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An Adenosine-Mediated Signaling Pathway Suppresses Prenylation of the GTPase Rap1B and Promotes Cell Scattering

Elizabeth Ntantie¹, Patrick Gonyo^{1,+}, Ellen L. Lorimer^{1,+}, Andrew D. Hauser¹, Nathan Schuld¹, Donna McAllister², Balaraman Kalyanaraman², Michael B. Dwinell³, John A. Auchampach¹, and Carol L. Williams^{1,*}

¹Department of Pharmacology and Toxicology, Cancer Center, and Cardiovascular Center, Medical College of Wisconsin, Milwaukee, WI, 53226

²Department of Biophysics and Cancer Center, Medical College of Wisconsin, Milwaukee, WI, 53226

³Department of Microbiology and Molecular Genetics and Cancer Center, Medical College of Wisconsin, Milwaukee, WI, 53226

Abstract

During metastasis, cancer cells acquire the ability to dissociate from each other and migrate, which is recapitulated in vitro as cell scattering. The small guanosine triphosphatase (GTPase) Rap1 opposes cell scattering by promoting cell-cell adhesion, a function that requires its prenylation, or posttranslational modification with a C-terminal isoprenoid moiety, to enable its localization at cell membranes. Thus, signaling cascades that regulate the prenylation of Rap1 offer a mechanism to control the membrane localization of Rap1. Here, we identified a signaling cascade initiated by adenosine A2B receptors that suppressed the prenylation of Rap1B through phosphorylation of Rap1B, which decreased its interaction with the chaperone protein SmgGDS (small G-protein dissociation stimulator). These events promoted the cytosolic and nuclear accumulation of non-prenylated Rap1B and diminished cell-cell adhesion, resulting in cell scattering. We found that non-prenylated Rap1 was more abundant in mammary tumors than in normal mammary tissue in rats, and that activation of adenosine receptors delayed Rap1B prenylation in breast, lung, and pancreatic cancer cell lines. Our findings support a model in which high concentrations of extracellular adenosine, such as those that arise in the tumor microenvironment, can chronically activate A2B receptors to suppress Rap1B prenylation and signaling at the cell membrane, resulting in reduced cell-cell contact and promoting cell scattering. Inhibiting A2B receptors may be an effective method to prevent metastasis.

*To whom correspondence should be addressed: williams@mcw.edu.

⁺These authors contributed equally to this study

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Introduction

The small GTPase Rap1 promotes the formation and maintenance of adherens junctions by localizing at the plasma membrane and interacting with membrane-localized regulators and effectors (1). Loss of Rap1 signaling at the plasma membrane diminishes cell-cell adhesion, promoting scattering of epithelial cells (1, 2) and enhancing invasion of carcinoma cells (3). These and other findings indicate that dissolution of cell-cell contacts and enhanced cell dispersion are induced by signaling events that diminish Rap1 activity at the plasma membrane (4, 5).

To localize at the plasma membrane, Rap1 must be post-translationally modified by the attachment of a geranylgeranyl isoprenoid to the C-terminal CAAX motif. Prenylation involving either geranylgeranylation or farnesylation occurs in most members of the Ras and Rho families of small GTPases, and is the major post-translational modification regulating the membrane localization of small GTPases (6-8). Signaling cascades that suppress the prenylation of Rap1 and thereby diminish its membrane localization might reduce cell-cell adhesion and promote cell scattering. However, there have been only a few reports of signaling events that affect prenylation, which describe signal-dependent changes in the activity or abundance of prenyltransferases, resulting in altered prenylation of multiple Ras and Rho family members simultaneously (9-11). Due to the lack of evidence that cells can regulate the selective prenylation of an individual Ras or Rho family member, it is generally assumed that prenylation occurs as soon as Rap1 and other small GTPases are synthesized, without input from signaling pathways.

Rap1B is phosphorylated by protein kinase A (PKA) at serines 179 and 180 (12). These serines are located in the C-terminal polybasic region (PBR), the positively charged region of small GTPases that promotes their electrostatic interaction with multiple forms of the chaperone protein SmgGDS (13-15), which associates with non-prenylated small GTPases and promotes their entrance into the prenylation pathway (15). Phosphorylation of the PBR might regulate the prenylation of Rap1B and other small GTPases by regulating their interactions with SmgGDS. Despite its potential importance, the role of phosphorylation in the prenylation of small GTPases has not been characterized.

In this study, we examined the regulation of Rap1B by adenosine, which acts as an autocrine or paracrine agonist to elicit sustained activation of adenosine receptors in multiple cell types (16-19). Activation of the adenosine A2B receptor (A2BR) generates cAMP (20) which can stimulate both PKA and EPAC, a guanine nucleotide exchange factor (GEF) for Rap1 (21). We report here that A2BR activation promotes Rap1B phosphorylation and delays its prenylation, resulting in reduced localization of Rap1B at the plasma membrane, diminished cell-cell contact, and initiation of cell scattering. The identification of adenosine as a suppressor of Rap1B prenylation and promoter of cell scattering is consistent with reports that autocrine activation of adenosine receptors promotes the metastatic phenotype in multiple forms of cancer (22-26).

Results

Activation of adenosine receptors or protein kinase A promotes Rap1B phosphorylation and suppresses Rap1B prenylation

To examine how adenosine signaling affects Rap1B, we utilized HEK293 cells, which have endogenous adenosine A2B receptors (A2BR) (20). Stimulation of A2BR in HEK293 cells with the agonist adenosine-5'-N-ethylcarboxamide (NECA) can activate PKA (20). To examine PKA-dependent regulation of Rap1B, we mutated serines 179 and 180 to generate phosphomimetic and phosphodeficient mutants of Rap1B (Fig. 1A). The cysteine in the CAAX motif was replaced with serine to generate the non-prenylated Rap1B-SAAX mutant (Fig. 1A). Myc-tagged versions of these small GTPases were immunoprecipitated from HEK293T cells labeled with ^{32}P and treated with or without NECA, immunoblotted, and subjected to phosphoimaging (Fig. 1B). Treatment with NECA promoted phosphorylation of wild-type Rap1B and the non-prenylated Rap1B-SAAX mutant, but not the phosphodeficient Rap1B(AA) or phosphomimetic Rap1B(EE) mutants (Fig. 1B and 1C).

Rap1 that has not been prenylated can be detected by its slowed migration in immunoblots, because non-prenylated small GTPases migrate more slowly than prenylated small GTPases in SDS-PAGE gels (27). The non-prenylated Rap1B-SAAX mutant appeared as a single, more slowly migrating protein in immunoblots (Figure 1B, lane 7), consistent with its lack of prenylation. Wild-type Rap1B was present as both slow and fast migrating forms in immunoblots (lane 1, Fig. 1B), consistent with wild-type Rap1B existing in both the non-prenylated and prenylated states. NECA promoted the accumulation of the slowly migrating form of Rap1B (lane 2, Fig. 1B), suggesting the accumulation of non-prenylated Rap1B. The non-prenylated state was promoted by phosphorylation at serines 179 and 180, because phosphomimetic Rap1B(EE) accumulated in the slow migrating form in the absence of NECA (lane 5, Fig. 1B), and phosphodeficient Rap1B(AA) did not exhibit a substantial NECA-induced mobility shift (lane 4, Fig. 1B). It is unlikely that these mobility shifts were caused simply by the higher molecular weight of phosphorylated proteins, because Rap1B(AA) and Rap1B(EE) exhibit different mobilities in the immunoblot even though neither of these proteins are phosphorylated (Fig. 1B). Alignment of the immunoblot with the phosphoimage (Fig. 1B) indicated that the slow migrating forms of Rap1B or Rap1B-SAAX were phosphorylated in NECA-treated cells, suggesting that the non-prenylated form of Rap1B was more sensitive than the prenylated form to A2BR-induced phosphorylation.

To determine the effects of directly activating PKA, we treated the cells with N6-benzoyladenosine-3', 5'-cyclic monophosphate (6-Bnz-cAMP) to activate PKA (28), which produced similar changes in Rap1B phosphorylation and mobility as those induced by NECA (fig. S1).

Due to differences in hydrophobicity, prenylated small GTPases fractionate into the detergent phase, whereas non-prenylated small GTPases fractionate into the aqueous phase (27). Thus, to further examine Rap1B prenylation, we lysed cells expressing the myc-tagged GTPases in Triton-X-114 (TX-114) to generate aqueous and detergent fractions. Both Rap1B and phosphodeficient Rap1B(AA) fractionated mainly into the detergent phase (lanes 2 and 6, Fig. 2A), whereas phosphomimetic Rap1B(EE) fractionated into both the

aqueous and detergent phases (lanes 9 and 10, Fig. 2A). As expected, the non-prenylated Rap1B-SAAX mutants fractionated solely into the aqueous phase (lanes 3, 7, and 11, Fig. 2A).

Treatment with NECA caused the accumulation of Rap1B in the aqueous phase of TX-114 fractions (fig. S2A), indicating that NECA decreased the steady state prenylation of Rap1B (Fig. 2B). This response involved Rap1B phosphorylation, because NECA did not diminish the prenylation of Rap1B(AA) or Rap1B(EA) (Fig. 2B and fig. S2A). Similar results were obtained by activating PKA directly with 6-Bnz-cAMP (Fig. 2C and fig. S2B). Unexpectedly, treatment with NECA or 6-Bnz-cAMP increased the total amount of the transiently expressed Rap1B proteins (fig. S2), suggesting that PKA activation stabilized the expressed Rap1B proteins. This enhanced stability did not require phosphorylation of serines 179 and 180, because it was exhibited by both Rap1B(AA) and Rap1B(EA) in addition to wild-type Rap1B (fig. S2).

To examine a potential role for EPAC, we treated cells with 8-CPT-2-OMe-cAMP (8-cpt-cAMP) to activate EPAC (28), which did not detectably alter the prenylation or stability of Rap1B (fig. 2D and fig. S2C) although it increased Rap1B activity (fig. S2D). As expected, the A2BR antagonist PSB-603 inhibited NECA-induced changes in Rap1 prenylation and stability (fig. S3). Together, these results indicated that A2BR activation induces the PKA-mediated phosphorylation of serines 179 and 180 in Rap1B and diminishes the prenylation of Rap1B, and other PKA-mediated events promote the stability of Rap1B.

Phosphorylation of Rap1B diminishes its interaction with SmgGDS and slows Rap1B entry into the prenylation pathway

Because prenylation is believed to be irreversible (6-8), the reduced prenylation of phosphorylated Rap1B was most likely caused by newly synthesized Rap1B undergoing a slower rate of prenylation after phosphorylation. To examine the rate of Rap1B prenylation, we utilized the previously described “prenylation block-and-release” assay in which prenylation is reversibly inhibited by treatment with mevastatin (29). When mevastatin is present, isoprenoid production is inhibited and newly synthesized small GTPases accumulate in the non-prenylated form. When mevastatin is removed by washing, isoprenoid production and prenylation rapidly resume (29). Immunoblotting of TX-114 fractions collected at hourly intervals after the removal of mevastatin provided an indication of how quickly small GTPases entered the prenylation pathway (fig. S4). Densitometric analysis of these immunoblots indicated that treatment with NECA slowed the prenylation of endogenous Rap1B (Fig. 3A and fig. S4A). The PKA-dependent phosphorylation of Rap1B contributed to this delay, as indicated by slower prenylation of phosphomimetic myc-Rap1B(EA) compared to myc-Rap1B or phosphodeficient myc-Rap1B(AA) (Fig. 3B and fig. S4B). Consistent with this finding, treatment with the PKA inhibitor KT5720 (30) accelerated prenylation of Rap1B (Fig. 3C and 3D, and fig. S4C).

We next examined the interactions of Rap1B with SmgGDS-607. The Rap1B-SAAX mutant co-precipitated with SmgGDS-607, indicating that non-prenylated Rap1B interacts with SmgGDS-607 (Fig. 4A). The interaction of SmgGDS-607 with Rap1B was decreased by the phosphomimetic mutation and by phosphorylation of Rap1B by PKA (Fig. 4B and 4C).

These results support our model that PKA-dependent phosphorylation of non-prenylated Rap1B diminishes its interaction with SmgGDS-607, delaying the entry of Rap1B into the prenylation pathway.

Phosphorylation of Rap1B promotes its cytosolic and nuclear accumulation, and promotes cell scattering

Suppressing prenylation is expected to diminish Rap1B membrane localization due to the absence of the geranylgeranyl moiety that helps anchor Rap1B at the plasma membrane (6-8). Confocal microscopy indicated that treatment with 6-Bnz-cAMP or NECA reduced the localization of GFP-tagged Rap1B at the cell membrane, and promoted its accumulation in the cytosol and nucleus (Fig. 5A). This distribution was due to phosphorylation of serines 179 and 180, because phosphodeficient Rap1B(AA) was membrane localized in the presence of 6-Bnz-cAMP or NECA treatment, and phosphomimetic Rap1B(EЕ) was cytosolic and nuclear in the absence of these drugs (Fig. 5A). Furthermore, the PKA inhibitor KT5720 promoted the membrane localization of Rap1B, and suppressed the NECA-induced accumulation of Rap1B in the cytosol and nucleus (Fig. 5B and fig. S5).

Non-prenylated GTPases are generally assumed to be in the inactive, GDP-bound state due to their diminished interaction with membrane-localized activators, such as guanine nucleotide exchange factors (GEFs) (6, 8). Unexpectedly, we found that a substantial proportion of non-prenylated Rap1B-SAAX was in the active, GTP-bound state, as indicated by its co-precipitation with the Rap1-GTP-binding domain of RalGDS (lane 7, Fig. 5C, and fig. S6). Our observation that both the non-prenylated and prenylated forms of Rap1B bound GTP (Fig. 5C and fig. S6) suggests that non-prenylated Rap1B interacted with GEFs and effectors located in the cytosol or nucleus, whereas prenylated Rap1B interacted with GEFs and effectors at cell membranes.

Reduced localization of Rap1B at the plasma membrane following A2BR activation is predicted to diminish cell-cell adhesion and promote cell scattering, similar to the effects of diminishing Rap1B signaling at the plasma membrane in other cell types (1, 2). Cell scattering involves the dispersion of epithelial cells from compacted colonies, and is accompanied by membrane ruffling, cell elongation, and loss of cell-cell contacts (31). We found that treatment with NECA promoted membrane ruffling and cell elongation, and diminished cell-cell contacts, indicating cell scattering (Fig. 6A and 6B, and fig. S7).

Our results suggested that NECA-treated cells scattered because phosphorylated Rap1B could not localize at the plasma membrane and promote cell-cell adhesion, and that expression of phosphodeficient Rap1B(AA) should enable cells to maintain contact with their neighbors and resist scattering in the presence of NECA. In the absence of NECA, cells expressing GFP-Rap1B or phosphodeficient GFP-Rap1B(AA) had greater contacts with their neighbors than did cells expressing phosphomimetic GFP-Rap1B(EЕ) (Fig. 6C and fig. S8). Cells expressing GFP-Rap1B lost contact with their neighbors when treated with NECA, consistent with GFP-Rap1B becoming phosphorylated and no longer promoting cell-cell adhesion. Moreover, cells expressing GFP-Rap1B(AA) maintained contacts with their neighbors after treatment with NECA (Fig. 6C and fig. S8).

Adenosine-mediated signaling regulates Rap1B prenylation in multiple cancer cell types

Together, our findings supported a model in which adenosine activates the A2BR to cause the PKA-mediated phosphorylation of serines in the PBR of Rap1B, resulting in diminished interaction with SmgGDS-607 and delayed prenylation of Rap1B (Fig. 7A). These events inhibited the ability of Rap1B to localize at the plasma membrane, resulting in reduced cell-cell contact and increased cell scattering. To determine the prevalence of this pathway in transformed cells, we examined Rap1B prenylation in cancer cell lines treated with or without NECA (Fig. 7B and fig. S9). In the absence of NECA, the rate of Rap1B prenylation was variable among the different cell lines, occurring relatively rapidly in MCF-7 breast cancer cells, and more slowly in MDA-MB-231 breast cancer cells and NCI-H23 lung cancer cells (Fig. 7B and fig. S9). We found that NECA delayed Rap1B prenylation in the NCI-H1703, MDA-MB-231, Panc-1, and MiaPaCa-2 cell lines (Fig. 7B and fig. S9). A2BR is reported to be present in these cell lines or their tissues of origin (22, 32-34).

Non-prenylated small GTPases are rarely detected in cells, consistent with the general view that prenylation is constitutive and is not regulated (35). Our findings suggest that signaling events that promote the accumulation of non-prenylated GTPases might occur in special conditions, such as those arising in the tumor microenvironment. Tumors generate high amounts of extracellular adenosine (19, 36), which can activate A2BR on tumor cells (17-19). This tumor-derived adenosine could chronically activate A2BR to promote the accumulation of non-prenylated Rap1. Consistent with this possibility, rat mammary tumors induced by 7,12-dimethylbenz(a)anthracene (DMBA) showed detectable amounts of non-prenylated Rap1 (Fig. 7C). Furthermore, these tumors showed an increase in total Rap1 protein (Fig. 7C), which is consistent with our observation that A2BR activation increased total Rap1 protein, as well as non-prenylated Rap1, in cultured cells (fig. S2). We are investigating whether non-prenylated Rap1 accumulates in these mammary tumors due to increased adenosine concentrations or increased abundance of A2BR, which is found in some forms of cancer (22). The prenylation of Rap1B was slowed in HEK293T stably expressing mouse A2BR, thus demonstrating that an increase in the abundance of A2BR can cause non-prenylated Rap1B to accumulate (fig. S10).

Discussion

The ability of A2BR activation to suppress the prenylation and localization of Rap1B suggests that in some cases, the multiple physiological effects of adenosine signaling (16-20, 23-26) might involve changes in Rap1B prenylation and localization. Increased adenosine generation occurs in tumors due to multiple factors, including increased abundance of the CD73 ecto-5'-nucleotidase that generates adenosine (19, 24, 25, 37). Adenosine promotes malignancy by activating A2BR on tumor cells (23-26) and by activating adenosine receptors on immune cells, which suppresses anti-tumor immune responses (16, 19). Our findings indicate that tumors that differ in their adenosine generation and A2BR abundance might differ in their Rap1B prenylation and signaling. These attributes might also regulate Rap1B prenylation and signaling in the cultured cell lines that we examined. For example, reports that MCF-7 cells have diminished adenosine generation (37) and reduced A2BR

abundance (34) might explain why Rap1B is prenylated rapidly in a NECA-insensitive manner in these cells.

Although non-prenylated small GTPases have been generally assumed to have few functions, there is evidence that non-prenylated, cytosolic small GTPases can actively signal (38, 39, 40). These findings are consistent with our observation that non-prenylated Rap1B actively bound GTP and was regulated by PKA, suggesting that Rap1B does not have to be prenylated nor associate with membranes to actively participate in signaling cascades, as well as with our discovery of a signaling cascade that suppressed Rap1B prenylation. The different subcellular distributions of non-prenylated and prenylated Rap1B probably means that these two forms of Rap1B participate in distinct signaling cascades.

It is possible that phosphorylation alters the functions of non-prenylated Rap1B in ways that extend beyond suppressing its prenylation. Non-prenylated Rap1B might participate in certain signaling cascades before being phosphorylated, and participate in other cascades after it is phosphorylated, just as phosphorylation affects signaling by prenylated Rap1B (12). Once non-prenylated Rap1B is phosphorylated, it might have to be de-phosphorylated by phosphatases before it can interact with SmgGDS-607 and enter the prenylation pathway.

Our findings support a model in which the suppression of Rap1B prenylation caused by chronic A2BR activation induces the dissociation of tumor cells and promotes their dispersion, resulting in enhanced metastasis. This model is consistent with reports that A2BR activation promotes the motile and invasive phenotype (23-26). Our model suggests that in cells with activated A2BR, the absence of prenylated Rap1B at the plasma membrane promotes invasion by reducing cell-cell adhesion. However, it is also intriguing to speculate that non-prenylated Rap1B in the cytosol or nucleus could promote invasion by initiating signaling cascades involved in epithelial-mesenchymal transition. This speculation is consistent with reports that Rap1 localizes in the nucleus in some tumor cell types (41) and that Rap1 participates in nuclear signaling cascades involving β -catenin (42).

In addition to the A2BR-mediated pathway described here, it is likely that additional receptor-mediated pathways regulate the prenylation and localization of other GTPases that have phosphorylation sites in their PBRs, including K-Ras4B (43), RhoA (44), Cdc42 (isoform 1) (44), and Rnd3 (also known as RhoE) (45). SmgGDS-607 associates with many of these PBRcontaining small GTPases before they are prenylated (15), and phosphorylation of the PBRs of these small GTPases might diminish their association with SmgGDS-607 and could alter their prenylation and trafficking. Therapeutically targeting the signaling pathways that regulate the prenylation of small GTPases, including the adenosine-mediated signaling cascade described here, could provide a new approach to diminish metastasis and other pathological conditions involving abnormal signaling by small GTPases.

Materials and Methods

Cell Culture and Transfection

Cell lines were obtained from the American Type Culture Collection (Manassas, VA). HEK293T cells were cultured in complete DMEM media (DMEM, 10% heat-inactivated

fetal bovine serum, and antibiotics). PANC-1, MiaPaCA-2, and MDA-MB-231 cells were cultured in complete DMEM media supplemented with 1X sodium pyruvate. MCF-7 cells were cultured in MEM, 10% heat-inactivated fetal bovine serum, 1X non-essential amino acids, 1X sodium pyruvate, and antibiotics. NCI-H23 and NCI-H1703 cells were cultured in RPMI 1640, 10% heat-inactivated fetal bovine serum, and antibiotics. cDNAs were generated as previously described (15, 47) or were purchased from the Missouri S&T cDNA Resource Center (www.cDNA.org) and were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Immunoprecipitation and Enhanced Chemiluminescence (ECL)-Immunoblotting

Immunoprecipitation and ECL-immunoblotting were conducted as previously described (15, 47) using the following primary antibodies: mouse myc antibody (Santa Cruz sc-40 or Abcam 18185), rabbit myc antibody (Covance PBR-150P or Sigma C3956), mouse HA antibody (Covance MMS-101P), rabbit HA antibody (Covance PRB-101P), mouse GAPDH antibody (Santa Cruz sc-32233), mouse β -actin antibody (Santa Cruz sc-47778), rabbit Lamin B1 antibody (Abcam ab16048), rabbit antibody that detects both the prenylated and non-prenylated forms of Rap1B (Cell signaling 36E1), and goat antibody that specifically recognizes only the non-prenylated forms of Rap1A and Rap1B (Santa Cruz sc-1482) (48). The optical densities of the proteins in immunoblots from independent experiments were obtained by densitometry, as described in the figure legends.

Analysis of Rap1B Phosphorylation by In-vivo ^{32}P Labeling

HEK293T cells were transiently transfected with cDNAs encoding myc-tagged Rap1B proteins. After 24 hours, the media was replaced with phosphate-free DMEM media containing 250 $\mu\text{Ci/ml}$ of ^{32}P -Orthophosphate (Perkin Elmer) with or without 6-Bnz-cAMP (1 mM) or NECA (10 μM). Cells were incubated for three hours, followed by lysis in 0.5% Nonidet P-40 in the presence of protease inhibitors and phosphatase inhibitors. The cleared lysates were immunoprecipitated, followed by SDS-PAGE and transfer to PVDF membranes. Phosphoimages of the PVDF membranes were obtained using a Storm 820 Phosphoimager, followed by immunoblotting of the PVDF membranes to detect expression of the myc-tagged Rap1B proteins. Densitometry of independent phosphoimages was conducted to obtain optical densities of the phosphorylated proteins.

Triton-X-114 (TX-114) Fractionation Assays

Untransfected HEK293T cells or cells transiently expressing different myc-tagged Rap1B proteins were treated with or without NECA (10 μM) or 6-Bnz-cAMP (1 mM) for 24 hours. In some experiments, KT5720 (10 μM) was added to the culture medium 30 minutes before the cells were exposed to NECA (10 μM). The cells were lysed with 1% Triton-X-114 in TBS (50 mM Tris, 150 mM NaCl, pH 7.5), and aqueous and detergent fractions were isolated as described previously (15). The fractions were subjected to ECL-immunoblotting, and optical densities of the proteins in the immunoblots were obtained to calculate the percentage of prenylated Rap1B in the different conditions.

Prenylation Block and Release Assay

A modification of a previously described technique (29) was used to assess the rate of Rap1B prenylation. HEK293T cells were untransfected, or transfected with cDNAs encoding myc-tagged Rap1B proteins, and 90 minutes later exposed to mevastatin (10 μ M). After 20-24 hours, mevastatin was removed by washing, and the cells were placed in fresh media in the presence or absence of NECA (10 μ M) or KT5720 (10 μ M). The cells were collected at different time points after the removal of mevastatin, and directly lysed in laemmli sample buffer, or lysed in Triton-X-114 to generate aqueous and detergent fractions. The samples were immunoblotted using either Rap1B antibody to detect endogenous Rap1B, or myc antibody to detect the expressed myc-tagged Rap1B proteins. Densitometry was conducted to assess the relative amount of prenylated and non-prenylated proteins that were detectable in independent immunoblots.

Analysis of Immunoprecipitates from Reticulocyte Lysates

Reticulocyte cell lysates (Promega) were used in *in vitro* transcription and translation assays as previously described (49) to generate 35 S-labelled SmgGDS-607-HA and 35 S-labelled myc-tagged Rap1B proteins. Combinations of the proteins were incubated in the absence or presence of 6-Bnz-cAMP (1 mM) for 1 hour, followed by immunoprecipitation using HA antibody (49). The immunoprecipitates were subjected to SDS-PAGE, followed by autoradiography.

Confocal Imaging

HEK293T cells were plated at a density of 1.5×10^5 cells on 35 mm glass-bottom culture dishes (MatTek Corp.) Forty-eight hours later, the cells were transiently transfected with cDNAs encoding GFP-tagged Rap1B proteins. Four hours after transfection, the media was replaced with fresh media with or without 6-Bnz-cAMP (1 mM) or NECA 10 μ M). The cells were incubated for an additional 20-24 hours before confocal images were obtained using a Leica SP- 5 microscope.

Morphometric Analysis of Cell-Cell Interactions and Rap1B Localization

For morphometric analysis of cell scattering, HEK293T cells were cultured in complete media on glass coverslips for 48 hours, and treated with or without NECA (10 μ M), 6-Bnz-cAMP (1 mM), or 8-CPT-cAMP (30 μ M) for another 24 hours. The cells were fixed with 4 % formaldehyde and 1% glutaraldehyde in PBS (10 min, 4 C) and incubated with 0.2 μ g DAPI/ml PBS for 5-10 minutes, before collection of brightfield images using a Nikon Eclipse E600 microscope. The coded images were analyzed, without knowledge of the drug treatments, using MetaMorph software to determine the parameters described in Figure S7. To analyze Rap1B localization and the regulation of cell-cell contacts by overexpressed Rap1B, HEK293T cells were cultured in complete media on glass coverslips for 48 hours and then transfected with a cDNA encoding either GFP or a GFP-tagged wild-type or mutant Rap1B protein. Four hours after transfection, the culture media was replaced with fresh media with or without 6-Bnz-cAMP (1 mM) or NECA (10 μ M). In some experiments, KT5720 (10 μ M) was added to the cultures 30 minutes before the addition of NECA (10 μ M). The cells were cultured another 24 hours before collection of fluorescence images

using a Nikon Eclipse E600 microscope. The coded images were analyzed, without knowledge of the transfected cDNAs or drug treatments, using the criteria described in Figures S5 and S8.

Rap1B Activity Assay using GST-RalGDS Pulldown

HEK293T cells transiently expressing myc-tagged wildtype or mutant Rap1B proteins were lysed in ice-cold lysis buffer (50 mM Tris pH 7.5, 10 mM MgCl₂, 0.3 M NaCl, 1% Triton-X-100). Cell lysates were collected and centrifuged (5000 rpm, 15 min, 4°C) to remove insoluble debris. Aliquots containing equal protein concentrations were incubated (1 hour, 4°C) with GST-beads conjugated to the Rap1-binding domain of RalGDS, followed by centrifugation. The precipitates and total cell lysates were subjected to SDS-PAGE, followed by immunoblotting to detect the myc-tagged Rap1B proteins. Densitometry of the immunoblots was conducted to obtain O.D. values of the immunoreactive proteins, and the ratio of GTP-bound Rap1B in the prenylated or non-prenylated forms was determined with respect to total Rap1B.

Analysis of Non-Prenylated and Total Rap1 in Tissues of DMBA-Treated Rats

Animal studies were conducted in accordance with institutional guidelines. Mammary tumors were induced in female Sprague Dawley rats by treatment with 7,12-dimethylbenz(a)-anthracene (DMBA) as previously described (50). Normal and tumor tissues were dissected from four rats and their identity confirmed by histology. Tissue sections were weighed and manually homogenized in 1X RIPA buffer at a ratio of 10 µl of RIPA buffer to 1 mg of tissue protein. Homogenization was done on ice and then tissue lysates were centrifuged at 10,000 rpm for 15 min at 4°C. The amount of protein in each sample was determined in protein assays using the BCA protein assay kit (Pierce Biotechnologies, Inc.) according to the manufacturer's protocol. Volumes of lysates containing equal protein concentrations were added to 2X Laemmli sample buffer, boiled, and subjected to SDS-PAGE. The amount of non-prenylated Rap1 and total Rap1 was determined by immunoblotting using antibody that detects non-prenylated Rap1 (Santa Cruz sc-1482) (48) and antibody that detects total Rap1 (Cell signaling 36E1), respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Activation of adenosine receptors induces phosphorylation of Rap1B. (A) C-terminal sequences of Rap1B phosphorylation and prenylation mutants are shown. (B) Phosphorylation of Rap1B is detectable in a phosphoimage (*top*) that was generated from an immunoblot (*bottom*) of myc-tagged Rap1B proteins that were immunoprecipitated from ³²P-labeled HEK293T cells treated with or without NECA. The immunoblot (*bottom*) indicates different migration rates of the immunoprecipitated myc-Rap1B proteins. Asterisks indicate migration of molecular weight markers (np = non-prenylated Rap1B, p = prenylated Rap1B). (C) The fold increase in Rap1B phosphorylation was determined by measuring the O.D. of proteins detected in the phosphoimages generated in experiments described in B. The values are presented as the fold increase in relation to the O.D. of the indicated proteins from untreated cells, and are the mean +/- SEM from three independent experiments.

Student's t-test was used for statistical analysis. The asterisks indicate that phosphorylated forms of myc-tagged Rap1B(AA) and Rap1B(EA) proteins were not detected in the phosphoimages.

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Fig. 2.

The prenylation of Rap1B is diminished by the phosphomimetic mutation and by treatment with NECA or 6-Bnz-cAMP. **(A)** HEK293T cells transiently expressing different myc-tagged Rap1B proteins were subjected to TX-114 fractionation to generate an aqueous phase “A” that contains non-prenylated (np) proteins, and a detergent phase “D” that contains prenylated (p) proteins. Cell fractions were immunoblotted with a myc antibody to detect the myc-tagged Rap1B proteins and a GAPDH antibody to confirm equal protein loading. An immunoblot of total cell lysates is shown beneath the TX-114 fractions. Results are representative of three independent experiments. **(B-D)** HEK293T cells transiently expressing different myc-tagged Rap1B proteins were treated for 24 hours with or without NECA (10 μ M) **(B)**, 6-Bnz-cAMP (1 mM) **(C)**, or 8-CPT-cAMP (30 μ M) **(D)**. The cells were subjected to TX-114 fractionation and immunoblotting as in A. The O.D. values of the

immunoblotted proteins from the aqueous and detergent phases were used to calculate the percentage of prenylated Rap1B. Values are the mean $\pm$ SEM calculated from four (*B*), five (*C*), and three (*D*) independent experiments. Statistical significance was determined by matched pairs Student's t-test with Bonferroni-adjusted p values (*, $p < 0.05$). Representative immunoblots are shown in Figure S2.

Fig. 3.

Rap1B prenylation is slowed by the phosphomimetic mutation or treatment with NECA, but accelerated by treatment with KT5720. Untransfected HEK293T cells (**A**) or HEK293T cells expressing myc-tagged Rap1B proteins (**B**) were incubated with mevastatin, washed, and placed in media with or without NECA. The cells were subjected to TX-114 fractionation at the indicated times, followed by immunoblotting using Rap1B antibody to detect endogenous Rap1B (**A**) or myc antibody to detect myc-tagged Rap1B proteins (**B**). Densitometry of the proteins in the immunoblotted aqueous and detergent fractions was performed to calculate the fraction of prenylated Rap1B. In each graph, the results are the mean $\pm$ SEM of densitometric analysis of three independent experiments. (**C**, **D**) HEK293T cells were incubated with mevastatin, washed, incubated with or without KT5720 or NECA for the indicated times, lysed, and immunoblotted with Rap1B antibody. The

fraction of prenylated Rap1B was calculated from densitometric analysis of the slowly migrating non-prenylated Rap1B and the faster migrating prenylated Rap1B detected in the immunoblots. In each graph, the results are the densitometric analysis of two independent experiments. Representative immunoblots are shown in Figure S4.

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Fig. 4. Phosphorylation of Rap1B diminishes its interaction with SmgGDS-607. **(A)** HA immunoprecipitates from HEK293T cells coexpressing HA-tagged SmgGDS-607 with myc-tagged Rap1B or Rap1B-Saax and total cell lysates were immunoblotted with HA and myc antibodies. **(B)** Similar to A, but the cells were transfected with cDNAs encoding myc-tagged phosphorylation mutants of Rap1B. **(C)** Reticulocyte lysates were used in *in vitro* transcription and translation assays to generate ³⁵S-labelled SmgGDS-607-HA and ³⁵S-labelled myc-tagged Rap1B or a dominant negative (DN) S17N Rap1B. The ³⁵S-labeled proteins were incubated together in the absence or presence of 6-Bnz-cAMP. Autoradiography was performed on HA immunoprecipitates from the *in vitro* assays. DN-Rap1B was used because it forms a more stable complex with SmgGDS-607 than does wild-type Rap1B (15). All results are representative of at least four independent experiments.

Fig. 5. Phosphorylation of Rap1B promotes its accumulation in the cytosol and nucleus. **(A)** HEK293T cells expressing GFP-tagged wildtype or mutant Rap1B proteins were treated with 6-Bnz-cAMP, NECA, or no drug and imaged with confocal microscopy. All images are at the same magnification and are representative of three independent experiments. The scale bar in the lower right corner represents 10 μ m. **(B)** HEK293T cells were transfected with a cDNA encoding GFP-Rap1B and treated with or without NECA in the presence or absence of KT5720. Images of the cells were collected by fluorescence microscopy, and the coded images were scored for membrane localization of GFP-Rap1B, as described in Figure S5. The results are the mean $\pm$ SEM from three independent experiments. Values (n) above the columns indicate the number of scored cells. The statistical difference between the drug-treated cells compared to the untreated cells was determined by repeated-measures ANOVA

with a secondary Dunnett's multiple comparison test. (C) HEK293T cells expressing the indicated myc-tagged Rap1B proteins were lysed and the amount of GTP-bound Rap1B was measured using pulldown assays. Multiple exposures of the immunoblots are shown, as indicated by times listed at the right of the figure. Densitometric analyses of immunoblots from three independent experiments are shown in Figure S6.

Fig. 6.

Activation of adenosine receptors promotes cell scattering, a response that is suppressed by expression of phosphodeficient Rap1B(AA). **(A)** Merged brightfield and DAPI images of HEK293T cells were collected after treatment with or without NECA, 6-Bnz-cAMP, or 8-CPT-cAMP. **(B)** Morphometric analysis of the cells described in A was conducted as explained in Figure S7. The results are the mean $\pm$ SEM from two independent experiments with 13 – 24 colonies scored in each sample. One-way ANOVA with a secondary Dunnett's multiple comparison test was used to determine significant differences between the drug-treated cells compared to the untreated cells. **(C)** HEK293T cells expressing either GFP or a GFP-tagged wild-type or mutant Rap1B protein were cultured with or without NECA before the collection of fluorescence images. The coded images were analyzed to define how many neighbors each cell contacted, as described in Figure S8.

Representative images of the cells are shown in Figure S8. The results are the mean $\pm$ SEM from two independent experiments with 18 – 21 colonies (representing 113 – 165 cells) scored in each sample. Matched-pairs Student's t-test with Bonferonni-adjusted p values was used for statistical analysis of the bracketed samples.

Fig. 7. Adenosine signaling slows Rap1B prenylation in multiple cell types. **(A)** Our model of how adenosine regulates Rap1B. In the absence of adenosine signaling, newly synthesized Rap1B interacts with SmgGDS-607, which facilitates prenylation. Prenylated Rap1B localizes to the plasma membrane, where it promotes cell-cell adhesion. When A2BR are activated, PKA-mediated phosphorylation of Rap1B diminishes its interaction with SmgGDS-607, thereby slowing Rap1B prenylation. Non-prenylated Rap1B accumulates in the cytosol and nucleus, resulting in the loss of Rap1B functions at the plasma membrane, promoting dissolution of cell contacts and initiation of cell scattering. **(B)** The lung cancer lines (NCI-H1703, NCI-H23), breast cancer lines (MCF-7, MDA-MB-231) and pancreatic cancer lines (PANC-1, MiaPaCa-2) were incubated with mevastatin, washed, and incubated with or without NECA. At the indicated times, cell lysates were immunoblotted to detect endogenous Rap1B. The fraction of prenylated Rap1B was calculated from densitometric analysis of the slowly migrating non-prenylated Rap1B and the faster migrating prenylated Rap1B detected in the immunoblots. Results are the mean $\pm$ SEM of densitometric analysis of immunoblots from 3 – 5 independent experiments (as indicated in each graph) for all cell

lines except NCI-H23 cells. Results for NCI-H23 cells were obtained by densitometric analysis of immunoblots from two independent experiments. Representative immunoblots are shown in Figure S9. (C) Cell lysates prepared from mammary tumors “T” or normal mammary tissue “N” from DMBA-treated rats were immunoblotted using antibodies that detect non-prenylated Rap1, total Rap1, or actin to confirm equal loading.