in Ca^{2+} signal in melanoma cells. We found that Epac increases Ca^{2+} in melanoma cells.

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activation, Ca\(^{2+}\) was released from the endoplasmic reticulum via the PLC/inositol triphosphate (IP\(_3\))/IP\(_3\) receptor pathway. In addition, Epac-induced Ca\(^{2+}\) signal is involved in melanoma cell migration, which was mediated by Ca\(^{2+}\)-induced actin assembly. We thus propose that, in addition to the heparan sulfate–related mechanism that we previously reported (9), a Ca\(^{2+}\)-dependent mechanism also participates in Epac-induced melanoma cell migration. Furthermore, expression of Epac1, a major isoform of Epac, was higher in human metastatic melanoma than in primary melanoma, suggesting that Epac plays a pivotal role in the acquisition of metastatic ability in melanoma.

**Materials and Methods**

**Reagents and cell lines**

Reagents were purchased from Sigma, unless otherwise specified, 1,2-Bis(\(\alpha\)-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM) and ryanodine were purchased from EMD Chemicals. 8-(4-Methylphenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8-pMeOPT) and 8-(4-chlorophenoxythio)adenosine-3',5'-cyclic monophosphate, acetoxymethyl ester (8-CPT-cAMP-AM) were purchased from Biolog. α-Melanin–stimulating hormone (α-MSH) was purchased from Calbiochem. Antibodies against non-muscle myosin heavy chain (MHC) IIα and S100A4 were purchased from Abcam. Antibodies against Epac1, Epac2, Rap1, PLC\(\beta\)1, PLC\(\beta\)2, PLC\(\gamma\)1, PLC\(\epsilon\), and IP\(_3\) receptors (IP\(_3\)R1, IP\(_3\)R2, and IP\(_3\)R3) were purchased from Santa Cruz Biotechnology. SK-Mel-2 and SK-Mel-24 melanoma cell lines were obtained from the American Type Culture Collection. The SK-Mel-187 melanoma cell line was kindly provided by Dr. Alan Houghton (Memorial Sloan-Kettering Cancer Center, New York, NY). The WM3248 cell line was kindly provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA). HEMA-LP, a normal human melanocyte cell line, was obtained from Invitrogen. All melanoma cell lines, with the exception of WM3248, were maintained in Medium 254 (Invitrogen) with the addition of human melanocyte growth supplement (Invitrogen).

**Tissue harvesting and total RNA isolation**

Tumor samples were obtained as we previously described (14) through the Tissue Retrieval Service, a shared resource of The Cancer Institute of New Jersey, in accordance with the guidelines of the UMDNJ Robert Wood Johnson Medical School Institutional Review Board, and in a Health Insurance Portability and Accountability Act–compliant fashion. Tumor specimens were fresh-frozen and stored in liquid nitrogen until RNA extraction. Frozen sections were prepared under RNase-free conditions. The melanoma regions were identified by microscopic inspection in adjacent sections by H&E and MART-1-stained sections. Melanoma regions were then dissected with a needle and transferred to a prechilled Eppendorf tube containing 100 μL of lysis buffer. Total RNA was isolated from samples using a standard, spin column–based kit (Nucleospin RNA XS, Clontech). First-strand synthesis was performed on total RNA using another standard kit (TaqMan Reverse Transcription reagents, Applied Biosystems) to reverse transcribe mRNA.

**Quantitative PCR**

Quantitative PCR was performed as we previously described (10, 11). Briefly, Epac1 and Epac2 mRNA expressions were quantified by real-time PCR using the ABI PRISM 7700 Sequence Detector (Perkin-Elmer/Applied Biosystems). The relative amounts of genes of interest were generated using the standard curve method and normalized to 18S rRNA.

**Immunohistochemistry**

Immunohistochemistry was performed as previously described (15) using the Histostain-Plus 3rd Gen IHC Detection kit (Invitrogen). After fixation of sections with acetone, endogenous peroxidase quenching and blocking were performed in 1.5% normal goat serum solution followed by incubation with the goat anti-mouse primary antibody (MART-1, Epac1, or Epac2). After washing with PBS, incubation with the secondary antibody (biotinylated goat anti-mouse IgG) was performed, followed by treatment with peroxidase-conjugated streptavidin and DAB chromogen. Sections were counterstained with hematoxylin to visualize nuclei.

### Table 1. mRNA expression of Epac in human melanoma tissues

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Epac1</th>
<th>Epac2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary melanoma</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Median (25th-75th percentiles)</td>
<td>0.07</td>
<td>1.03</td>
</tr>
<tr>
<td>Range</td>
<td>0.02–1.4</td>
<td>0.07–9.3</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Median (25th-75th percentiles)</td>
<td>0.18*</td>
<td>0.18*</td>
</tr>
<tr>
<td>Range</td>
<td>0.01–1.83</td>
<td>0.01–1.83</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Median (25th-75th percentiles)</td>
<td>0.20</td>
<td>0.33</td>
</tr>
<tr>
<td>Range</td>
<td>0.03–1.11</td>
<td>0.2–11.5</td>
</tr>
</tbody>
</table>

NOTE: Epac mRNA expression in human melanoma tissues from primary melanoma, regional dermal metastasis, lymph node metastasis, and distant organ metastasis.

*P < 0.05 vs. primary melanoma.
performed. Then, streptavidin-peroxidase conjugate was applied followed by diaminobenzidine chromogen development of the staining.

**Fluorescence imaging of intracellular calcium**

Measurement of intracellular calcium release was performed as we previously described (16, 17) with some modifications. Cells were incubated with HEPES buffer containing 10 μmol/L of Fura2-AM or 2 μmol/L of Fluo-4AM (Figs. 2D, 4B, and D; Supplementary Fig. S2C) followed by washing and incubation with HEPES buffered saline containing 1.8 mmol/L of CaCl₂. Either a monochromator-equipped imaging system (TILL-Photonics) for Fura-2AM or an iXon+885 charge-coupled device camera (Andor Technology) for Fluo-4AM was used to monitor fluorescence changes using a PL Fluotar 40× oil immersion objective. Exposure times were 100 to 1,000 ms, depending on loading of the dyes. Full images were collected every 4 seconds. The field was illuminated by two wavelengths in rapid succession. Fura-2 was excited alternately at 340 and 380 nm and emission fluorescence was expressed as a ratio (F340/F380). Fluo-4 was excited at 488 nm, and data were expressed as normalized changes in background-corrected fluorescence emission (F/F₀). Data were analyzed using TILLvisION software (version 3.02) for Fura-2AM or Imaging Workbench (INDEC Biosystems) for Fluo-4.

**Short hairpin RNA transduction**

Short hairpin RNA (shRNA) transductions with lentivirus (Santa Cruz Biotechnology) were performed according to the protocols of the manufacturer. Cells were incubated with 8 μg/mL of Polybrene (Santa Cruz Biotechnology) and lentiviral particles harboring each shRNA followed by selection with puromycin dihydrochloride (Santa Cruz Biotechnology) for 1 week. Fresh puromycin-containing medium was replaced every 3 to 4 days. Established cell lines are as follows:

- Epac expression in human melanoma tissues and in human melanoma cell lines. A, representative immunohistochemical images for MART-1, Epac1, and Epac2 in human melanoma tissues from regional dermal metastatic melanoma tissue (arrows, melanoma tumor nest). B, protein expression of Epac1 and Epac2 in melanocyte and melanoma cell lines. C, migration assay in the presence of BAPTA-AM (5 μmol/L) and EGTA (5 mmol/L) in SK-Mel-2 cells with adenoviral LacZ or Epac1 overexpression (n = 4).

Immunocytochemistry

Immunocytochemistry was performed as we previously described (9). Cells grown on glass coverslips were fixed, permeabilized, and incubated sequentially with primary antibodies for MHCIHA or S100A4 and respective secondary antibodies. Alexa Fluor 488- and 594-conjugated goat anti-rabbit and anti-mouse antibodies (Molecular Probes) were used. Filamentous actin (F-actin) staining was performed by incubation with rhodamine phalloidin (Invitrogen). Pictures were taken with a digital camera operated on a Nikon Eclipse TE200. Quantification of actin filaments were performed as previously described (18, 19) by calculating total intensity of staining with ImageJ software (NIH).

Data analysis and statistics

Statistical comparisons among groups, except pixel intensity analysis for actin assembly and mRNA expression in melanoma tissues, were performed using Student’s t test or one-factor ANOVA with Bonferroni post hoc test. For pixel intensity analysis for actin assembly, two-factor factorial ANOVA with Bonferroni post hoc test was used. The Wilcoxon rank-sum test was used for comparison of mRNA expression in melanoma tissues. Statistical significance was set at the 0.05 level.

Results

Epac1 mRNA expression is increased in metastatic melanoma

mRNA expression of Epac1 and Epac2 was examined in human melanoma specimens. As shown in Table 1, Epac1 mRNA expression was higher in regional dermal metastasis (P = 0.017; also see Fig. 1A for immunohistochemistry) and distant metastasis (P = 0.013) than in primary melanoma.
By contrast, there was no significant difference in Epac2 mRNA expression between primary and metastatic melanoma tissues. Lymph node metastasis showed a tendency toward increased mRNA expression of Epac1, but it did not reach statistical significance \((P = 0.059)\), presumably due to the small number of samples. Western blotting in melanocyte and melanoma cell lines showed higher expression of Epac1 in a primary melanoma cell line (WM3248 from vertical growth phase melanoma) and in metastatic melanoma cell lines (SK-Mel-2, SK-Mel-24, and SK-Mel-187) than in a melanocyte cell line (HEMA-LP; Fig. 1B). Activated Rap1 was obvious in WM1552C, SK-Mel-2, and SK-Mel-187 cells (Supplementary Fig. S1A), cAMP-producing agents, i.e., α-MSH, a MSH receptor agonist, and isoproterenol, a β-adrenergic receptor agonist, activated Rap1 in both SK-Mel-2 cells and SK-Mel-187 cells (Supplementary Fig. S1B). Furthermore, 8-pMeOPT increased Rap1 activation in a dose-dependent manner in SK-Mel-2 cells (Supplementary Fig. S1C). These data suggest that Rap1 is involved in Epac signaling in melanoma. To examine the role of Ca\(^{2+}\) in melanoma cell migration, we performed a migration assay in SK-Mel-2 cells with Ca\(^{2+}\) depletion. Chelating Ca\(^{2+}\) with BAPTA-AM and EGTA inhibited Epac1 overexpression–induced cell migration (Fig. 1C), but not basal migration. These data suggest that the Ca\(^{2+}\) signal is involved in Epac-induced melanoma cell migration.

**Epac increases intracellular Ca\(^{2+}\) in melanoma cells**

We next examined whether endogenous Epac increases cytoplasmic Ca\(^{2+}\). 8-pMeOPT, a specific Epac agonist, increased the Ca\(^{2+}\) signal in WM3248, SK-Mel-2, SK-Mel-24, and SK-Mel-187 cells, whereas no change was observed in HEMA-LP cells (Fig. 2A). 8-CPT-cAMP-AM, another specific agonist for Epac, showed Ca\(^{2+}\) elevation in SK-Mel-2 and SK-Mel-187 cells (Fig. 2B). These data suggest that endogenous Epac evokes Ca\(^{2+}\) elevation in melanoma cells but not in melanocytes. When Epac1 was ablated by shRNA (Fig. 2C), 8-pMeOPT did not increase the Ca\(^{2+}\) signal (Fig. 2D). In contrast, ablation of Epac2 did not affect 8-pMeOPT–induced Ca\(^{2+}\) elevation.

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**Figure 3.** Epac mediates Ca\(^{2+}\) elevation through IP\(_3\) receptors.

Ca\(^{2+}\) signal was measured with the addition of 8-pMeOPT (100 μmol/L, arrows) in SK-Mel-2 cells. A, depletion of Ca\(^{2+}\) from endoplasmic reticulum with thapsigargin (1 μmol/L) abolishes 8-pMeOPT–induced Ca\(^{2+}\) elevation. B, IP\(_3\) receptor antagonists, 2-APB (1 μmol/L, top) and Xestopongin C (1 μmol/L, middle), inhibit 8-pMeOPT–induced Ca\(^{2+}\) elevation. Blocking ryanodine receptor by ryanodine (10 μmol/L) does not inhibit 8-pMeOPT–induced Ca\(^{2+}\) elevation (bottom). C, chelating Ca\(^{2+}\) in the extracellular buffer with EGTA (5 mmol/L) does not inhibit 8-pMeOPT–induced Ca\(^{2+}\) elevation (top). Neither verapamil (25 μmol/L, middle) nor nifedipine (250 μmol/L, bottom) inhibit 8-pMeOPT–induced Ca\(^{2+}\) elevation. D, PLC inhibition with U73122 (10 μmol/L) negates 8-pMeOPT–induced Ca\(^{2+}\) elevation.
Additionally, Epac1 ablation with shRNA was sufficient to abolish Ca^{2+} elevation in SK-Mel-187 cells (data not shown). These data suggest that Epac1, but not Epac2, plays a major role in Ca^{2+} elevation in melanoma cells.

**Epac increases Ca^{2+} from the endoplasmic reticulum via IP3 receptors**

We next explored the mechanism by which Epac increases Ca^{2+} in melanoma cells. When Ca^{2+} was depleted from the endoplasmic reticulum with thapsigargin, a sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase inhibitor, the Epac agonist failed to increase Ca^{2+} signal (Fig. 3A), suggesting that the Epac mobilized Ca^{2+} from intracellular stores. We thus examined Ca^{2+}-regulating molecules located in the endoplasmic reticulum. Pretreatment with 2-APB, an IP3 receptor antagonist, abolished Epac-mediated Ca^{2+} signal (Fig. 3B, top). In addition, Xestopongin C, another IP3 receptor antagonist, also negated Epac-mediated Ca^{2+} signal (Fig. 3B, middle). By contrast, inhibition of ryanodine receptors did not inhibit Epac-mediated Ca^{2+} increase (Fig. 3B, bottom). These data suggest that Epac mobilized Ca^{2+} via the IP3 receptors. On the other hand, depletion of extracellular Ca^{2+} with EGTA did not inhibit Epac-induced Ca^{2+} signal (Fig. 3C, top). Furthermore, because the expression of L-type calcium channels was reported in melanoma cells (20), we examined the effect of inhibition of these calcium channels on Epac-induced Ca^{2+} signal. Neither verapamil nor nifedipine, L-type Ca^{2+} channel blockers, inhibited Epac-induced Ca^{2+} signal (Fig. 3C, middle and bottom). A previous report suggested the involvement of PLC in Epac-induced Ca^{2+} release (12). We thus examined the effect of PLC inhibition on Epac-induced Ca^{2+} elevation. U73122, a PLC inhibitor, prevented 8-pMeOPT-induced Ca^{2+} elevation (Fig. 3D), suggesting that Epac increases Ca^{2+} via the PLC/IP3 receptor signaling pathway.

**The PLCε/IP3/IP3R1 pathway mediates Epac-induced Ca^{2+} elevation**

It is known that there are three isoforms of the IP3 receptor, i.e., IP3R1, IP3R2, and IP3R3 (21). However, the expressions and roles of IP3R isoforms in melanoma cells have not been studied. We thus examined the expression of the IP3R isoforms, and found that IP3R1 is a major isoform in melanoma cells.
We next examined the involvement of IP3R1 in Epac-induced Ca2+ elevation. When IP3R1 was ablated by shRNA in SK-Mel-2 cells (Fig. 4B, top), 8-pMeOPT failed to increase Ca2+ signals (Fig. 4B bottom), suggesting that IP3R1 is involved in the Epac-induced Ca2+ elevation. In addition, 8-pMeOPT increased IP3 production in SK-Mel-2 cells (Fig. 4C, bottom), and it was inhibited by the PLC inhibitor. We then investigated a specific PLC isoform which is involved in the Epac signaling pathway. Among PLC isoforms, PLCγ1 (Supplementary Fig. S2A) and PLCε (Fig. 4C) are abundantly expressed in SK-Mel-2 cells. Ablation of PLCε (Fig. 4C, top) inhibited Epac-induced IP3 production (Fig. 4C, bottom) and Ca2+ release (Fig. 4D), whereas ablation of PLCγ1 (Supplementary Fig. S2B) did not inhibit Ca2+ release (Supplementary Fig. S2C). This is in accordance with a previous report demonstrating that PLCγ is activated by Epac (12). Taken together, these data suggest that Epac mediates Ca2+ elevation via the PLCε/IP3/IP3R signaling pathway in melanoma.

Epac-induced Ca2+ increases melanoma cell migration

Because Ca2+ is a major mediator of cell migration (10, 11), we examined whether Epac-induced Ca2+ increases melanoma cell migration. As shown in Fig. 5A, 8-pMeOP–induced cell migration was inhibited by the IP3 receptor inhibitor, 2-APB and Xestopongin C. In addition, 2-APB and Xestopongin C inhibited cell migration induced by Epac1 overexpression. These data suggest that IP3 receptor–mediated Ca2+ release is involved in Epac-induced cell migration. Interestingly, neither 2-APB nor Xestopongin C reduced basal cell migration, suggesting that Ca2+ mediates melanoma cell migration only when the Epac/Ca2+ signaling pathway is activated. Consistent with the results shown in Fig. 3B and C, neither inhibition of ryanodine receptor nor L-type Ca2+ channel blockers inhibited Epac-induced cell migration. We next examined the effects of ablation of the signaling molecules involved in Epac-induced Ca2+ elevation, i.e., Epac1, PLCε, and IP3R1, on cell migration. Ablation of Epac1 and IP3R1 did not reduce basal migration whereas ablation of Epac1 did, suggesting the involvement of another mechanism in Epac1-induced cell migration, i.e., the heparan sulfate–related mechanism (9). Taken together, these data suggested that Epac increases melanoma cell migration via the PLCε/IP3R/Ca2+ signaling pathway, and it occurs only under Epac-activated conditions.

Epac increases cell migration via actin assembly

We next studied the mechanism by which Epac-induced Ca2+ regulates melanoma cell migration. It is known that S100A4, a Ca2+ binding protein, activates MHCIIA, leading to actin assembly (22–25) and thus enhanced cell migration through lamellipodia formation (26). We therefore hypothesized that Epac enhances the interaction between S100A4 and MHCIIA, and thus, actin assembly. We first examined
Figure 6. Epac-induced Ca\textsuperscript{2+} increases actin assembly. A, migration assay was performed in the presence of cytochalasin D (Cyto D, 1 \mu mol/L) and/or 8-pMeOPT (100 \mu mol/L) in SK-Mel-2 cells. Cytochalasin D inhibits 8-pMeOPT-induced cell migration, but not basal cell migration (n = 4). B, actin assembly with Epac activation and/or IP\textsubscript{3} receptor inhibition. Top, immunocytochemistry for F-actin staining shows that incubation with 8-pMeOPT (100 \mu mol/L) for 30 min increases actin fiber formation, and is inhibited by 2-APB (1 \mu mol/L). Bottom, a bar graph showing quantification of actin assembly using the pixel intensity. 8-pMeOPT increases F-actin staining and is inhibited by 2-APB (n = 49–50). C, immunocytochemistry of double staining with S100A4 (red) and MHCIIA (green; top). Merged image shows that colocalization (yellow) of S100A4 and MHCIIA at the edge of the lamellipodium (arrow). Immunoprecipitation for MHCIIA and S100A4 was performed (bottom). Incubation with 8-pMeOPT (100 \mu mol/L) for 30 min increases physical interaction between MHCIIA and S100A4, and is inhibited by 2-APB (1 \mu mol/L). IB, immunoblot; IP, immunoprecipitation. D, top, immunocytochemistry of double staining with F-actin (red) and MHCIIA (green). Merged image shows the colocalization (yellow) of S100A4 and MHCIIA at the edge of the lamellipodium. Arrow denotes actin-rich membrane processes. Bottom, quantitative analysis of the number of cells with more than one lamellipodium. Lamellipodia were identified by colocalization of MHCIIA with actin. Cells with more than one lamellipodium were counted under the microscope [n = 353 cells (control), n = 342 cells (2-APB), n = 373 cells (8-pMeOPT), and n = 383 cells (8-pMeOPT + 2-APB)].
the involvement of actin assembly in Epac-induced cell migration. Incubation with cytochalasin D, an inhibitor of actin assembly, abolished 8-pMeOPT–induced cell migration (Fig. 6A), suggesting that actin assembly is indeed involved in Epac-induced cell migration. 8-pMeOPT increased F-actin content, and it was inhibited by 2-APB (Fig. 6B), suggesting that Epac-induced Ca²⁺ mediates actin assembly. We next examined the effect of Epac on the interaction of S100A4 with MHCIIA. It was shown that S100A4 and MHCIIA are colocalized on the edges of lamellipodia, at least, in breast cancer cells (23). Such colocalization was also observed in SK-Mel-2 cells (Fig. 6C, top). Furthermore, immunoprecipitation showed that Epac agonist increases physical interactions between S100A4 and MHCIIA, and the interaction was inhibited by 2-APB (Fig. 6C, bottom). These findings suggest that Ca²⁺ released from IP₃ receptors, which is evoked by Epac, mediates the interaction between S100A4 and MHCIIA. When we quantified the number of cells with lamellipodia, which are formed during the process of cell migration (Fig. 6D, top; ref. 27), we found that 8-pMeOPT increased the number of lamellipodia-positive cells (Fig. 6D, bottom), and it was inhibited by 2-APB. These data suggest the involvement of Ca²⁺ in Epac-induced lamellipodia formation. Consistent with cell migration results (Fig. 5A), 2-APB did not inhibit basal actin assembly and lamellipodia formation. Taken together, these data suggested that Epac-induced Ca²⁺, which is released from IP₃ receptors, mediates melanoma cell migration through the interaction between S100A4 and MHCIIA, and the resultant actin assembly.

Discussion

Previous studies including ours showed the roles of Epac in melanoma cell migration with the heparan sulfate–related mechanism (9) and with activation of extracellular signal–regulated kinase (28), and formation of embryonic vasculogenic networks in melanoma cells (8). In the present study, we showed that Epac1 expression is increased in metastatic melanoma compared with primary melanoma. We also showed that Epac increases melanoma cell migration through the Ca²⁺ signaling pathway. Although Ca²⁺ is one of the major second messengers (10, 11), the role of Ca²⁺ in cancer cells has not been well studied. Ca²⁺–mediated cell migration through calpain activation was shown in fibrosarcoma cells (29), and through CDC42 and Rac in neutrophils (30). Also, in lung embryonic fibroblasts, a role of transient receptor potential ion channels (TRPM, the "M" stands for "melastatin") in Ca²⁺–mediated cell migration has been shown (31). We showed that Epac increases Ca²⁺ in multiple melanoma cell lines. This Ca²⁺ elevation was mediated by the PLC/IP₃/IP₃R1 signaling pathway. Furthermore, Epac-induced Ca²⁺ mediates actin assembly, presumably through a physical interaction between S100A4 and MHCIIA, which leads to enhanced melanoma cell migration. Because cell migration is a key process in cancer metastasis, our findings suggest that Epac is a potential target for inhibition/prevention of melanoma metastasis. Several reports have shown that Epac increases Ca²⁺ via PLC in non-cancer cells, i.e., sperm (32), rat inner medullary collecting duct epithelial cells (33), neuroblastoma cells (12), and cardiac myocytes (13). The involvement of a specific subtype of PLC, i.e., PLCε, in the Epac signaling pathway was suggested in cardiac myocytes (13) and in HEK293 cells by adenosinergic overexpression of PLCε (12). Our study showed the involvement in melanoma using ablation of PLCε, suggesting that Epac-induced Ca²⁺ release in cardiac myocytes (13), we showed that, in melanoma cells, the IP₃R1 is involved in Epac-induced Ca²⁺ release. From these findings, it is likely that Epac-mediated Ca²⁺ signaling differs among cell types. Meanwhile, it was reported that Epac activates PLC via Rap1 (34). Similarly, we found that the Epac agonist activates Rap1 in melanoma cells (Supplementary Fig. S1C), suggesting that Rap1 mediates Epac-induced PLC activation in melanoma cells. The variation of Rap1 activity in Supplementary Fig. S1A is presumably due to the expression of Rap1, and/or GTPase activity by Rap1GAP in melanoma (35). The increase of Rap1 activation over basal with physiologic stimuli was higher in SK-Mel-187 than in SK-Mel-2 (Supplementary Fig. S1B). Also, 50 μmol/L of 8-pMeOPT did not evoke Ca²⁺ elevation in SK-Mel-2, although it did in SK-Mel-187 (data not shown). Because Epac1 expression was higher in SK-Mel-187 than in SK-Mel-2, these data suggest that Epac1 expression positively correlates, at least in part, with Rap1 activation, and the resultant Ca²⁺ elevation in melanoma.

We previously reported that heparan sulfate is involved in Epac-induced melanoma cell migration (9). Using SK-Mel-2 cells, we showed that Epac mediates cell migration via increased heparan sulfate production. Therefore, our current and prior findings indicate that Epac increases melanoma cell migration through two different mechanisms; the heparan sulfate–related and the Ca²⁺–dependent mechanism. However, there are two potential differences between these mechanisms: (a) timing in the process of migration and (b) status of Epac activation. In regards to the timing in the process of migration, the heparan sulfate–related mechanism occurs in the relatively late phase after Epac activation. Epac-induced increase in heparan sulfate did not occur until after 48 hours of incubation. In addition, the effect of heparitinase, a heparan sulfate–degrading enzyme, on Epac-induced cell migration was more obvious after 48 hours of incubation than after short-time incubation (within 24 hours; ref. 9). In contrast, Ca²⁺–dependent mechanisms occur in the relatively early phase. The resultant Ca²⁺–mediated actin assembly also occurs within 30 minutes, and inhibition of actin assembly almost completely inhibits Epac agonist–induced migration. These results suggest that Ca²⁺–dependent cell migration occurs first, followed by heparan sulfate–related cell migration. Regarding the status of Epac activation, our data suggest that the heparan sulfate–related mechanism is involved in both basal and Epac-activated conditions (9), but the Ca²⁺–dependent mechanism participates only in an Epac-activated condition. Melanoma cell migration was reduced under both basal and Epac-activated conditions with inhibition of molecules involved in the heparan sulfate–related mechanism (9). In contrast, abrogation of the Ca²⁺–dependent mechanism, such as pharmacologic inhibition of PLC, IP₃R and actin assembly, or ablation of PLCε or...
Results

Our results showed the major role of Epac in Ca\textsuperscript{2+} regulation; however, it is not yet clear which upstream G protein–coupled receptor(s) plays a major role in Epac-induced Ca\textsuperscript{2+} release and cell migration. MSH receptor and β-adrenergic receptor potentially regulate Epac-mediated effects because of effective Rap1 stimulation in melanoma (Supplementary Fig. S1). A recent study suggested the involvement of the Epac-IP\textsubscript{3} pathway in serotonin 5-hydroxytryptamine (5-HT4) receptor signaling (36). No prior reports in melanoma cells have been published, thus further investigation is required to identify a G protein–coupled receptor(s), which could selectively stimulate Epac to evoke Ca\textsuperscript{2+} elevation. We have previously shown that Epac1 overexpression increases lung metastasis in mice (9). The next step is to examine the effect of ablation of Epac in melanoma metastasis in vivo. It will be also necessary to determine the role of the heparan sulfate–related and the Ca\textsuperscript{2+}-dependent mechanisms in melanoma metastasis using an animal model. Further-more, investigation for the expression of Epac protein, in addition to mRNA, in human melanoma samples is also required to obtain conclusive evidence for the role of Epac in melanoma metastasis. In conclusion, the robust findings of our current study suggest that Epac may serve as a molecular target for the treatment of melanoma, and the Epac-dependent mechanism of cell migration might occur in other cancers as well.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Exchange Protein Directly Activated by Cyclic AMP Increases Melanoma Cell Migration by a Ca\textsuperscript{2+}-Dependent Mechanism

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