HeLa Human Cervical Cancer Cell Migration Is Inhibited by Treatment with Dibutyryl-cAMP

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Abstract. *Cyclic AMP (cAMP) activates both protein kinase A (PKA) and guanine-nucleotide exchange factor exchange protein directly activated by CAMP (EPAC)-mediated Ras-related Protein[1] (RAP[1]) GTPase that regulates various cellular functions including cell migration. Herein, we investigated whether cAMP-mediated PKA and EPAC1/RAP1 pathways differentially control HeLa cervical cancer cell migration. Although HeLa cell migration was reduced by dibutyryl-cAMP, we observed an increase in cAMP/PKA, cAMP/EPAC1/RAP1-GTPase, and RAC1-GTPase. HeLa cell migration and RAC1-GTPase were increased by treatment with 8-(4-chloro-phenylthio)-2'-O-methyladenosine-3',5'-cAMP analogue to activate EPAC-specific signaling pathways. When HeLa cells were treated with H-89, a PKA inhibitor, cell migration was enhanced but RAC1-GTPase was inhibited. In addition, cell migration induced by dibutyryl-cAMP was reversed but the activity of Rac1-GTPase was inhibited by H-89 treatment. Taken together, these data demonstrate that cAMP/PKA and cAMP/EPAC1/RAP1-GTPase might inversely control cervical cancer cell migration, although both signaling pathways may up-regulate RAC1-GTPase. It also suggests that cAMP-mediated cancer cell migration was independent of RAC1-GTPase activation.*

Cell migration plays an important role in diverse cellular physiological and pathological process including tumor metastasis. Cell motility is stimulated by several growth factors, cytokines and extra-cellular signaling (1-4). Cell migration accompanied the re-organization of actin cytoskeleton (5, 6). Cytoskeletal re-organization is dependent on the activation of small GTPases, including RAC1, CDC42, and RAP1 (7-9). RAC1 is a member of the Rho-family GTPases that is ubiquitously expressed and required for lamellipodia formation by the actin polymerization (10, 11). RAC1 is activated by the interaction with calmodulin (12) or by non-receptor tyrosine kinase ACK-2 (12). When RAC1 is activated, it binds to P21-activated kinase 1 (PAK1) binding domain (PBD) (8, 13, 14), which leads to the formation of lamellipodia and filopodia at the protrusion of the cells leading edge (9, 13). Lamellipodia are the migration force of motile and invasive cells (15, 16). RAC1 activity is also necessary for ADP-ribosylation factor GTPase-activating proteins to promote the growth of focal adhesions (17).

Cyclic AMP (cAMP) is a second messenger and mediates various cellular signaling events including cell migration (18). cAMP regulates intracellular signaling pathways through cAMP-dependent protein kinase A (PKA), and guanine-nucleotide exchange factor exchange protein directly activated by cAMP (EPAC)-mediated RAP1 (19-21). The effect of cAMP on cell migration can be promotive or inhibitory depending on cell types and extracellular matrix. cAMP/PKA has an inhibitory role in migration of mouse embryonic fibroblasts and 4T1 breast cancer cells (5, 22). A positive role of cAMP/PKA in cell migration was also reported in epidermal growth factor (EGF)-induced MDA-MB435 human breast carcinoma cells (2) and A431 squamous carcinoma cells (23). While EPAC1/RAP1 inhibits cell migration in hepatocyte growth factor (HGF)-induced epithelial cells (24) and thyroid cancer cells (25), EPAC1/RAP1 increases melanoma cell migration (26). Although cAMP regulates cell migration through PKA and EPAC1/RAP1 activation, little is known about how cAMP/PKA and cAMP/EPAC1/RAP1 differentially regulate cell migration through their interactions.

In the present study, we investigated whether cell migration might be differentially regulated through interactions between cAMP/PKA and cAMP/EPAC1/RAP1 using HeLa human cervical cancer cells.

Materials and Methods

Reagents. Dibutyryl-cAMP was obtained from Calbiochem (San Diego, CA, USA). 8-(4-Chloro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic cAMP (8-CPT-2me-cAMP) was purchased from Axxora Biolog (LLC, NY, USA). Antibodies to cAMP response element-

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binding protein (CREB) and phospho-CREB (Ser133) came from Cell Signaling (Beverly, MA, USA). Anti-mouse RAC1 was purchased from Millipore (Billerica, MA, USA). Anti-rabbit RAP1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Except where indicated, all other materials were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Cell culture. Human cervical cancer HeLa (ATCC CCL-2) cell line was obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB) cell bank (Daejeon, Korea). Cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, Grand Island, NY, USA) supplemented with 5 % fetal-bovine serum (GIBCO), 2 mM L-glutamine, 100 units/ml of penicillin and 100 units/ml of streptomycin (GIBCO). The cells were incubated at 37˚C in a humidified incubator supplemented with 5 % CO_2 and 95 % air.

Wound healing and Boyden chamber assay. Cell migration was assessed by wound healing and Boyden chamber assay. For wound healing assay, 8×10^5 HeLa cells were seeded into a 35 mm^2 culture dish. When cells were confluent, three wound lines in the form of a cross were made by scratching the cellular monolayer with a 200 μl pipette tip. Cell debris was then washed off serveral times with culture medium. During subsequent incubation, the wound closure was recorded by taking photographs under phase-contrast microscopy. Boyden chamber assay was performed by using transwell plate with 8 μm pore size (Costar, Corning, NY, USA). The upper transwell was filled with 1.5×10^4 cells in 100 μl and culture medium of 600 μl was added into the lower chambers. Cells in the upper transwell were treated with each agent for 15 h and then stained with 0.1% crystal violet dye solution containing 20% methanol. Non-migrated cells on the upper face of the transwell membrane were wiped off with a cotton swab. Migrated cells adhered to the lower face of the transwell membrane were counted under a magnifying lens.

Measurement of RAP1- or RAC1-GTPase activation. The level of active GTP-bound small GTPase was determined by using glutathione (GST)-pulldown assay. In brief, 500 μg of cell lysates were clarified with sepharose beads (Amersham Biosciences, Uppsala, Sweden) and incubated with GST-Ral guanine nucleotide dissociation stimulator (GDS) and GST-PAK1PBD for RAP1-GTP and RAC1-GTP, respectively at 4˚C for 1h with rotation. RAP1-GTP or RAC1-GTP was collected by incubation with GST sepharose beads. Unbound residual proteins were washed out with cell lysis buffer three times. GTP-bound RAP1 or RAC1 were released from the beads by adding 3× protein sample buffer, and boiled for 10 min. The amounts of active RAP1-GTP or RAC1-GTP were measured by western blot analysis.

Reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated from HeLa cells using TRIZOL reagent (Invitrogen, Calsbad, CA, USA). cDNA was synthesized from 1μg of total RNA, using oligo dT18 Primer and RT PreMix (Bioneer, Taejeon, Republic of Korea). cDNA product of 1 μl was amplified by using Taq DNA polymerase and oligonucleotides specific for human EPAC1 (Forward: TGC CAT GTG AAA CAC GAC TG, Reverse: TTG TCT TCT CGC AGG ATG AT) as follows: 30 thermocycles of 95˚C for 30 s, 58˚C for 30 s, and 72˚C for 50 s. PCR products were detected by running 1.2% agarose gel electrophoresis.

Western blot analysis. Cells were harvested and lysed in ice-cold lysis buffer containing 0.5 % Nonidet P-40(v/v) in 20 mM Tris-HCl, at pH of 8.3; 150 mM NaCl; protease inhibitors (2 μg/ml aprotinin, pepstatin, and chymostatin; 1 μg/ml leupeptin; 1 mM phenylmethyl sulfonyl fluoride (PMSF); and 1 mM Na_3VO_4). All samples of lysate protein were seperated by SDS-polyacrylamide gel electrophoresis and were then transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween, and then incubated with appropriate antibodies. Bound antibodies were visualized with horse-radish peroxidase-conjugated secondary antibodies with the use of enhanced chemiluminescence (Pierce, Rockford, IL, USA).

Statistical analyses. Experimental differences were tested for statistical significance using ANOVA and Student's *t*-test. A *p*-value of 0.05 and 0.01 or less was considered to be significant.

Results

Dibutyryl-cAMP-mediated HeLa cell migration is independent of RAC1-GTPase activation. Given that cAMP regulates cell migration through PKA and EPAC1/RAP1 activation (2, 5, 19, 22-26), we investigated whether cAMP/PKA and cAMP/EPAC1/RAP1 differentially regulate cell migration through their effects on each other using dibutyryl-cAMP, cAMP analogue. As shown in Figure 1A and 1B, HeLa cervical cancer cell migration after 18 h was reduced by the treatment with 100 μM dibutyryl-cAMP by 25% compared to the untreated control group. No changes in cell growth were detected when dibutyryl-cAMP was used from 50 to 400 μM as compared to the control group (data not shown). We confirmed the effect of cAMP on cancer cell migration by Boyden chamber assay. Cell migration decreased by treatment with dibutyryl-cAMP by about 30% (Figure 1C and 1D). This is consistent with the result of the wound repair assay. The results suggest that cAMP might inhibit cervical cancer cell migration.

It has been reported that RAC1 plays a role in driving cells to move forward by the formation of lamellipodia (9, 13, 15, 16). Our data showed that RAC1 was activated by treatment of 100 μM dibutyryl-cAMP, time-dependently (Figure 1E). This demonstrates that cAMP-mediated cervical cancer cell migration might be independent of RAC1 activation.

Dibutyryl-cAMP increased PKA and EPAC1-mediated RAP1 activity in cervical cancer cells. Since cAMP regulates cAMP-dependent PKA and EPAC (19, 27-29), we measured PKA and EPAC activity in HeLa cervical cancer cells. EPAC1 gene was detected in these cells (Figure 2A). EPAC1-mediated RAP1 was activated by the treatment with dibutyryl-cAMP, dose- and time-dependently (Figure 2B and 2C). In addition, we assessed PKA activity as judged by CREB phosphorylation at Ser133. As shown in Figure 2D and 2E, dibutyryl-cAMP enhanced the level of Ser133 phosphorylation of CREB in a dose- and time-dependent

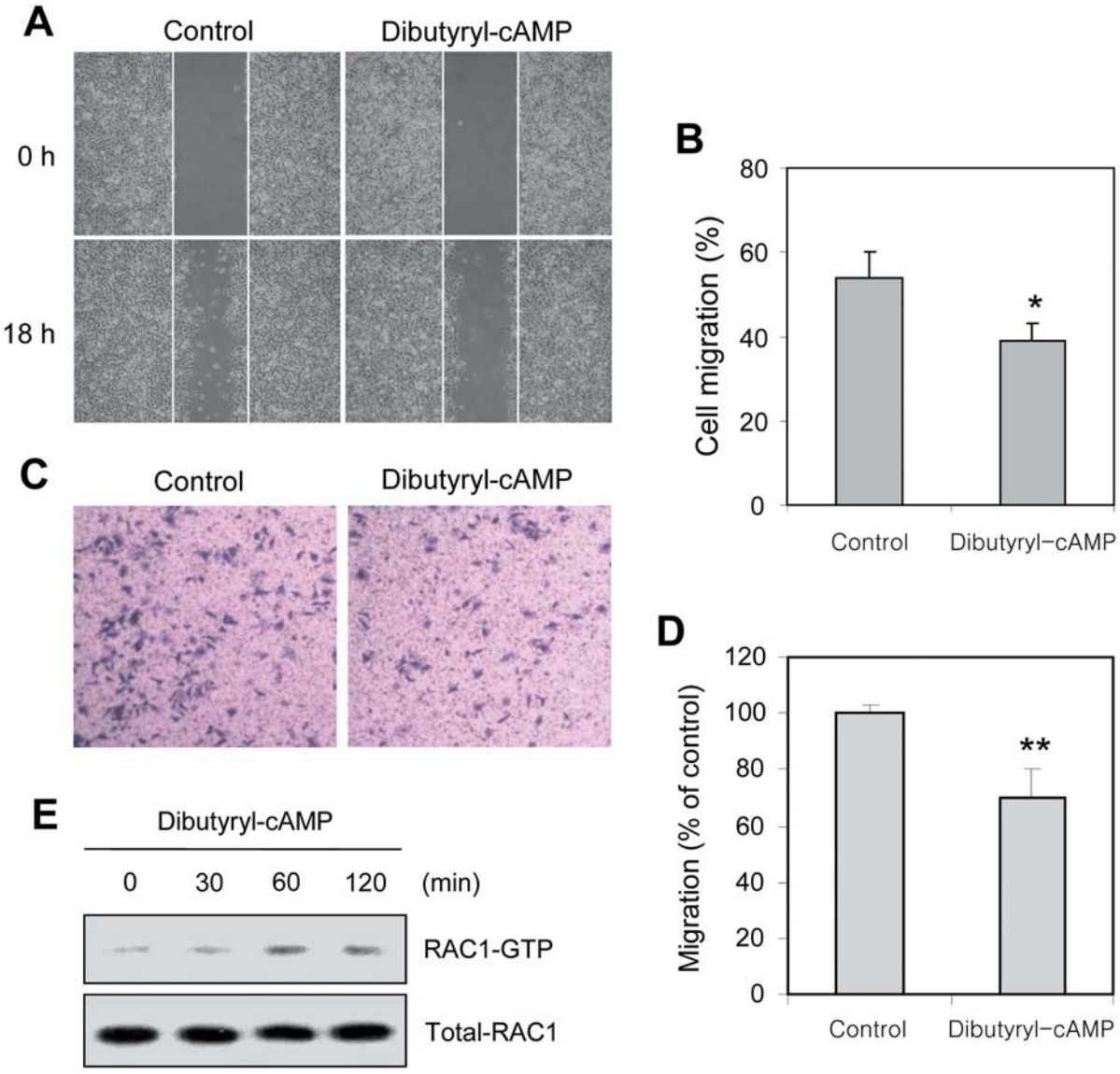

Figure 1. *Cancer cell migration was inhibited by treatment with dibutyryl-cAMP. A straight wound line was made in HeLa cells confluent in a 35 mm^2 culture dish and the cells were then incubated in the absence and presence of dibutyryl-cAMP (100 μM) for 18 h. Wound closure was recorded and photographed under phase-contrast microscopy at the indicated time (A). The empty area at each time point was quantified with NIH image analysis software (version 1.62) and compared with that in the initiation of cancer cell migration. Relative cell migration is represented with a bar graph (B). HeLa cells (1.5×10^4) suspended in medium were seeded in the upper chamber, treated with dibutyrylb-cAMP (100 μM) and incubated for 15 h. The filter membrane of the upper chamber was fixed and dyed with crystal violet solution and non-migrated cells on the upper face of the insert were removed using a cotton swab. The remaining cells migrating on lower face of upper chamber were photographed under phase-contrast microscopy (C). Migrated cells were counted and are represented with a bar graph (D). Data are means±SED representative of three independent experiments. *p≤0.05 and **p≤0.01, significant difference as compared to the control. E: HeLa cells were treated with dibutyryl-cAMP (100 μM) for the indicated times. RAC1 activity was analyzed by pulldown assay as described in Materials and Methods.*

manner. The two different cAMP-mediated pathways might affect RAC1 activity in cervical cancer cell migration.

RAC1-GTPase and cell migration were enhanced by EPAC1/RAP1-GTPase. To investigate the effect of cAMP/EPAC-mediated RAP1 pathway on cell migration and RAC1-GTPase, we treated HeLa cells with 8CPT-2me-cAMP, another cAMP analogue. As shown in Figure 3A and 3B, cell migration was increased by about 50% at 15 h. No changes in cell growth were detected by the treatment with 8CPT-2me-cAMP (data not

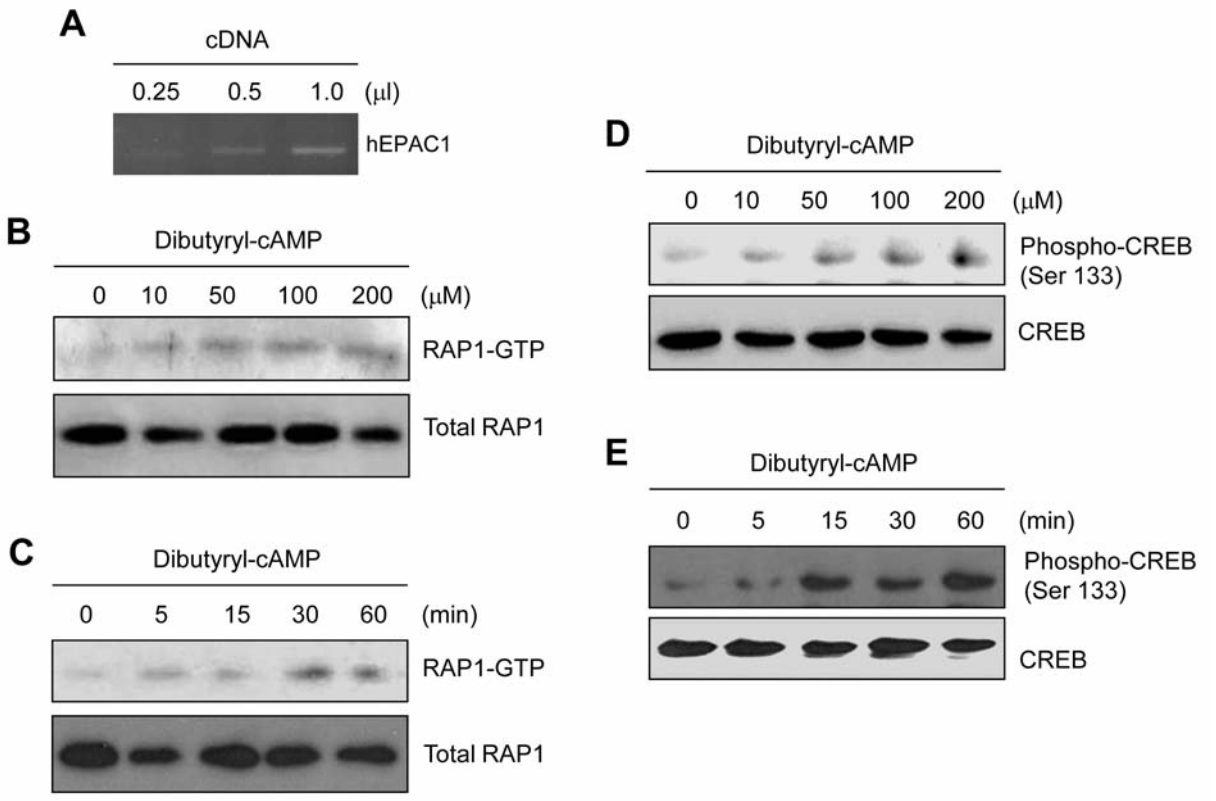

Figure 2. *PKA and EPAC1-mediated RAP1-GTPase were activated by the treatment with dibutyryl-cAMP. A: RNA was prepared from HeLa cells using Trizol and EPAC1 gene in HeLa cells was detected by RT-PCR. B and C: HeLa cells were treated with different concentrations of dibutyryl-cAMP for the indicated times. EPAC1-mediated RAP1-GTPase activity was measured by pulldown assay as described in the Materials and Methods. D and E: Protein kinase A (PKA) activation was judged by cAMP response element-binding protein (CREB) phosphorylation at serine 133 (Ser133) using western blot analysis. Data are representative of three independent experiments.*

shown). While RAP1-GTPase was activated by 8CPT-2me-cAMP treatment, no changes were found in Ser133 phosphorylation of CREB (Figure 3C). In addition, RAC1-GTPase was increased by the treatment with 8CPT-2me-cAMP (Figure 3D). This suggests that cAMP/EPAC-mediated RAP1 activation increases cell migration and up-regulates RAC1-GTPase in cervical cancer cells.

Treatment with H-89 inhibited RAC1-GTPase but enhanced cell migration. Given that PKA activity regulates actin cytoskeleton and cell migration (30), we examined the effect of PKA on cervical cancer cell migration and RAC1-GTPase. When HeLa cells were treated with H-89, a PKA inhibitor, cell migration was increased by about 20% at 18 h (Figure 4A and 4B). In contrast, H-89 effectively inhibited not only Ser133 phosphorylation of CREB but also RAC1-GTPase activity at the same concentration (Figure 4C). No changes in cell growth were detected by the treatment with less than 500 nM of H-89 for 18 h (data not shown). These data suggest that cAMP/PKA pathway negatively regulates HeLa cell migration irrespective of RAC1 activation.

To confirm the effect of cAMP-induced PKA activity on cell migration and RAC1-GTPase, HeLa cells were treated with dibutyryl-cAMP in the presence of H-89. Our results showed that cell migration attenuated by dibutyryl-cAMP treatment was significantly increased by pre-treatment with H-89 (Figure 5A and 5B). H-89 treatment inhibited not only dibutyryl-cAMP-induced Ser133 phosphorylation of CREB but also RAC1-GTPase (Figure 5C). This implies that cervical cancer cell migration is independent of cAMP-mediated RAC1 up-regulation by PKA.

Discussion

Cell migration is a multi-step process and regulates many physiological and pathological cellular functions (31-33). Cells in migration move forward by lamellipodia formation which is regulated by actin polymerization and small GTPases, RAC1 (5, 6, 8, 10, 11, 15, 16). cAMP, an intracellular second messenger, mediates various cellular signaling events, including cell migration (2, 5, 18, 22, 24,

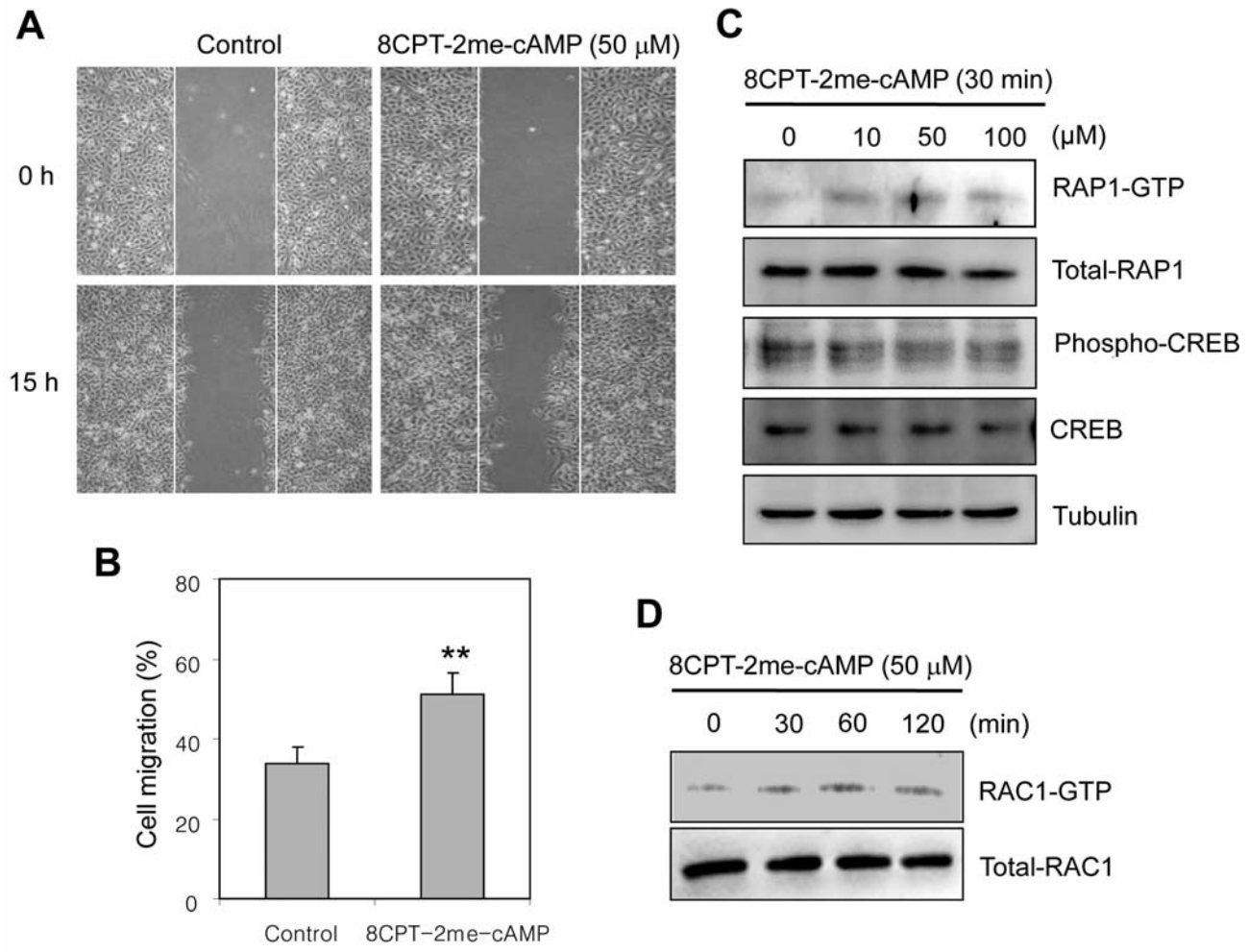

Figure 3. *8-(4-Chloro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8CPT-2me-cAMP), a cAMP analogue, increased HeLa cell migration with the activation of EPAC-specific RAP1 signaling pathways. A straight wound line was in HeLa cells confluent in a 35 mm^2 culture dish, and incubated in the absence and presence of 8CPT-2me-cAMP (50 μM) for 15 h. Wound closure was recorded and photographed under phase-contrast microscopy at the indicated time (A). The empty area at each time point was quantified with NIH image analysis software (version 1.62) and compared with that in the initiation of cancer cell migration. Relative cell migration is represented with bar graph. Data in bar graph represent the means±SED. **p≤0.01, significant difference as compared to control (B). RAC1-GTPase activity was measured by pulldown assay as described in the Materials and Methods (C). cAMP response element-binding protein (CREB) phosphorylation at serine 133 (Ser133) was detected by using western blot analysis. RAP1-GTPase activity was measured by pulldown assay, as described in the Materials and Methods (D). Data are representative of three independent experiments.*

26, 30, 34) through the activation of PKA and EPAC1/RAP1 (19-21). Here, we investigated whether cAMP-mediated pathways might be differentially required for cell migration of HeLa human cervical cancer cells that express EPAC1 gene (Figure 2A). Our data showed that HeLa cell migration decreased and RAC1-GTPase was activated by treatment with dibutyryl-cAMP under our experimental condition (Figure 1). We also observed that dibutyryl-cAMP increased PKA which was inhibited by H-89, PKA inhibitor and EPAC1-mediated RAP1 activity, which is stimulated by 8CPT-2me-cAMP, an EPAC agonist (Figures 2-4). Cell migration was increased by treatment with both H-89 and 8CPT-2me-cAMP (Figures 3 and 4). We confirmed that the cAMP-mediated decrease in cell migration might be a result of PKA by the pre-treatment of cells with H-89 prior to addition of dibutyryl-cAMP (Figure 5). The data demonstrate that cAMP-mediated HeLa cell motility can be positively-regulated by cAMP/EPAC-mediated RAP1 pathway but it may be negatively controlled by PKA activation. This suggests that PKA and EPAC-mediated RAP1 pathway play opposing roles in cell migration. It also suggests that the effect of the cAMP/PKA pathway could be more dominant

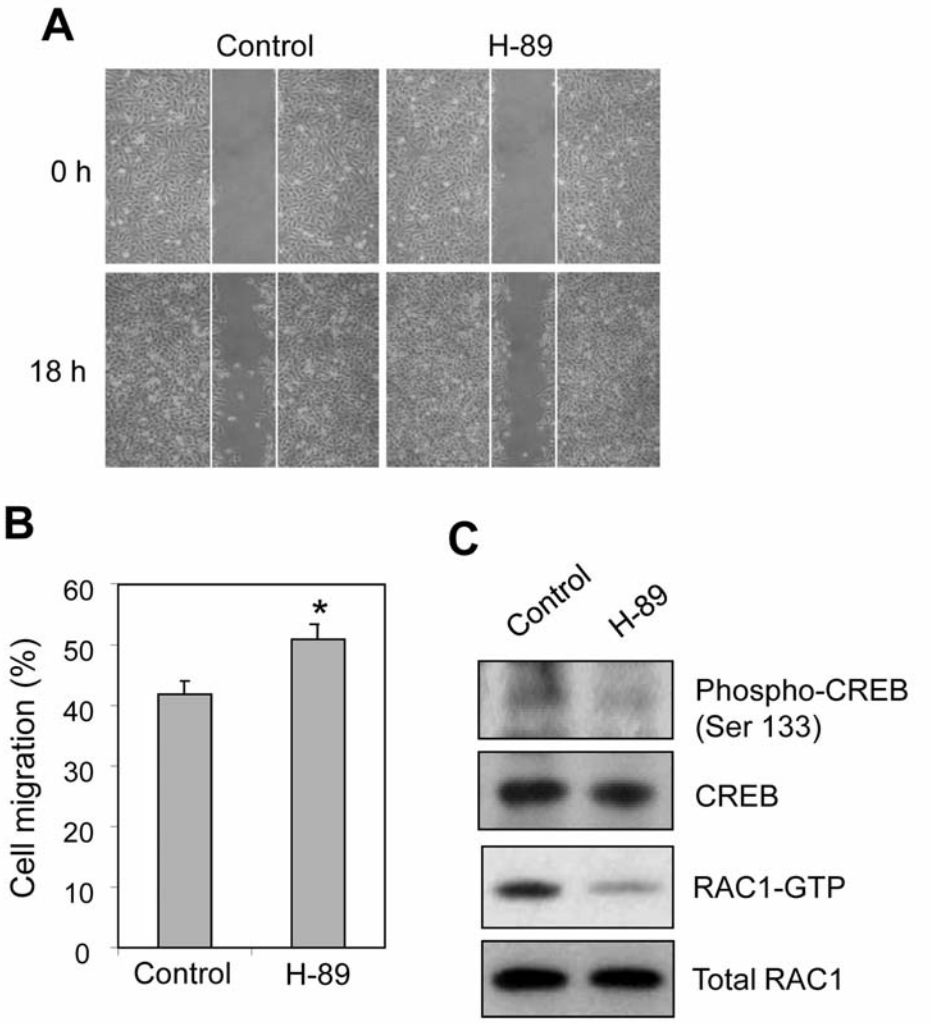

Figure 4. *H-89, protein kinase A (PKA) inhibitor, increased cell migration with the inhibition of RAC1-GTPase. A straight wound line was made in HeLa cells confluent in a 35 mm^2 culture dish and cells were incubated in the absence and presence of dibutyryl-cAMP (100 μM) for 18 h. Wound closure was recorded and photographed under phase-contrast microscopy at the indicated time (A). The empty area at each time point was quantified with NIH image analysis software (version 1.62) and compared with that in the initiation of cancer cell migration. Relative cell migration is represented with a bar graph. Data in the bar graph represent the means±SED. *p≤0.05, significant difference as compared to the control (B). cAMP response element-binding protein (CREB) phosphorylation at serine 133 (Ser133) was detected by western blot analysis at 2 h after the treatment with H-89. RAC1-GTPase activity was measured by pulldown assay, as described in the Materials and Methods. Data are representative of three independent experiments (C).*

than the effect of cAMP/EPAC1-mediated RAP1, leading to a decrease in cAMP-mediated HeLa cervical cancer cell migration.

RAC1 GTPase activation can enhance cell migration (11). Activation of RAC1 plays a key role in lamellipodia formation (35, 36). Lamellipodia drive cancer cells to move forward and increase invasiveness during tumor metastasis (16, 37). Our data showed that 8CPT-2me-cAMP enhanced RAC1-GTPase activity and cell migration (Figure 3). This means that 8CPT-2me-cAMP-mediated cell migration might be dependent on lamellipodia formation. In contrast, H-89 by inhibiting RAC1-GTPase increased cell migration (Figure 4). This shows that H-89-mediated cell migration might be independent of lamellipodia formation. In addition, RAC1-GTPase activated by treatment with dibutyryl-cAMP was related to the decrease in HeLa cell migration (Figure 1), which was reversed by pre-treatment with H-89 (Figure 5). We, therefore, speculate that lamellipodia formation is not the only factor controling cell migration but other factors are also involved in cAMP-mediated cell migration. Lamella which are more stable and less dynamic structures than lamellipodia may play a role in cell migration (38). It may

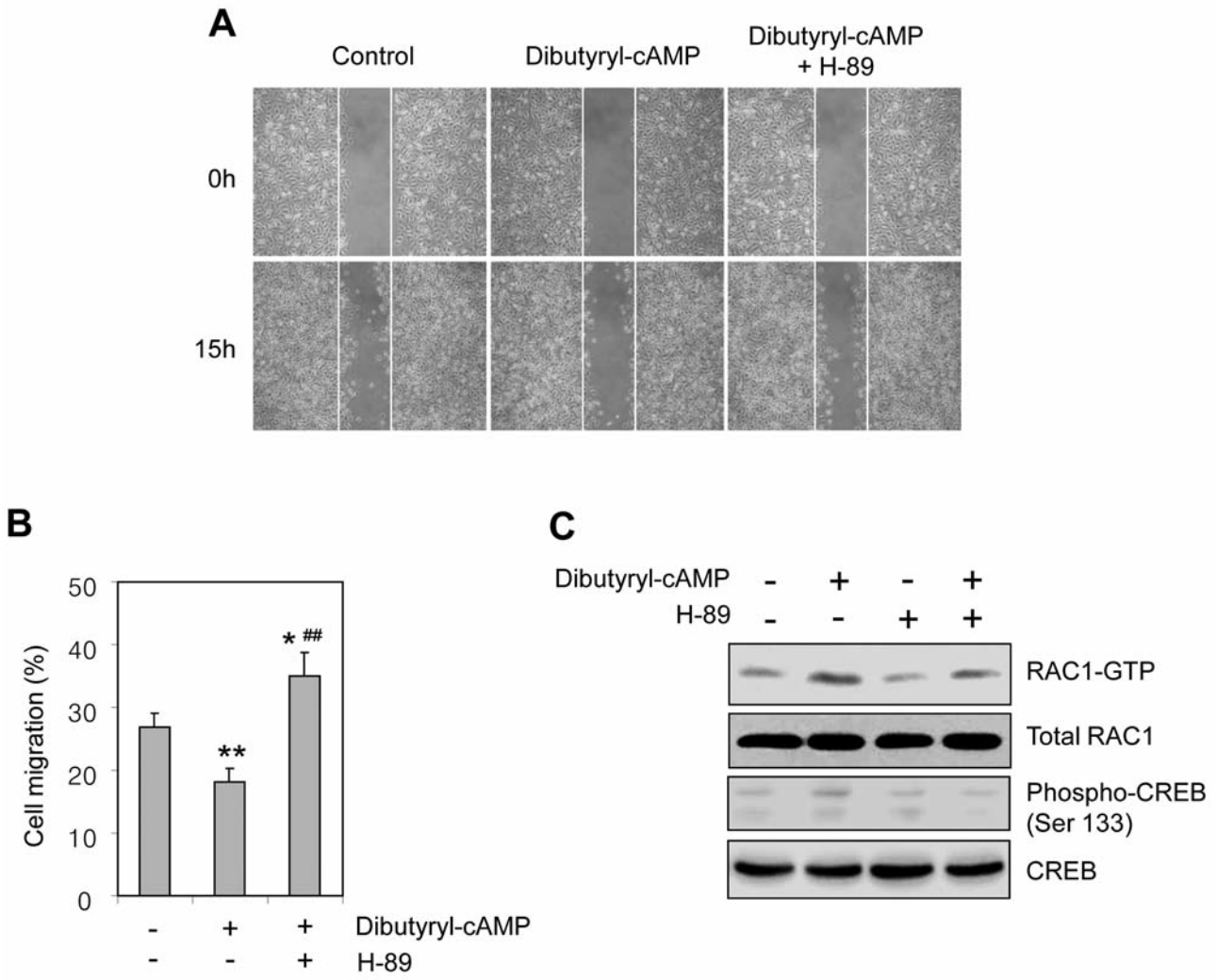

Figure 5. *Cell migration inhibited by dibutyryl-cAMP was restored by the pre-treatment with H-89. A straight wound line was made in HeLa cells confluent in a 35 mm^2 culture dish, and cells were treated with dibutyryl-cAMP (100 μM) in the absence and presence of H-89 and incubated for 18 h. Wound closure was recorded and photographed under phase-contrast microscopy at the indicated time (A). The empty area at each time point was quantified with NIH image analysis software (version 1.62) and compared with that in the initiation of cancer cell migration. Relative cell migration is represented with a bar graph. Data represent the means±SED. *p≤0.05 and **p≤0.01, significant difference compared to the control. ##p≤0.01, significant difference compared to dibutyryl-cAMP-treated and H-89-untreated control (B). cAMP response element-binding protein (CREB) phosphorylation at serine 133 (Ser133) was detected by western blot analysis. RAC1-GTPase activity was measured by pulldown assay, as described in the Materials and Methods (C). Data are representative of three independent experiments.*

also be possible for other signaling molecules to be involved in preventing cAMP-mediated cell migration.

Cell body translocation is controlled by a contraction of the actin-myosin II (39, 40). Cell body translocation continues after the arrest of lamellipodia and is independent of actin polymerization (39). Therefore, when dibutyryl-cAMP reduced cell migration with the activation of RAC1-GTPase, we assumed that cell body translocation could be another possible indicator to interpret the decreased cell migration by dibutyryl-cAMP.

In conclusion, although we can not explain all of the mechanisms involved in the regulatory effect of cAMP on cell migration, our data demonstrate that dibutyryl-cAMP-mediated HeLa cell migration might be the combined result of the inhibitory effect of cAMP/PKA and stimulatory effect of EPAC-mediated RAP1 pathway. We depict a possible scheme of the differential roles of cAMP-mediated PKA and EPAC1/RAP1 pathways on cell migration (Figure 6), Our findings suggest that cAMP-dependent signal transduction pathways may differentially regulate cancer cell migration.

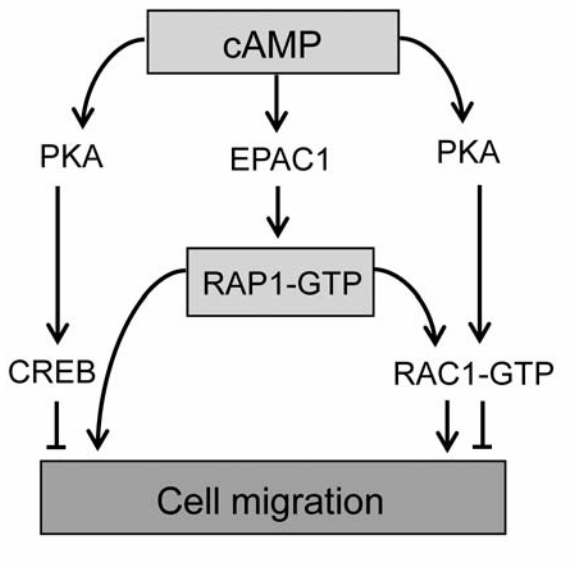

Figure 6. *Possible scheme of the role of cAMP-mediated signaling pathways in cell migration. cAMP-mediated cancer cell migration might be associated with a 'tug-of-war' between PKA and EPAC1/RAP1 pathways. In addition, cancer cell migration should be independent of RAC1-GTPase activation, which is up-regulated by cAMP/EPAC1/RAP1 and down-regulated by cAMP/PKA pathways.*

In addition, they also suggest that cAMP-mediated PKA and EPAC1-mediated RAP1 could be targets for developing therapeutics to control cancer cell migration.

Conflicts of interest

All Authors declare no conflicts of interest.

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