Cyclic AMP induces integrin-mediated cell adhesion through Epac and Rap1 upon stimulation of the β2-adrenergic receptor

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CAMP controls many cellular processes mainly through the activation of protein kinase A (PKA). However, more recently PKA-independent pathways have been established through the exchange protein directly activated by cAMP (Epac), a guanine nucleotide exchange factor for the small GTPases Rap1 and Rap2. In this report, we show that cAMP can induce integrin-mediated cell adhesion through Epac and Rap1. Indeed, when Ovcar3 cells were treated with cAMP, cells adhered more rapidly to fibronectin. This cAMP effect was insensitive to the PKA inhibitor H-89. A similar increase was observed when the cells were transfected with Epac. Both the cAMP effect and the Epac effect on cell adhesion were abolished by the expression of Rap1–GTPase-activating protein, indicating the involvement of Rap1 in the signaling pathway. Importantly, a recently characterized cAMP analogue, 8-(4-chloro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate, which specifically activates Epac but not PKA, induced Rap-dependent cell adhesion. Finally, we demonstrate that external stimuli of cAMP signaling, i.e., isoproterenol, which activates the Gαs-coupled β2-adrenergic receptor can induce integrin-mediated cell adhesion through the Epac-Rap1 pathway. From these results we conclude that cAMP mediates receptor-induced integrin-mediated cell adhesion to fibronectin through the Epac-Rap1 signaling pathway.

Introduction

cAMP is a common second messenger controlling many cellular processes. Protein kinase A (PKA)* is a general receptor for cAMP, resulting in the phosphorylation of a large variety of cellular targets. Specificity is regulated by A kinase anchoring proteins that target PKA to specific regions in the cell. A few years ago we discovered an additional cAMP target, exchange protein directly activated by cAMP (Epac)1. This protein and its close relative Epac2 contain cAMP-binding domains very similar to the cAMP-binding domains in the regulatory subunit of PKA and are exchange factors of the small GTPases Rap1 and Rap2 (de Rooij et al., 1998, 2000; Kawasaki et al., 1998).

Rap1 is a GTPase of the Ras superfamily, which functions as a molecular “switch,” cycling between inactive GDP- and active GTP-bound forms. Specific guanine nucleotide exchange factors are the “on switches,” and GTPase-activating proteins (GAPs) are the “off switches” (for review see Bos et al., 2001). Rap1 was initially identified in a screen for proteins that can suppress the transformed phenotype of fibroblasts transformed by oncogenic K-Ras (Kitayama et al., 1989), providing a model in which Rap1 functions as an antagonist of Ras signaling mainly by trapping Ras effectors (Raf-1) in an inactive complex. However, from numerous reports accumulated so far it is evident that Rap1 signaling is important in itself and independently of Ras regulates several important cellular processes (Bos et al., 2001). One of the most consistent findings is the involvement of Rap1 in integrin-mediated...
cell adhesion (Caron et al., 2000; Katagiri et al., 2000; Reedquist et al., 2000; Arai et al., 2001; Ohba et al., 2001; de Bruyn et al., 2002; Sebzda et al., 2002). Integrins are heterodimeric cell adhesion molecules consisting of one of several different α chains and one of at least five different β chains. One of the first indications was that in 32D cells granulocyte colony stimulating factor-induced cell adhesion could be abolished by the introduction of Spa1, a GAP for Rap proteins (Tsukamoto et al., 1999). This finding was followed by three independent observations showing a role for Rap1 in the inside-out signaling to integrins. First, in Jurkat cells introduction of Rap1 induced integrin αLβ2 (LFA1)-mediated adhesion to the intercellular adhesion molecule. Importantly, adhesion induced by ligation of the T cell receptor was inhibited by introduction of an interfering mutant of Rap1 (Katagiri et al., 2000). Second, also in Jurkat cells ligation of the adhesion molecule CD-31 induced activation of αLβ2, which was inhibited by blocking Rap1 signaling (Reedquist et al., 2000). Finally, in a macrophage cell line, complement-mediated phagocytosis, which requires activated αMβ2, was abolished by inhibition of Rap1 signaling (Caron et al., 2000). Other studies reached the same conclusion for integrins with a β1 chain, i.e., α5β1 (Arai et al., 2001) and for integrins with a β3 chain, i.e., αIIbβ3 (Bertoni et al., 2002). Recently, it was shown that in mice expressing active Rap1 in their T cell compartment both the thymocytes and mature T cells exhibited increased integrin-mediated cell adhesion. In addition, these cells showed enhanced T cell receptor–mediated responses (Sebzda et al., 2002). From the above results we hypothesized that cAMP or signals that raise cAMP levels may regulate integrin-mediated cell adhesion through Epac and Rap1. We have tested this model and found that indeed cAMP was able to induce integrin-mediated cell adhesion to fibronectin.

Results and discussion

To investigate whether cAMP could induce integrin-mediated cell adhesion, we used ovarian carcinoma cells (Ovcar3), since these cells express the β1 integrin chain in association with α chains 1–6 and αv with the α5β1 and αvβ3 integrins mediating binding to fibronectin (Cannistra...
Cytomegalovirus-luciferase–transfected cells were detached with trypsin and allowed to reexpress cell surface markers. The cells were seeded onto fibronectin-coated multiwell plates in the presence or absence of 8-Br-cAMP, and the amount of cells that adhered after a certain period of time was quantified. We observed that 8-Br-cAMP augmented cell adhesion and activated Rap1 in a concentration-dependent manner to fibronectin (EC50, \(11601\) \(0.2–0.5\) mM) (Fig. 1, A and B). Rap1 was activated rapidly and remained active for at least 3 h. 8-Br-cAMP–induced adhesion was also observed using a different promoter (thymidine kinase [TK]–luciferase) driving luciferase expression and a direct method of measuring adhesion by counting cells (unpublished data). Cell adhesion induced by cAMP was insensitive to the PKA inhibitor H-89 when cells were pretreated for a short time just before adhesion (Fig. 1 C, Short). It has been reported that detachment of cells rapidly and transiently activates PKA, one of the well-established targets of cAMP (Howe and Juliano, 2000), raising the possibility that if a potential PKA substrate with a sustained phosphorylation profile was involved, addition of H-89 at a later time (post-PKA activation) may falsely imply a PKA-independent mechanism. However, when cells were treated with H-89 before trypsinization and throughout the recovery period, we found that cAMP-induced adhesion was not blocked (Fig. 1 C, Long), indicating that indeed PKA was not involved. As a control for H-89 activity, we measured cAMP-induced phosphorylation of the direct PKA target CREB (Gonzalez and Montminy, 1989) and ERK, which is also PKA-dependent (Fig. 1 D). Activation of Rap1, which is independent of PKA (de Rooij et al., 1998; Kawasaki et al., 1998; Enserink et al., 2002) was measured also. From these results we conclude that in Ovcar3 cells cAMP can induce cell adhesion to fibronectin independently of PKA.

Our finding that the induction of integrin-mediated cell adhesion by cAMP is independent of PKA suggested that Epac-Rap1 might be mediating this effect. To further test this idea, Ovcar3 cells were transiently transfected with Epac1. This resulted in an increase in basal adhesion to fibronectin, which was further increased by stimulation with 8-Br-cAMP (Fig. 2 A), suggesting that Epac mediates cAMP-
induced cell adhesion. This observation was further strengthened by the introduction of Rap1GAPII, an inhibitor of Rap1 (Mochizuki et al., 1999), which attenuated Epac-induced cell adhesion (Fig. 2 A). These results show that ectopic expression of Epac is sufficient to induce Rap1-dependent cell adhesion to fibronectin, which can be enhanced by additional stimulation with cAMP. It should be noted that although Rap1GAPII is more effective on Rap1 than on Rap2, we cannot exclude a role for Rap2 in this process.

To formally exclude the possibility that cAMP and Epac are on parallel pathways, both of which would be required for the induction of cell adhesion, we used a newly characterized analogue of cAMP, 8CPT-2Me-cAMP, which specifically activates Epac but not PKA even at high concentrations (Enserink et al., 2002). As observed with 8-Br-cAMP, stimulation of Epac1-transfected cells with 8CPT-2Me-cAMP further increased cell adhesion to fibronectin (Fig. 2 B) and raised Rap1GTP levels (Fig. 2 C). Expression of Rap1GAPII inhibited adhesion of cells to fibronectin and completely abolished Rap1GTP levels (Fig. 2, B and C), indicating that Rap1 is critically involved in cAMP-induced cell adhesion.

We next investigated whether activation of endogenous Epac is sufficient to induce adhesion to fibronectin. Ovcar3 cells were treated with 8CPT-2Me-cAMP to activate endogenous Epac, which is abundantly expressed in ovary tissue...
motif bind to peptide. Peptides containing the RGD amino acid sequence were used in this study. The concentration of RGD peptides was determined by the activity of PKA and Epac1-Rap1 signaling cascades resulting in elevation of intracellular cAMP levels and subsequent activation of PKA and Epac1-Rap1 signaling cascades. The percentage of adherent cells was quantified and plotted relative to unstimulated cells (range from 2–10%). Summarizing data of four (for the left half of the plot) and two (for the right half of the plot) independent experiments performed in triplicate are shown with error bars representing SD.

(A) Isoproterenol induces adhesion to fibronectin. Ovcar3 cells transiently transfected with CMV-luciferase plasmid were treated with increasing concentrations of isoproterenol, and cells adhering to fibronectin (2 μg/ml) were quantified as described in Materials and methods. (B) Isoproterenol induces activation of Rap1 and CREB. (Top) Ovcar3 cells were treated with increasing concentrations of isoproterenol for 5 min. Cells were lysed, and equal amounts of cell lysate were analyzed for activation of Rap1 (top) and CREB (bottom). Total levels of Rap1 in cell lysates are shown (middle blot). (Bottom) Cells were treated with 10 μM of isoproterenol for the indicated times. Cells were lysed, and equal amounts of cell lysate were analyzed for activation of Rap1 (top blot) and CREB (bottom blot). Total levels of Rap1 in cell lysates are shown (middle blot). (C) Isoproterenol-induced adhesion to fibronectin is independent of PKA. (Top) Ovcar3 cells were pretreated with H-89 as described in the legend to Fig. 1 C and seeded onto wells in the absence or presence of isoproterenol (100 μM). Cells were allowed to adhere for 1 h, and nonadherent cells were removed. The percentage of adherent cells was quantified and plotted relative to unstimulated, mock-transfected cells (range from 5–15%). Summarizing data of four (for the left half of the plot) and two (for the right half of the plot) independent experiments performed in triplicate are shown with error bars representing SD.

Figure 4. Stimulation of the β2-AR with isoproterenol induces cell adhesion.

(A) Isoproterenol induces adhesion to fibronectin. Ovcar3 cells transiently transfected with CMV-luciferase plasmid were treated with increasing concentrations of the β2-AR agonist isoproterenol, and cells adhering to fibronectin (2 μg/ml) were quantified as described in Materials and methods. (B) Isoproterenol induces activation of Rap1 and CREB. (Top) Ovcar3 cells were treated with increasing concentrations of isoproterenol for 5 min. Cells were lysed, and equal amounts of cell lysate were analyzed for activation of Rap1 (top) and CREB (bottom). Total levels of Rap1 in cell lysates are shown (middle blot). (Bottom) Cells were treated with 10 μM of isoproterenol for the indicated times. Cells were lysed, and equal amounts of cell lysate were analyzed for activation of Rap1 (top blot) and CREB (bottom blot). Total levels of Rap1 in cell lysates are shown (middle blot). (C) Isoproterenol-induced adhesion to fibronectin is independent of PKA. (Top) Ovcar3 cells were pretreated with H-89 as described in the legend to Fig. 1 C and seeded onto wells in the absence or presence of isoproterenol (100 μM). Cells were allowed to adhere for 1 h, and nonadherent cells were removed. The percentage of adherent cells was quantified and plotted relative to unstimulated, mock-transfected cells (range from 5–15%). Summarizing data of four (for the left half of the plot) and two (for the right half of the plot) independent experiments performed in triplicate are shown with error bars representing SD.

experiments, each in triplicate. Error bars represent SD. (Bottom) Cells were pretreated with either DMSO or H-89 for 30 min before trypsinization and during the recovery period (DMSO and long H-89 treatment, respectively) or during the last 30 min of recovery (short H-89 treatment). Then cells were stimulated with either 50 μM 8CPT-2Me-cAMP for 10 min or isoproterenol for 2 min, respectively. Cells were centrifuged, cell pellets were lysed, and equal amounts of cell lysate were incubated with precoupled GST-RalGDS-RBD, and activation of Rap1 was analyzed on Western blot using a Rap1 antibody. (D) Isoproterenol-induced adhesion to fibronectin is inhibited by Rap1GAPs or RBD of RalGDS. Isoproterenol-treated cells were transfected with either mock DNA (Vector) or HA-Rap1GapII alone or in combination with a β2-AR expression vector. Adhesion of cells to fibronectin in the absence or presence of isoproterenol was quantified. The percentage of adherent cells was plotted relative to unstimulated, mock-transfected cells (range from 5–15%). Summarizing data of four (for the left half of the plot) and two (for the right half of the plot) independent experiments performed in triplicate are shown with error bars representing SD.

(Kawasaki et al., 1998). Indeed, 8CPT-2Me-cAMP significantly induced cell adhesion to fibronectin (Fig. 2 D). To investigate whether cAMP-induced cell adhesion is indeed mediated by integrins, we pretreated Ovcar3 cells with the β1-integrin–binding arginine, glycine, aspartic acid (RGD) peptide. Peptides containing the RGD amino acid sequence motif bind to β1 integrins and have been shown to block fibronectin binding in ovarian carcinoma cells (Buczko-Thomas et al., 1998). As expected, 8CPT-2Me-cAMP–induced attachment to fibronectin was abolished (Fig. 2 D). 8CPT-2Me-cAMP did not increase the integrin-independent adhesion of Ovcar3 cells to poly-1-lysine (Fig. 2 E). From these results we conclude that activation of endogenous Epac induces integrin-mediated cell adhesion to fibronectin.

8CPT-2Me-cAMP enhanced cell adhesion to fibronectin and induced Rap1 activation at comparable concentrations (EC50, ~30 μM) (Fig. 3 A). In a time-course analysis, we noted that increased adhesion was already observed after 30 min, which correlated with a rapid and sustained Rap1 activation (Fig. 3 B). As expected, the induction of adhesion and activation of Rap1 were insensitive to the PKA inhibitor H-89 (Fig. 3 C). However, even low levels of Rap1GAPII completely inhibited cAMP-induced adhesion of Ovcar3 cells to fibronectin (Fig. 3 D, left plot). Furthermore, the Rap1-inhibitory proteins Rap1GAPI and Ras-binding domain (RBD) of Ras guanine nucleotide dissociation stimulator (RalGDS) (Reedquist et al., 2000) also inhibited adhesion to fibronectin (Fig. 3 D, left plot). Transfection of cells with Rap1GAPs or RBD of RalGDS did not affect luciferase expression (Fig. 3 D, right plot).

Our observations that cAMP analogs could induce adhesion of Ovcar3 cells to fibronectin prompted us to test whether cAMP-elevating receptors could also mirror the same effect, thereby linking an in vivo cAMP signaling system to integrin activation. The β2-adrenergic receptor (β2-AR) couples to Gs, type of heterotrimeric G proteins, resulting in elevation of intracellular cAMP levels and subsequent activation of PKA and Epac1-Rap1 signaling cascades (Marinissen and Gutkind, 2001; Neves et al., 2002). Ovcar3 cells endogenously express the β2-AR and stimulation with isoproterenol, a ligand for the β2-AR receptor, significantly increased adhesion to fibronectin (Fig. 4 A). Treatment with isoproterenol also induced both activation of Rap1 and phosphorylation of CREB (Fig. 4 B). Treatment with isoproterenol and adhesion, ~0.05 μM). Isoproterenol-induced adhesion was insensitive to short pretreatments with H-89 (Fig. 4 C, Short) but was partially inhibited when exposed very early to H-89 (Fig. 4 C, Long). Therefore, we looked at
activation of Rap1 under similar conditions. We observed that after early (Fig. 4 C, Long) pretreatment with H-89, isoproterenol-induced Rap1 activation was clearly inhibited, whereas 8CPT-2Me-cAMP–induced Rap1 activation was not (Fig. 4 C, bottom). Since both 8-Br-cAMP–induced and 8CPT-2Me-cAMP–induced adhesion were not blocked by H-89 (Fig. 1 C and Fig. 3 C), the effect of very early treatment of H-89 on β2-AR signaling could likely be attributed to slow recovery and expression of the β2-AR on the cell surface. This possibility is consistent with the observation that PKA is involved in vesicle fusion (Morgan et al., 1993). Transient transfection of Ovcar3 cells with the β2-AR receptor further enhanced the isoproterenol-induced adhesion to fibronectin, which was sensitive to the Rap1-inactivating protein, Rap1GAP (Fig. 4 D), showing a critical involvement of Rap1.

Our results demonstrate a clear connection between cell surface receptors that induce cAMP, cAMP signaling, and integrin-mediated cell adhesion and show that this pathway is independent of PKA but mediated by the cAMP target Epac and the small GTPase Rap1. This conclusion is based on the observations that isoproterenol, which raises cAMP levels through activation of endogenous β2-AR, is able to induce integrin-mediated cell adhesion to fibronectin in a Rap1-dependent, PKA-independent manner. Furthermore, importantly, a cAMP analogue that specifically activates Epac but not PKA is also able to induce cell adhesion. However, our results do not entirely exclude a role for PKA in this process. Both Rap1 and Rap1GAP are substrates for PKA (Bos et al., 2002), whereas in megakaryocytes Rap1 increases the affinity of αIIbβ3 (Bertoni et al., 2002). In addition, Rap1 is required for the direct activation of integrins by integrin-activating antibodies or manganese ions (de Bruyn et al., 2002). Apparently, Rap1 modulates a process before integrin activation, for instance, the recruitment of an essential cofactor. Interestingly, it has been reported recently that Rap1 is essential in the formation of adherens junctions, though it is less clear whether the process involves integrin-mediated signaling (Knox and Brown, 2002).

Materials and methods

Cells, plasmids, and transfections

NIH-OVCA3 (Ovcar3) cells were maintained at 37°C in RPMI 1640 supplemented with 10% heat-inactivated (30 min at 56°C) FBS and 0.05% glutamine in the presence of penicillin and streptomycin. Hemagglutinin (HA)-tagged constructs of Epac1 and Rap1GAP in the PM2HA expression vector have been described previously (de Rooij et al., 1998; de Bruyn et al., 2002). Transient transfection of Ovca3 cells was performed using the FuGENE 6 transfection reagent (Roche Diagnostics Corporation) according to the manufacturer’s procedures using 6 μg total DNA including either a Tk-luciferase plasmid (1 μg) or CMV-luciferase plasmid (0.2 μg) as indicated. Cells were starved at least 16 h before stimulation.

Reagents

Western blotting of protein samples was performed using polyvinylidene difluoride membranes. Antibodies against dual phosphorylated p42/44MAPK and phosphorylated CREB (directed against phosphorylated Ser 133) were obtained from Cell Signaling, and antibodies against K-Ras/Rap1 and polyclonal anti-HA were obtained from Santa Cruz Biotechnology, Inc. The following inhibitor and stimuli were used at the indicated concentrations: RGD peptide (100 μM) and H-89 (10 μM), obtained from Biomol Research Laboratories Inc. (Plymouth Meeting, Isoproterenol (10 μM, unless indicated otherwise) was obtained from Sigma-Aldrich and 8-Br-cAMP (10 μM, unless indicated otherwise) and 8CPT-2Me-cAMP (100 μM, unless indicated otherwise) was obtained from Biolog Life Science Institute.

Adhesion assay

24-well plates were coated overnight with fibronectin (Sigma-Aldrich; 1–5 μg/ml as indicated) in sodium bicarbonate buffer (Sigma-Aldrich). Poly-L-lysine was coated for 2 h at RT (0.1% wt/vol in water), washed twice with water, and dried overnight. Plates were washed in TSM buffer (20 mM Tris-phosphate, pH 7.8, 8 mM MgCl2, 1 mM DTT) at 4°C for 1.5–2 h with gentle rotation in suspension. Plates were centrifuged, counted, and resuspended at 3 × 105 cells/ml in serum-free RPMI with 0.5% BSA. The experiment was performed in triplicates, and to each well 150 μl of cells was added to 150 μl of medium with or without stimulus. In studies with H-89 (10 μM), cells were either preincubated at 37°C for 30 min with the inhibitor before seeding the wells (short pretreatment), or H-89 was added before trypsinization, during the recovery period, and before seeding wells (long pretreatment). Cells were allowed to adhere for 1 h at 37°C, and non-adherent cells were removed by gently washing plates three times with warmed 0.5% BSA/TSM. Adherent cells were lysed in luciferase lysis buffer (15% glycerol, 25 mM Tris-phosphate, pH 7.8, 1% Triton X-100, 8 mM MgCl2, 1 mM DTT) at 4°C for 30 min, and units of luciferase activity were quantified with addition of equal volume of luciferase assay buffer (25 mM Tris-phosphate, pH 7.8, 8 mM MgCl2, 1 mM DTT, 1 mM ATP, pH 7, 1 mM luciferin) using a luminometer (Lumat LB9507; Berthold Technologies). Unseeded cells (150 μl) were lysed separately to determine lu-
ciferase counts in the total input cells. Specific adhesion (%) was determined (counts in cells bound/counts in total input x 100) and plotted either directly or relative to the basal adhesion of HA vector-transfected cells. Error bars represent average deviation among experiments, and where representative experiments are depicted error bars represent average SD within each experiment. The expression of transfected constructs was confirmed by immunoblotting of total cell lysates.

**Rap1 activation assay and phosphorylation of ERK and CREB**

Rap1 activation assays were performed as described previously (Franke et al., 1997; van Triest et al., 2001). Briefly, adherent cells (unless stated otherwise) were serum starved overnight, treated, and lysed in 750 μl lysis buffer (10% glycerol, 1% Nonidet P-40, 50 mM Tris-Cl, pH 7.5, 200 mM NaCl, 2 mM MgCl2, 1 mM leupeptin, 0.1 mM aprotinin, 5 mM NaF, 1 mM NaVO3). Lysates were centrifuged, and 30 μl of lysate was incubated with GST-tagged RBD of RaGLDS precoupled to glutathione beads to specifically pull down the GTP-bound forms of Rap1. Samples were incubated for 1 h at 4°C while tumbling. Beads were washed four times in lysis buffer, and remaining fluid was removed with an insulin syringe. Proteins were eluted with Laemmli sample buffer and analyzed by SDS-PAGE and Western blotting using Rap1 antibodies (Santa Cruz Biotechnology, Inc.). To 100 μl of 1% sodium dodecyl sulfate, 5 μl of Laemmli sample buffer was added, and phosphorylation of ERKs was analyzed by Western technology, using the phospho-specific antibody against p42/44MAPK. Phosphate buffer was added, and phosphorylation of ERKs was analyzed by Western blotting using Rap1 antibodies (Santa Cruz Biotechnology, Inc.). Proteins were eluted with Laemmli sample buffer and analyzed by SDS-PAGE and Western blotting using Rap1 antibodies (Santa Cruz Biotechnology, Inc.). To 100 μl of 1% sodium dodecyl sulfate, 5 μl of Laemmli sample buffer was added, and phosphorylation of ERKs was analyzed by Western blotting using the phospho-specific antibody against p42/44MAPK. Phosphorylation of CREB was analyzed by Western blotting using a phospho-specific antibody directed against phosphorylated Ser133.

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