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Rap1 and p38 MAPK Mediate 8-Chloro-cAMP-Induced Growth Inhibition in Mouse Fibroblast DT Cells

YOUNG-HO AHN, JEE HAE HAN, AND SEUNG HWAN HONG*

School of Biological Sciences, and Institute of Molecular Biology and Genetics, Seoul National University, Seoul, Korea

8-Cl-cAMP, which is known to induce differentiation, growth inhibition, and apoptosis in various cancer cells, has been investigated as a putative anti-cancer drug. Previously, we reported that 8-Cl-cAMP and its metabolite 8-Cl-adenosine induce growth inhibition and apoptosis through p38 mitogen-activated protein kinase (MAPK) activation. To further investigate the signal mechanisms that regulate the cellular effects of 8-Cl-cAMP, we focused on a small GTPase Rap1 that is known to be involved in growth inhibition and reverse-transformation. 8-Cl-cAMP and 8-Cl-adenosine could increase Rap1 activity, which was blocked by ABT702—an adenosine kinase inhibitor. This suggests that 8-Cl-cAMP-induced Rap1 activation is also dependent on the metabolic degradation of 8-Cl-cAMP. Overexpression of a constitutively active mutant form of Rap1 (Rap1V12) attenuated cellular growth and soft-agar colony formation, which was basically the same effect as that observed with the 8-Cl-cAMP treatment. Furthermore, the Rap1V12 transfectant showed a high level of p38 MAPK activation. However, 8-Cl-cAMP-induced Rap1 activation was not diminished by SB203580, a p38 MAPK inhibitor, suggesting that Rap1 activation might act upstream of p38 MAPK activation during 8-Cl-cAMP-induced growth inhibition. J. Cell. Physiol. 209: 1039–1045, 2006.

8-Chloro-cyclic AMP (8-Cl-cAMP) is a membrane-permeable cAMP analogue that inhibits cellular proliferation and induces apoptosis in various cancer cell lines (Kim et al., 2001). Owing to its growth inhibitory effects, 8-Cl-cAMP is undergoing clinical testing as a potential anti-cancer therapeutic drug (Tortora et al., 1995; McDaid and Johnston, 1999; Propper et al., 1999). Previously, it was reported that the activities of 8-Cl-cAMP were mediated by differential regulation of type I and type II PKA (Cho-Chung et al., 1989). Type I PKA activation or upregulation results in proliferation and transformation; however, type II PKA is correlated with growth arrest and differentiation (Beebe et al., 1989; Cho-Chung et al., 1989). After treatment with 8-Cl-cAMP, the expression level of the type I regulatory subunit of PKA decreased, which was regarded as the major cause of 8-Cl-cAMP-induced growth inhibition (Rohlff et al., 1993; Noguchi et al., 1998).

However, some researchers have suggested that the key molecule in the cytotoxic effect is not 8-Cl-cAMP but its metabolite 8-Cl-adenosine. In a murine leukemia L1210 variant lacking adenosine kinase that participates in the metabolic conversion of 8-Cl-cAMP, both 8-Cl-cAMP and 8-Cl-adenosine could not induce growth inhibition (Gandhi et al., 2001). Further, addition of adenosine deaminase, which inactivates 8-Cl-adenosine by deamination to 8-Cl-inosine, prevented the reduction of nucleic acid synthesis and cellular proliferation (Langeveld et al., 1997; Halgren et al., 1998). In addition, chemical inhibitors of adenosine kinase or adenosine transporter attenuated the cytotoxicity induced by both drugs (Halgren et al., 1998; Lamb and Steinberg, 2002). We previously reported that 8-Cl-cAMP-induced growth inhibition and apoptosis is dependent upon its metabolic conversion (Ahn et al., 2004) and is mediated by PKC and p38 mitogen-activated protein kinase (MAPK) activation (Ahn et al., 2005). In this report, we investigated the upstream factor(s) that might transduce the growth-inhibitory signal of 8-Cl-cAMP to p38 MAPK activation.

Rap1, a Ras superfamily of small G proteins, was isolated as a revertant of the *K*-Ras-transformed phenotype of fibroblasts (Kitayama et al., 1989). Rap1 is known to be activated by signals that increase the intracellular level of cAMP (Altschuler et al., 1995). Further, Rap1 stimulates cell growth under conditions where cAMP exerts positive effects; however, it inhibits cell growth under conditions where cAMP has a negative effect on the growth of other cells (Stork and Schmitt, 2002). For example, Rap1 has oncogenic potential in Swiss 3T3 fibroblasts, a system in which cAMP is known to be a positive regulator of cell growth (Altschuler and Ribeiro-Neto, 1998). Additionally, Rap1 is indispensable in the process of cAMP-induced growth inhibition of NIH3T3 cells (Schmitt and Stork, 2001). Furthermore, one of the guanine nucleotide exchange factors (GEFs) for Rap1, that is, exchange protein directly activated by cAMP (Epac) was found to be another intracellular cAMP-binding target (Kawasaki et al., 1998). Thus, the cAMP-Epac-Rap1 pathway has been regarded as an alternative cAMP-signaling pathway to the cAMP-PKA pathway, even though it is still debatable whether the catalytic activity of PKA is necessary for Rap1 activation (Schmitt and Stork, 2001, 2002; Mei et al., 2002; Fukuhara et al., 2005).

Abbreviations: 8-Cl-cAMP, 8-chloroadenosine-3′,5′-cyclic monophosphate; GEF, guanine nucleotide exchange factor; Epac, exchange protein directly activated by cAMP; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; NBTI, nitrobenzyl thioinosine; FBS, fetal bovine serum; Sp-8-Cl-cAMPS, 8-chloroadenosine-3′,5′-cyclic monophosphorothioate Sp-isomer; 8-CPT-2-Me-cAMP, 8-(*p*-chlorophenylthio)-2′-*O*-methyl cAMP.

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*Correspondence to: Seung Hwan Hong, Institute of Molecular Biology and Genetics, Seoul National University, Seoul 151-742, Korea. E-mail: shong@plaza.snu.ac.kr

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Rap1 is known to exert its effect by regulating MAPKs. Growth hormone-stimulated Rap1 activation suppressed extracellular signal-regulated kinase (ERK) activity and enhanced Jun N-terminal kinase (JNK) activity (Cook et al., 1993; Ling et al., 2003). Rap1 is also known to be involved in mechanical force-induced fibroblast stretching (Sawada et al., 2001) and AMPA receptor endocytotic trafficking in hippocampal synapses (Huang et al., 2004) through the activation of p38 MAPK. In this study, we attempted to elucidate the roles of Rap1 during the 8-Cl-cAMP-induced growth inhibition and p38 MAPK activation.

MATERIALS AND METHODS

Chemicals and drugs

8-Cl-cAMP, 8-Cl-adenosine, 8-Cl-AMP, 8-Cl-ATP, Sp-8-Cl-cAMPS, Rp-8-Cl-cAMPS, and 8-CPT-2-Me-cAMP were purchased from Biolog (Bremen, Germany). SB203580 (a p38 MAPK inhibitor) was obtained from A. G. Scientific (San Diego, CA). A134974 and ABT-702 (adenosine kinase inhibitors), and nitrobenzyl thioinosine (NBTI, an adenosine transporter inhibitor) were purchased from Sigma-Aldrich (St. Louis, MO). All the other chemicals used in this study were obtained from Sigma-Aldrich.

Cell culture and transfection

DT (K-*ras*-transformed NIH3T3) cells were grown in Dulbecco's modified Eagle's medium (HyClone, Logan, UT), and HL60 (human acute promyelocytic leukemia) cells were cultured in RPMI 1640 medium (HyClone). The culture media were supplemented with 10% fetal bovine serum (FBS, HyClone), 100 units/ml penicillin G, and 100 μg/ml streptomycin. For the heat-treated serum condition, FBS was heated to 58°C for 1 h. The cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. Cell counting, MTT assay, and soft-agar culture were performed as previously described (Ahn et al., 2004). DT cells were transfected with Rap1V12 and N17 (obtained from Dr. P. J. S. Stork, Oregon Health Sciences University, OR) that had been cloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA) using the FuGENE6™ reagent (Roche, Basel, Switzerland), according to the manufacturer's protocol.

Rap1 activity assay

Cells were lysed with RIPA buffer (100 mM Tris-Cl, pH 7.4; 150 mM NaCl; 2% NP-40; 1% sodium deoxycholate; 0.2% SDS; 1 mM PMSF; 1 μg/ml each of aprotinin, pepstatin A, antipain, and leupeptin) on ice for 15 min. After centrifugation at 14,000*g* for 15 min at 4°C, supernatant was removed and used as the cell extract. Ten micrograms of RalGDS-RBD (a gift from Dr. J. L. Bos, Utrecht University, The Netherlands) precoupled to GSH-Sepharose beads (Amersham, Piscataway, NJ) was added to the cell extract (0.5–1 mg) and incubated at 4°C for 2 h with slight agitation. The beads were washed with RIPA buffer and PBS, and the proteins were then separated on 12% SDS/PAGE. Western blot analysis was performed using the anti-Rap1 antibody (BD Transduction Laboratory, San Diego, CA). The cell extract (50 μg) that had not been incubated with the beads was also loaded on SDS/PAGE in order to measure the total Rap1 protein.

Western blot analysis

Cells were harvested by centrifugation and washed with PBS. The cell pellet was suspended in an extraction buffer (20 mM Tris-Cl, pH 7.4; 100 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; 5 mM $MgCl_2$; 1 mM PMSF; 1 μg/ml each of aprotinin, pepstatin A, antipain, and leupeptin; 1 mM Na_3VO_4; 50 mM NaF; 10 mM pyrophosphate) and incubated on ice for 15 min. After centrifugation at 14,000*g* for 15 min at 4°C, the supernatant was removed and used as the cell extract. The extracts were separated on 10% SDS/PAGE and transferred onto a PVDF membrane. The protein-bound membrane was incubated with the appropriate antibodies followed by horseradish peroxidase-conjugated anti-mouse or rabbit IgG antibody (Bio-Rad, Hercules, CA). The relevant protein bands were then visualized using the ECL™ detection kit (Amersham). The antibody against phospho-p38 was obtained from BD Transduction Laboratories, while the p38 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

RESULTS

8-Cl-cAMP induced growth inhibition through its metabolite 8-Cl-adenosine

Treatment of mouse fibroblast DT cells (K-*ras*-transformed NIH3T3 cells) with 8-Cl-cAMP inhibited cellular proliferation. However, 8-Cl-cAMP did not cause cell death or apoptosis (Kim et al., 2001; Ahn et al., 2004). As previously reported (Ahn et al., 2004), 8-Cl-cAMP began to induce growth inhibition in DT cells 2 or 3 days after the treatment (Fig. 1A) in a dose-dependent manner (Fig. 1B). This growth inhibition did not appear to be caused by 8-Cl-cAMP itself because an adenosine kinase inhibitor (A134974, 10 μM) and an adenosine transporter inhibitor (NBTI, 100 μM) could completely block the effect of 8-Cl-cAMP (Fig. 1C). In addition, 8-Cl-adenosine, a metabolite of 8-Cl-cAMP, also induced growth inhibition, which was attenuated by A134974 or NBTI co-treatment (Fig. 1C). These results suggest that 8-Cl-cAMP is converted to 8-Cl-adenosine in culture media, and then transported into the cells. To further confirm the importance of the metabolic conversion of 8-Cl-cAMP on the induction of growth inhibition, DT cells were cultured in the media supplemented with heat-treated serum. Heat treatment might eliminate the enzymatic activity present in the serum; this enzymatic activity mediates the conversion of 8-Cl-cAMP to 8-Cl-adenosine. As expected, 8-Cl-cAMP could not induce growth inhibition of DT cells cultured with heat-treated serum; however, 8-Cl-adenosine could still exert its toxicity (Fig. 1C). In addition, unhydrolyzable analogues of 8-Cl-cAMP, such as 8-chloroadenosine-3′,5′-cyclic monophosphorothioate, Sp-isomer (Sp-8-Cl-cAMPS) and Rp-8-Cl-cAMPS (Rp-isomer), which are not hydrolyzed by phosphodiesterases, had no effect on cellular proliferation (Fig. 1D). Based on these data, it is plausible to state that 8-Cl-cAMP should be converted to its metabolites in order to induce growth inhibition.

8-Cl-cAMP increased Rap1 activity

Rap1, a small GTPase, is known to be a mediator involved in growth inhibition caused by cAMP signals (Schmitt and Stork, 2001; Stork and Schmitt, 2002). Therefore, we decided to study whether Rap1 also has any significant roles in the process of 8-Cl-cAMP-induced growth inhibition. To measure Rap1 activity after 8-Cl-cAMP treatment, the GST-pull down assay was performed using the Rap1-binding domain of RalGDS as the bait for the activated (GTP-bound) Rap1 (Franke et al., 1997). The activated form of Rap1 increased in time- and dose-dependent manners after treatment with 8-Cl-cAMP (Fig. 2A,B). However, more than 2 days were required for 8-Cl-cAMP to augment Rap1 activity; this incubation time is slightly longer than that required for other Rap1-activating signals (Posern et al., 1998; Sawada et al., 2001; Schmitt and Stork, 2001; Mei et al., 2002; Huang et al., 2004). Based on this time gap, it can be surmised that 8-Cl-cAMP might induce Rap1 activation via an indirect route instead of direct binding to Epac (Kawasaki et al., 1998; Qiao et al., 2002; Fukuhara et al., 2005; Gupta and Yarwood, 2005).

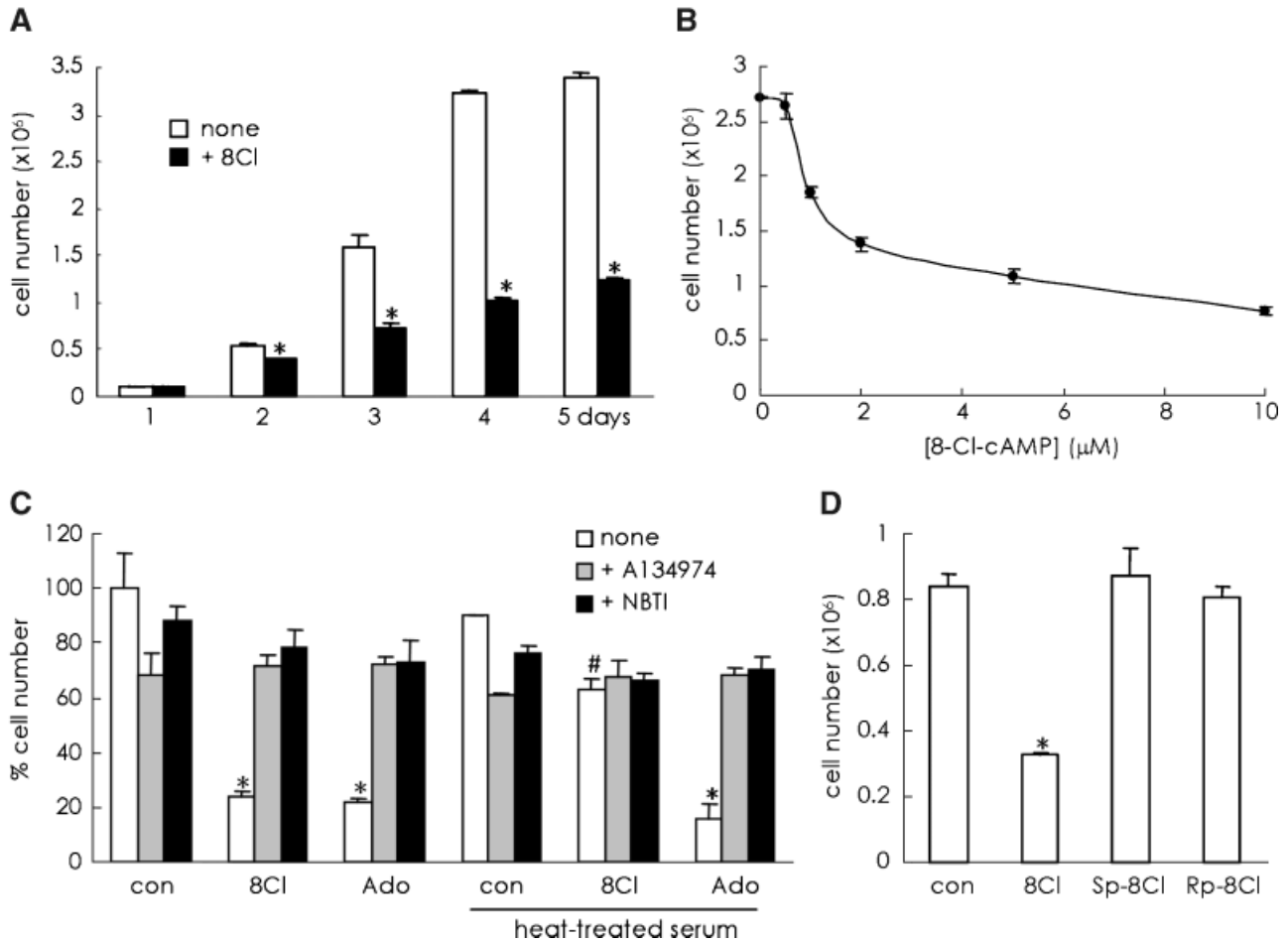

Fig. 1. 8-Cl-cAMP- and 8-Cl-adenosine-induced growth inhibition. **A**,**B**: Cell counting after 8-Cl-cAMP (8Cl) treatment. DT cells were seeded at a concentration of 1×10^5 per well on 6-well plates. In **part A**, 10 μM of 8-Cl-cAMP was added for the indicated times. In **part B**, the cells were incubated with 0, 0.5, 1, 2, 5, and 10 μM of 8-Cl-cAMP for 4 days. After the treatment, the cell numbers were measured using a Coulter counter. Graphs denote the cell number (mean + SD, n = 3), and asterisks represent the differences from the control group ($P < 0.01$, two-tailed t-test). **C**: The MTT assay after 8-Cl-cAMP (8Cl) and 8Cl-adenosine (Ado) treatment. DT cells were seeded at a concentration of 3×10^4 per well on 24-well plates, and 8-Cl-cAMP (10 μM) and 8-Cl-adenosine (2 μM) were then added in combination with NBTI (100 μM) or A134974 (10 μM) for 3 days under normal conditions or with heat-treated serum. After this treatment, the MTT assay was carried out. Bars denote the cell numbers normalized to the control group (mean + SD, n = 4). Asterisks (*) represent the differences from the control group, and sharp (#) represents the difference from the 8-Cl-cAMP-treated group under normal conditions ($P < 0.01$, two-tailed t-test). **D**: Cell counting after treatment with the 8-Cl-cAMP analogues. DT cells were seeded at a concentration of 5×10^4 per well on 12-well plates. Each culture was treated with 10 μM of 8-Cl-cAMP (8Cl), Sp-8Cl-cAMPS (Sp-8Cl), or Rp-8Cl-cAMPS (Rp-8Cl) for 3 days, and the cell number was then counted as in **part A**. Bars denote the cell numbers normalized to the control group (mean + SD, n = 3). Asterisk represents the difference from the control group ($P < 0.01$, two-tailed t-test).

As mentioned above, 8-Cl-cAMP induces growth inhibition and apoptosis through its metabolites. To test whether metabolic degradation is also indispensable for 8-Cl-cAMP to increase Rap1 activity, DT cells were treated with the metabolites of 8-Cl-cAMP, and Rap1 activity was then measured (Fig. 2C,D). Similar to the results of the cell growth test and phospho-p38 Western blotting (Ahn et al., 2004, 2005), 8-Cl-adenosine and 8-Cl-AMP could activate Rap1, and Rap1 activation caused by 8-Cl-cAMP or 8-Cl-adenosine was blocked by co-treatment with another adenosine kinase inhibitor ABT-702 (10 μM, Fig. 2C). Moreover, Sp-8-Cl-cAMPS had no effect on Rap1 activation (Fig. 2D). These data clearly support the hypothesis that 8-Cl-cAMP induces Rap1 activation through its metabolites.

8-Cl-cAMP-induced growth inhibition was mediated by Rap1 activation

It has been reported that Rap1 activation resulted in the inhibition of cellular growth or suppression of tumorigenesis (Lin et al., 2000; Schmitt and Stork, 2001; D'Silva et al., 2003). Accordingly, it was necessary to check whether augmentation of Rap1 activity was also a critical event during 8-Cl-cAMP-induced growth inhibition. For this, we transfected DT cells with either a constitutively active (Rap1V12) or a dominant-negative (Rap1N17) mutant form of Rap1 (Fig. 3A,D) (Lou et al., 2002; Ling et al., 2003; Gupta and Yarwood, 2005). Compared with the parental DT cells or the pcDNA3 vector-transfected cells, the Rap1V12 transfectant exhibited growth retardation (Fig. 3B). In soft-agar cultures, the ability for colony formation, a measure of transformed cell phenotype, was also reduced by Rap1V12 overexpression, suggesting that Rap1 has reverse-transforming activity (Fig. 3C). Furthermore, the enforced expression of Rap1N17 attenuated the growth inhibitory effect of 8-Cl-cAMP (Fig. 3D), although the effect was not 100%. These results imply that Rap1 activation plays a significant role in 8-Cl-cAMP-induced growth inhibition.

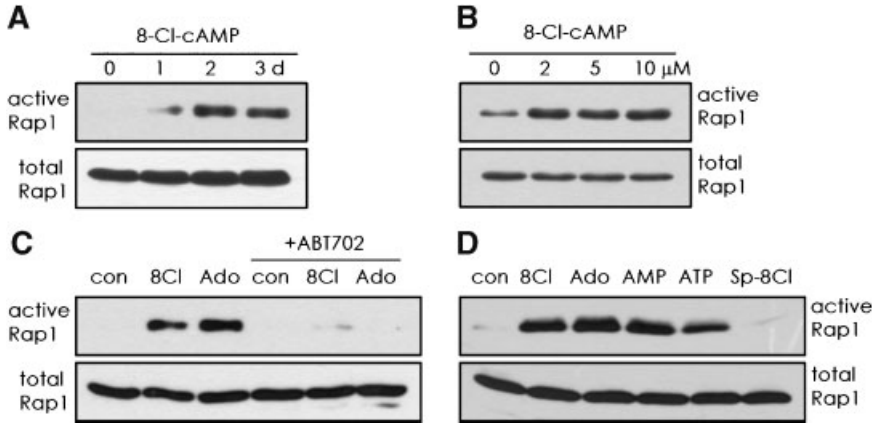

Fig. 2. 8-Cl-cAMP increased Rap1 activity. **A**,**B**: Rap1 activity after 8-Cl-cAMP treatment. In **part A**, 8-Cl-cAMP (10 μM) was added for the indicated time. In **part B**, the cells were incubated with 0, 2, 5, and 10 μM of 8-Cl-cAMP for 3 days. After the treatment, active or total Rap1 was assessed by Western blot analysis as described in Materials and Methods. **C**: Rap1 activity after 8-Cl-cAMP and 8-Cl-adenosine treatment. Cells were incubated with 8-Cl-cAMP (8-Cl, 10 μM) and 8-Cl-adenosine (Ado, 2 μM) in the presence or absence of ABT702 (10 μM) for 3 days. The Rap1 activity assay was then carried out. **D**: Rap1 activity after treatment with the metabolites of 8-Cl-cAMP. The cells were treated with 8-Cl-cAMP (8Cl, 10 μM), 8-Cl-adenosine (Ado, 2 μM), 8-Cl-AMP (AMP, 10 μM), 8Cl-ATP (ATP, 10 μM), or Sp-8-Cl-cAMPS (Sp-8Cl, 10 μM) for 3 days, and Rap1 activity was then measured.

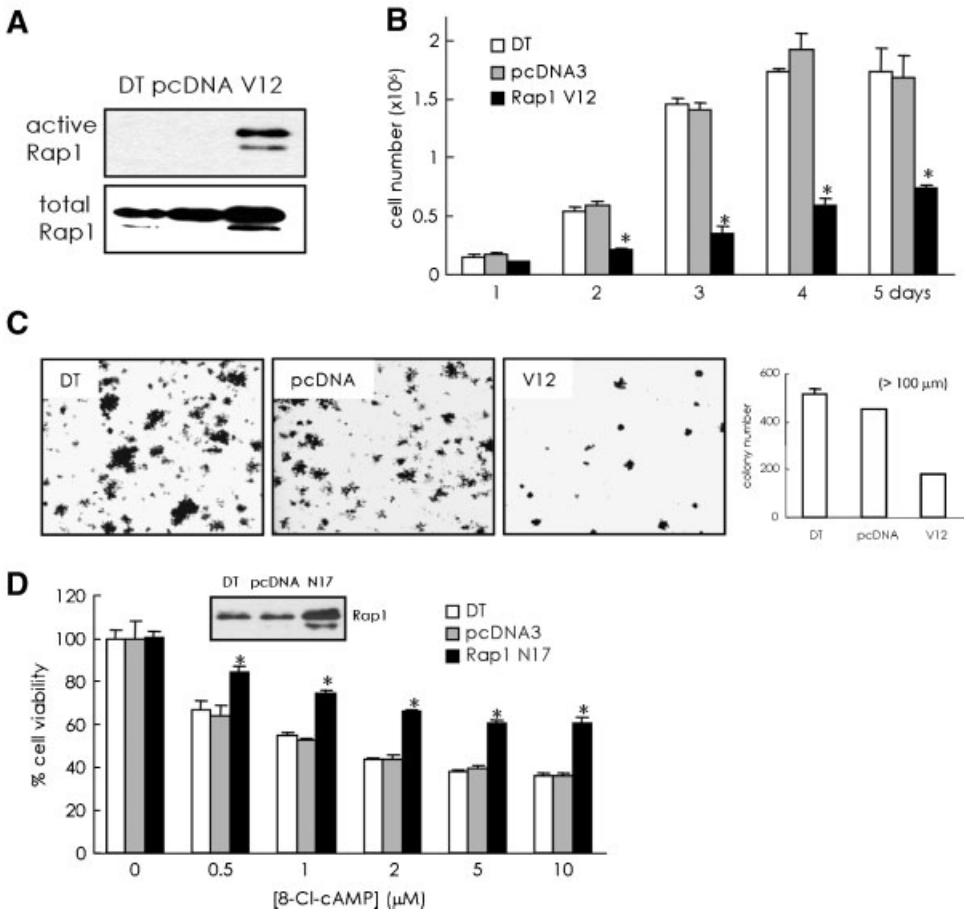

Fig. 3. Rap1 activation mediated the growth inhibition. **A**: The Rap1 activity assay was performed on the Rap1V12 transfectant. Rap1V12 transfection increased Rap1 activation. **B**: Cell growth rate of the Rap1V12 transfectant. DT cells transfected with the vector (pcDNA3) or Rap1V12 were seeded at a concentration of 1×10^5 per well on 6-well plates, and the cell numbers were counted daily for 5 days. Bars denote the cell numbers (mean + SD, n = 3) and asterisks represent the differences from the parental DT cells ($P < 0.01$, two-tailed t-test). **C**: The soft-agar colony formation assay carried out on the Rap1V12 transfectant. Top agar (0.3%) medium containing 5×10^4 cells (DT, vector, and Rap1V12) was layered over 1 ml of 0.8% bottom agar medium in a 35-mm culture dish. After 7 days, the colonies were stained overnight with nitro blue tetrazolium and then photographed under a microscope. The graph shows the number of colonies with a diameter larger than 100 μm that were counted under the microscopic field (mean + SD, n = 3). **D**: The MTT assay after treatment of the Rap1N17 transfectant with 8-Cl-cAMP. DT, pcDNA3-transfected, or Rap1N17-transfected cells were seeded at a concentration of 3×10^4 per well on 24-well plates, and 8-Cl-cAMP was then added at the indicated concentrations for 3 days. Rap1N17 significantly attenuated 8-Cl-cAMP-induced growth inhibition. Asterisks represent the differences from the parental DT cells ($P < 0.01$, two-tailed t-test). The immunoblot in the inset shows the expression level of Rap1 in each cell.

Rap1 activation caused p38 MAPK activation

Previously, we found that 8-Cl-cAMP and 8-Cl-adenosine could activate p38 MAPK while exerting a growth-inhibitory effect in HL60 cells (Ahn et al., 2005). In DT cells, 8-Cl-cAMP could also activate the p38 MAPK (Fig. 4A), and 8-Cl-cAMP-induced growth inhibition was attenuated by SB203580 (10 μM), a p38 MAPK inhibitor (Fig. 4B). It has been reported that many cellular functions of Rap1 are associated with the activation of MAPKs (Sawada et al., 2001; Schmitt and Stork, 2001; Ling et al., 2003; Huang et al., 2004; Dillon et al., 2005). Thus, we tried to investigate the influence of Rap1 activation on p38 MAPK during 8-Cl-cAMP-induced growth inhibition. First, p38 MAPK phosphorylation was measured in a Rap1V12 transfectant (Fig. 4C). The level of phospho-p38 MAPK was greatly increased as a result of Rap1V12 overexpression, which indicates that Rap1 activation might evoke p38 MAPK activation. Next, the Rap1 activity assay was carried out after the treatment with 8-Cl-cAMP or 8-Cl-adenosine along with SB203580 (10 μM) treatment. Rap1 activation was not affected by the presence of the p38 MAPK inhibitor (data not shown). These results suggest that Rap1 might regulate p38 MAPK activation at an upstream step during the course of 8-Cl-cAMP-induced growth inhibition.

8-Cl-cAMP induces not only growth retardation but also cell death in HL60 cells (Ahn et al., 2005). To determine whether Rap1 activity also increased during 8-Cl-cAMP-induced apoptosis, HL60 cells were incubated with 8-Cl-cAMP for 3 days (Fig. 4D). Similar to the tests performed on DT cells, 8-Cl-cAMP began to induce Rap1 activation in HL60 cells after 2 days of incubation. This activation was also blocked by ABT-702 (Fig. 4E) but not by SB203580 (data not shown), which was the same as the result presented above using DT cells, that is, Rap1 acts upstream of p38 MAPK.

DISCUSSION

A small G protein Rap1 was first isolated as one of the cDNA expression library clones that has the potential to suppress the transformed phenotype associated with activated *ras* (Ki-*ras*) genes in mouse fibroblast DT cells (Kitayama et al., 1989; Noda et al., 1989). These authors selected flat revertants from a DT cell population transfected with a human fibroblast cDNA expression library and then obtained the cDNA clones. 8-Cl-cAMP is also known to possess the reverse-transforming activity, that is, when transformed NIH3T3 cells were treated with 8-Cl-cAMP, it induced growth inhibition and a morphological change to a flat non-transformed shape (Tagliaferri et al., 1988; Budillon et al., 1995; Ahn et al., 2004). In this report, we intended to investigate the possibility of whether Rap1 plays a meaningful role during 8-Cl-cAMP-induced growth inhibition in mouse fibroblast DT cells. As expected, 8-Cl-cAMP could activate Rap1 (Fig. 2A,B), and this activation resulted in the activation of p38 MAPK (Fig. 4) as well as growth inhibition (Fig. 3).

Many other researchers reported that the modulation of cellular proliferation by cAMP is mediated by Rap1 (Altschuler et al., 1995; Altschuler and Ribeiro-Neto, 1998; Schmitt and Stork, 2001; Stork and Schmitt, 2002). As a signaling linker between cAMP and Rap1, Epac has drawn attention since it was identified as a new intracellular cAMP receptor and a GEF for Rap1 (Kawasaki et al., 1998; Mei et al., 2002; Schmitt and Stork, 2002; Fukuhara et al., 2005). cAMP can directly bind to Epac and induce its GDP/GTP exchange activity. Epac then converts Rap1 to a GTP-bound activated state. However, due to the following reasons, it might not be plausible to believe that Epac is a mediator for Rap1 activation in the 8-Cl-cAMP-induced growth inhibition system presented in this study. First, the time interval between the 8-Cl-cAMP treatment and Rap1 activation required 2 days or more (Fig. 2A); this is

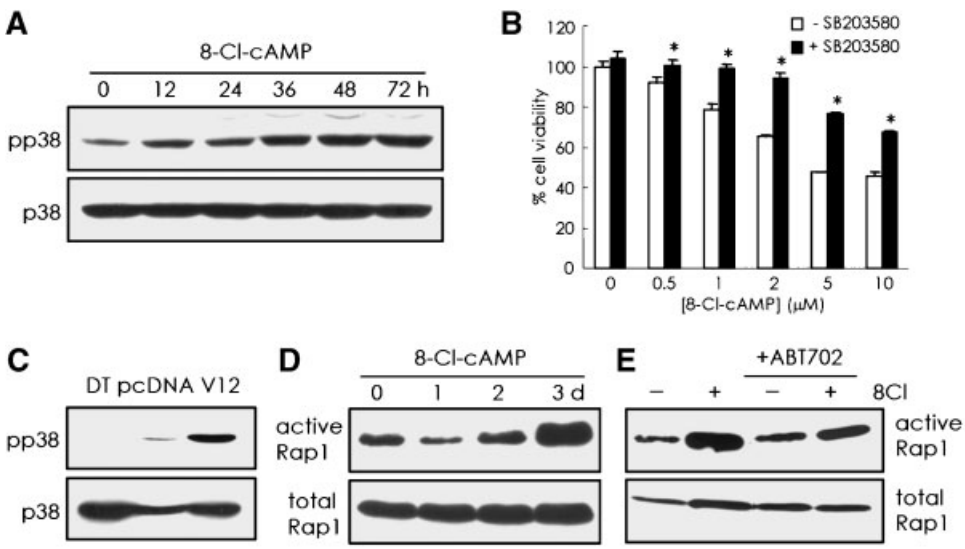

Fig. 4. Rap1 regulated p38 MAPK activation at an upstream step. **A**: p38 Western blotting after 8-Cl-cAMP treatment. The phosphorylated (active) or total p38 protein level was measured by immunoblotting using a specific antibody. p38 phosphorylation was increased in a time-dependent manner after 8-Cl-cAMP treatment. **B**: The MTT assay after 8-Cl-cAMP treatment in the presence or absence of the p38 inhibitor (SB203580, 10 μM). Co-treatment of SB203580 attenuated 8-Cl-cAMP-induced growth inhibition. Asterisks represent the differences from the control (−SB203580) group ($P < 0.01$, two-tailed t-test). **C**: p38 Western blotting performed with the Rap1V12 transfectant. p38 protein level was measured as in **part A**. p38 phosphorylation was greatly increased after Rap1V12 transfection. **D**,**E**: Rap1 activity after 8-Cl treatment of HL60 cells. In **part D**, 8-Cl-cAMP (10 μM) was added to HL60 cells for the indicated number of days. In **part E**, 8-Cl-cAMP (8Cl) was added with or without ABT-702 (10 μM) for 3 days. After the treatment, Rap1 activity was measured as described above.

unusually longer than that of other experimental systems, which do not require more than 5 min to elicit Rap1 activation (Posern et al., 1998; Sawada et al., 2001; Schmitt and Stork, 2001; Mei et al., 2002; Huang et al., 2004). Second, a novel Epac-specific cAMP analogue 8-CPT-2-Me-cAMP (8-(*p*-chlorophenylthio)-2'-*O*-methyl cAMP) (Enserink et al., 2002) could not exert a growth inhibitory effect on mouse fibroblasts (data not shown). Finally, it was not 8-Cl-cAMP itself but its metabolites that could increase Rap1 activity because 8-Cl-adenosine was also able to activate Rap1, and an adenosine kinase inhibitor completely blocked 8-Cl-cAMP-induced Rap1 activation (Fig. 2C). Therefore, GEFs other than Epac might mediate Rap1 activation by 8-Cl-cAMP treatment.

One GEF candidate that influences 8-Cl-cAMP-induced Rap1 activation is C3G, a Crk SH3 domain-binding guanine nucleotide-releasing factor. C3G was shown to stimulate the nucleotide exchange reaction of Rap1 (Gotoh et al., 1995), and the enforced expression of active C3G in transformed fibroblasts provoked a reverse-transformation similar to the results observed upon 8-Cl-cAMP treatment or Rap1V12 overexpression (Gotoh et al., 1995; Guerrero et al., 2004). However, whether 8-Cl-cAMP could influence the C3G-Rap1 signaling pathway is a point that requires further investigation.

It has been established that Rap1 could bind to Raf-1 such that it can interfere with the association of Ras and Raf-1, which caused the inhibition of ERK activation and cellular proliferation (Schmitt and Stork, 2001). In this study, we have shown another mechanism of MAPK regulation by Rap1 during cAMP-induced growth inhibition, namely, Rap1 can activate p38 MAPK—an important MAPK participating in the inhibition of proliferation and the induction of apoptosis (Brenner et al., 1997; Assefa et al., 2000; Zhang et al., 2006). In fact, Rap1 activation could also be observed upon induction of apoptosis by 8-Cl-cAMP in HL60 cells (Fig. 4D,E).

Overexpression of a Rap1 dominant-negative mutant (Rap1N17) mitigated the effect of 8-Cl-cAMP; however, it could not completely reverse the growth inhibition (Fig. 3D). It is possible that the expression of Rap1N17 is not sufficient for complete ablation of wild-type Rap1. Therefore, residual Rap1 may take over the function of growth inhibition. Another possible explanation for this result is that in addition to Rap1 activation, other signaling mechanisms might become operational after treatment with 8-Cl-cAMP, such as the differential modulation of PKA isozymes (Rohlff et al., 1993; Noguchi et al., 1998).

Previously, we reported that 8-Cl-cAMP-induced growth inhibition through its metabolites (Ahn et al., 2004, 2005). In this report, we reconfirmed that 8-Cl-cAMP should be modified in order to induce the growth inhibitory signal. NBTI, an adenosine transporter inhibitor, blocked 8-Cl-cAMP-induced growth inhibition, which implies that 8-Cl-cAMP is converted to 8-Cl-adenosine in the culture medium and then enters the cells through the adenosine transporter (Fig. 1C). Furthermore, when the enzymatic activity present in the serum, which might be important for the conversion of 8-Cl-cAMP, was ablated by heat treatment, 8-Cl-cAMP lost its growth inhibitory effect (Fig. 1C).

In summary, we have shown that 8-Cl-cAMP could activate Rap1 and this activated Rap1 transmitted the signal to p38 MAPK, thereby causing inhibition of cellular growth. Further, this Rap1 activation by 8-Cl-cAMP was also mediated by its metabolites such as 8-Cl-adenosine. The precise mechanisms by which 8-Cl-cAMP provides an impetus to the GEFs for Rap1 activation and the manner in which Rap1 induces the p38 MAPK signaling pathway still remain to be elucidated.

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LITERATURE CITED

Ahn YH, Jung JM, Hong SH. 2004. 8-Cl-cAMP and its metabolite, 8-Cl-adenosine induce growth inhibition in mouse fibroblast DT cells through the same pathways: Protein kinase C activation and cyclin B down-regulation. J Cell Physiol 201:277–285.

Ahn YH, Jung JM, Hong SH. 2005. 8-Chloro-cyclic AMP-induced growth inhibition and apoptosis is mediated by p38 mitogen-activated protein kinase activation in HL60 cells. Cancer Res 65:4896–4901.

Altschuler DL, Ribeiro-Neto F. 1998. Mitogenic and oncogenic properties of the small G protein Rap1b. Proc Natl Acad Sci USA 95:7475–7479.

Altschuler DL, Peterson SN, Ostrowski MC, Lapetina EG. 1995. Cyclic AMP-dependent activation of Rap1b. J Biol Chem 270:10373–10376.

Assefa Z, Vantieghem A, Garmyn M, Declercq W, Vandenabeele P, Vandenheede JR, Bouillon R, Merlevede W, Agostinis P. 2000. p38 mitogen-activated protein kinase regulates a novel, caspase-independent pathway for the mitochondrial cytochrome c release in ultraviolet B radiation-induced apoptosis. J Biol Chem 275:21416–21421.

Beebe SJ, Segaloff DL, Burks D, Beasley-Leach A, Limbird LE, Corbin JD. 1989. Evidence that cyclic adenosine 3′,5′-monophosphate-dependent protein kinase activation causes pig ovarian granulosa cell differentiation, including increases in two type II subclasses of this kinase. Biol Reprod 41:295–307.

Brenner B, Koppenhoefer U, Weinstock C, Linderkamp O, Lang F, Gulbins E. 1997. Fas- or ceramide-induced apoptosis is mediated by a Rac1-regulated activation of Jun N-terminal kinase/p38 kinases and GADD153. J Biol Chem 272:22173–22181.

Budillon A, Cereseto A, Kondrashin A, Nesterova M, Merlo G, Clair T, Cho-Chung YS. 1995. Point mutation of the autophosphorylation site or in the nuclear location signal causes protein kinase A RII beta regulatory subunit to lose its ability to revert transformed fibroblasts. Proc Natl Acad Sci USA 92:10634–10638.

Cho-Chung YS, Clair T, Tagliaferri P, Ally S, Katsaros D, Tortora G, Neckers L, Avery TL, Crabtree GW, Robins RK. 1989. Site-selective cyclic AMP analogs as new biological tools in growth control, differentiation, and proto-oncogene regulation. Cancer Invest 7:161–177.

Cook SJ, Rubinfeld B, Albert I, McCormick F. 1993. RapV12 antagonizes Ras-dependent activation of ERK1 and ERK2 by LPA and EGF in Rat-1 fibroblasts. EMBO J 12:3475–3485.

D'Silva NJ, Mitra RS, Zhang Z, Kurnit DM, Babcock CR, Polverini PJ, Carey TE. 2003. Rap1, a small GTP-binding protein is upregulated during arrest of proliferation in human keratinocytes. J Cell Physiol 196:532–540.

Dillon TJ, Carey KD, Wetzel SA, Parker DC, Stork PJ. 2005. Regulation of the small GTPase Rap1 and extracellular signal-regulated kinases by the costimulatory molecule CTLA-4. Mol Cell Biol 25:4117–4128.

Enserink JM, Christensen AE, de Rooij J, van Triest M, Schwede F, Genieser HG, Doskeland SO, Blank JL, Bos JL. 2002. A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. Nat Cell Biol 4:901–906.

Franke B, Akkerman JW, Bos JL. 1997. Rapid Ca^{2+}-mediated activation of Rap1 in human platelets. EMBO J 16:252–259.

Fukuhara S, Sakurai A, Sano H, Yamagishi A, Somekawa S, Takakura N, Saito Y, Kangawa K, Mochizuki N. 2005. Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling pathway. Mol Cell Biol 25:136–146.

Gandhi V, Ayres M, Halgren RG, Krett NL, Newman RA, Rosen ST. 2001. 8-chloro-cAMP and 8-chloro-adenosine act by the same mechanism in multiple myeloma cells. Cancer Res 61:5474–5479.

Gotoh T, Hattori S, Nakamura S, Kitayama H, Noda M, Takai Y, Kaibuchi K, Matsui H, Hatase O, Takahashi H, Kurata T, Matsuda M. 1995. Identification of Rap1 as a target for the Crk SH3 domain-binding guanine nucleotide-releasing factor C3G. Mol Cell Biol 15:6746–6753.

Guerrero C, Martin-Encabo S, Fernandez-Medarde A, Santos E. 2004. C3G-mediated suppression of oncogene-induced focus formation in fibroblasts involves inhibition of ERK activation, cyclin A expression and alterations of anchorage-independent growth. Oncogene 23:4885–4893.

Gupta M, Yarwood SJ. 2005. MAP1A light chain 2 interacts with exchange protein activated by cyclic AMP 1 (EPAC1) to enhance Rap1 GTPase activity and cell adhesion. J Biol Chem 280:8109–8116.

Halgren RG, Traynor AE, Pillay S, Zell JL, Heller KF, Krett NL, Rosen ST. 1998. 8Cl-cAMP cytotoxicity in both steroid sensitive and insensitive multiple myeloma cell lines is mediated by 8Cl-adenosine. Blood 92:2893–2898.

Huang CC, You JL, Wu MY, Hsu KS. 2004. Rap1-induced p38 mitogen-activated protein kinase activation facilitates AMPA receptor trafficking via the GDI.Rab5 complex. Potential role in (S)-3,5-dihydroxyphenylglycene-induced long term depression. J Biol Chem 279:12286–12292.

Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE, Graybiel AM. 1998. A family of cAMP-binding proteins that directly activate Rap1. Science 282:2275–2279.

Kim SN, Ahn YH, Kim SG, Park SD, Cho-Chung YS, Hong SH. 2001. 8-Cl-cAMP induces cell cycle-specific apoptosis in human cancer cells. Int J Cancer 93: 33–41.

Kitayama H, Sugimoto Y, Matsuzaki T, Ikawa Y, Noda M. 1989. A ras-related gene with transformation suppressor activity. Cell 56:77–84.

Lamb D, Steinberg RA. 2002. Anti-proliferative effects of 8-chloro-cAMP and other cAMP analogs are unrelated to their effects on protein kinase A regulatory subunit expression. J Cell Physiol 192:216–224.

Langeveld CH, Jongenelen CA, Theeuwes JW, Baak JP, Heimans JJ, Stoof JC, Peters GJ. 1997. The antiproliferative effect of 8-chloro-adenosine, an active metabolite of 8-chloro-cyclic adenosine monophosphate, and disturbances in nucleic acid synthesis and cell cycle kinetics. Biochem Pharmacol 53:141–148.

Lin Y, Mettling C, Chou C. 2000. Rap1-suppressed tumorigenesis is concomitant with the interference in ras effector signaling. FEBS Lett 467:184–188.

Ling L, Zhu T, Lobie PE. 2003. Src-CrkII-C3G-dependent activation of Rap1 switches growth hormone-stimulated p44/42 MAP kinase and JNK/SAPK activities. J Biol Chem 278:27301–27311.

Lou L, Urbani J, Ribeiro-Neto F, Altschuler DL. 2002. cAMP inhibition of Akt is mediated by activated and phosphorylated Rap1b. J Biol Chem 277:32799–32806.

McDaid HM, Johnston PG. 1999. Synergistic interaction between paclitaxel and 8-chloro-adenosine 3′,5′-monophosphate in human ovarian carcinoma cell lines. Clin Cancer Res 5:215–220.

Mei FC, Qiao J, Tsygankova OM, Meinkoth JL, Quilliam LA, Cheng X. 2002. Differential signaling of cyclic AMP: Opposing effects of exchange protein directly activated by cyclic AMP and cAMP-dependent protein kinase on protein kinase B activation. J Biol Chem 277:11497–11504.

Noda M, Kitayama H, Matsuzaki T, Sugimoto Y, Okayama H, Bassin RH, Ikawa Y. 1989. Detection of genes with a potential for suppressing the transformed phenotype associated with activated ras genes. Proc Natl Acad Sci USA 86: 162–166.

Noguchi K, Murata T, Cho-Chung YS. 1998. 8-chloroadenosine 3′,5′-monophosphate (8-Cl-cAMP) selectively eliminates protein kinase A type I to induce growth inhibition in c-ras-transformed fibroblasts. Eur J Cancer 34:1260–1267.

Posern G, Weber CK, Rapp UR, Feller SM. 1998. Activity of Rap1 is regulated by bombesin, cell adhesion, and cell density in NIH3T3 fibroblasts. J Biol Chem 273:24297–24300.

Propper DJ, Saunders MP, Salisbury AJ, Long L, O'Byrne KJ, Braybrooke JP, Dowsett M, Taylor M, Talbot DC, Ganesan TS, Harris AL. 1999. Phase I study of the novel cyclic AMP (cAMP) analogue 8-chloro-cAMP in patients with cancer: Toxicity, hormonal, and immunological effects. Clin Cancer Res 5: 1682–1689.

Qiao J, Mei FC, Popov VL, Vergara LA, Cheng X. 2002. Cell cycle-dependent subcellular localization of exchange factor directly activated by cAMP. J Biol Chem 277:26581–26586.

Rohlff C, Clair T, Cho-Chung YS. 1993. 8-Cl-cAMP induces truncation and down-regulation of the RI alpha subunit and up-regulation of the RII beta subunit of cAMP-dependent protein kinase leading to type II holoenzyme-dependent growth inhibition and differentiation of HL-60 leukemia cells. J Biol Chem 268: 5774–5782.

Sawada Y, Nakamura K, Doi K, Takeda K, Tobiume K, Saitoh M, Morita K, Komuro I, De Vos K, Sheetz M, Ichijo H. 2001. Rap1 is involved in cell stretching modulation of p38 but not ERK or JNK MAP kinase. J Cell Sci 114: 1221–1227.

Schmitt JM, Stork PJ. 2001. Cyclic AMP-mediated inhibition of cell growth requires the small G protein Rap1. Mol Cell Biol 21:3671–3683.

Schmitt JM, Stork PJ. 2002. PKA phosphorylation of Src mediates cAMP's inhibition of cell growth via Rap1. Mol Cell 9:85–94.

Stork PJ, Schmitt JM. 2002. Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol 12:258–266.

Tagliaferri P, Katsaros D, Clair T, Neckers L, Robins RK, Cho-Chung YS. 1988. Reverse transformation of Harvey murine sarcoma virus-transformed NIH/3T3 cells by site-selective cyclic AMP analogs. J Biol Chem 263:409–416.

Tortora G, Ciardiello F, Pepe S, Tagliaferri P, Ruggiero A, Bianco C, Guarrasi R, Miki K, Bianco AR. 1995. Phase I clinical study with 8-chloro-cAMP and evaluation of immunological effects in cancer patients. Clin Cancer Res 1: 377–384.

Zhang R, Murakami S, Coustry F, Wang Y, de Crombrugghe B. 2006. Constitutive activation of MKK6 in chondrocytes of transgenic mice inhibits proliferation and delays endochondral bone formation. Proc Natl Acad Sci USA 103:365–370.