The cAMP-Epac-Rap1 pathway regulates cell spreading and cell adhesion to laminin-5 through the $\beta 3\beta 1$ integrin but not the $\beta 6\beta 4$ integrin

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Abstract

Laminin-5 is an important constituent of the basal lamina. The receptors for laminin-5, the integrins β3β1 and β6β4, have been associated with epithelial wound migration and carcinoma invasion. The signal transduction mechanisms that regulate these integrins are not well understood. We report here that the small GTPase Rap1 regulates the adhesion of a number of cell lines to various extracellular matrix proteins including laminin-5. cAMP also mediates cell adhesion and spreading on laminin-5, a process that is independent of protein kinase A but rather dependent on Epac1, a cAMP-dependent exchange factor for Rap. Interestingly, while both β3β1 and β6β4 mediate adhesion to laminin-5, only β3β1-dependent adhesion is dependent on Rap1. These results provide evidence for a function of the cAMP-Epac-Rap1 pathway in cell adhesion and spreading on different extracellular matrix proteins. They also define different roles for the laminin-binding integrins in regulated cell adhesion and subsequent cell spreading.
**Introduction**

Laminins are extracellular matrix proteins important for epithelial cell migration and adhesion. Laminin-5 (Ln-5), a dynamic component of the basement membrane, is a ligand for integrins β3β1 and β6β4 (1). β3β1 couples to the actin cytoskeleton, and its function in epithelial cell adhesion and migration is established (2). Recently, β6β4 has received much attention, since it was found to function as a signaling adaptor for growth factor-induced invasive growth (3-5). In contrast to all other integrins, β6β4 couples to the intermediate filament cytoskeleton, and its regulation is likely to be different from other integrins. β6β4 is a major constituent of hemidesmosomes (6-8), but in aggressive, late-stage tumors, β6β4 relocalizes from hemidesmosomes to membrane protrusions associated with cell migration (9,10). The upstream factors that regulate adhesion and migration to Ln-5 are not well understood, although cAMP has been shown to regulate the small GTPases RhoA and Rac in a protein kinase A (PKA)-dependent manner, thereby affecting cell migration on Ln-5 (for a review see (11)).

CAMP is a common second messenger that regulates many cellular processes. Until recently, PKA was thought to be the main target of cAMP in eukaryotic cells. However, exchange factor directly activated by cAMP (Epac), a widely expressed exchange factor for the small GTPases Rap1 and Rap2, has been shown to be a receptor for cAMP as well (12,13). Importantly, Epac controls a number of cellular processes previously attributed to PKA (for a review, see (14)). Rap1 has recently attracted much attention, as it was shown to be involved in the regulation of cell adhesion in a variety of cell types (for reviews see (15,16)). cAMP also controls cell adhesion in many cell types, and recently a link between cAMP, Epac-Rap1, and regulation of cell adhesion has been

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established (17). Furthermore, camp is known to control cell spreading, which is thus far thought to be mediated by PKA (18).

Although cAMP has previously been shown to regulate specific integrin functions on Ln-5 in a PKA-dependent manner, the involvement of the Epac-Rap1 pathway has not been appreciated in these studies. Therefore, the goal of this research was to test the role of the cAMP-Epac-Rap1 pathway in the regulation of adhesion to Ln-5. Furthermore, since β3β1 and β6β4 are regulated by two separate mechanisms, close investigation of Rap1-mediated adhesion to Ln-5 provides an excellent opportunity to gain more insight into how Rap1 may regulate integrins in general. Here we present evidence that cAMP acts as an upstream regulator of cell adhesion and cell spreading to Ln-5 through an Epac-Rap1-dependent pathway. Importantly, we found that while both β3β1 and β6β4 mediate adhesion to Ln-5, only β3β1-dependent adhesion is dependent on Rap1, showing that Rap1 specifically regulates β3β1 and not β6β4. This suggests that Rap1 exclusively regulates factors that couple to the actin cytoskeleton, without affecting the intermediate filament cytoskeleton. In addition, these results define different roles for the laminin-binding integrins in cAMP-regulated cell adhesion and subsequent cell spreading.
**Materials and Methods**

*Cell culture, constructs, transfections* - 804G and HaCaT cells were maintained in DMEM (4.5 g/liter glucose), and K562 cells, NIH3T3:Ovcar-3 cells and HEK293T cells were maintained in RPMI. All cell media were supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 50 units/ml penicillin and 50 units/ml streptomycin. Cells were transfected with cDNAs for CMV-luciferase (17), or HA-tagged cDNAs in the pMT2-HA expression vector for Rap1V12 (19), Epac1 (13), PDZ-GEF1 (20), or Rap1GAPI (21), using the FuGENE6 transfection reagent (Roche Diagnostics Corporation) according to the manufacturer’s procedures, whereas K562 cells were transfected by electroporation.

*Antibodies and Reagents* – Monoclonal antibodies against HA were from Babco, phospho-specific polyclonal antibodies against CREB were from Cell Signaling, function-blocking β3 antibodies (P1B5, 10 µg/ml) were from CHEMICON International, function-blocking antibodies against β6 integrin (GoH3, 10 µg/ml) were from BD Biosciences, function-blocking antibodies against β1 integrin (AIIB2) were kindly obtained from Dr. C.H. Damsky (UCSF, San Francisco, CA), and Rap1 antibodies were from Santa Cruz Biotechnology, Inc. H-89 (10 µM) and forskolin (10 µM) were from Calbiochem, isoproterenol (10 µM) was from Sigma, 8CPT-2Me-cAMP (8-(4-chlorophenylthio)-2-O-methyladenosine-3,5 cyclic monophosphate) (50 µM) was from Biolog Life Science Institute, and phalloidin-TRITC (50 µg/ml) was obtained from Sigma-Aldrich.

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Extracellular matrix preparation – All cell adhesion assays – except K562 cells – were carried out using the laminin-5-rich matrix of 804G cells, which was prepared as described previously (22). Briefly, 804G cells were plated on 6-well plates or 24-well plates (Costar) and allowed to adhere overnight. After removal of the culture medium, the cells were washed in PBS, and removed from their matrix by treatment with 20 mM NH₄OH for 10 minutes at room temperature. The plates were then washed four times with PBS, and complete removal of any residual cells was confirmed by microscopy. Alternatively, plates were coated overnight at 4°C with conditioned medium from 804G cells, yielding identical results. Plates were blocked with serum-free DMEM containing 20 mM Hepes pH 7.4 and 1% fatty acid-free bovine serum albumin (BSA) for 1 hour at 4°C. K562 cell adhesion assays were performed using the laminin-5 matrix of Rac-11P/SD cells as described previously (23). Briefly, cells were grown in 96-well tissue culture plates for 2 days until confluent. Cells were then detached with 10 mM EDTA overnight at 4°C. Resulting laminin-5-coated wells were washed 3 times with PBS and checked under the microscope to ensure that all cells had been removed. Coated wells were then blocked with 1% heat-denatured BSA for 1 hour at 37°C. Fibronectin matrix (5 μg/ml) was prepared as previously described (17).

Adhesion assays – Adhesion assays were performed in triplicates as described previously (17,24), with minor modifications. Briefly, as indicated, transfected or untransfected adherent cells were trypsinized and recovered in serum-free DMEM containing 20 mM Hepes pH 7.4 and 1% fatty acid-free BSA for 1.5 hours at 37°C while rocking gently, to allow re-expression of cell surface markers. Serum-starved K562 cells, which grow in

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Rap1 regulates β3β1 and not β6β4 suspension, were not trypsinized but centrifuged and resuspended at a concentration of 1 x 10^6 cells ml\(^{-1}\) and rolled gently at 37\(^0\)C for one hour. In studies with H-89 (10 µM), cells were pre-incubated with the inhibitor during the final 40 minutes of the recovery period, whereas stimuli like forskolin, 8CPT-2Me-cAMP, isoproterenol, and PMA were added during the final 10 minutes of the recovery period. After the recovery period, cells (2.5 x 10^4 per well for 24-well plates and 10 x 10^4 per well for 6-well plates in case of adherent cells; 5 x 10^5 cells per well of 96-well plates (Nunc Maxisorp) in case of K562 cells) were directly transferred to plates. Cells were allowed to adhere at 37\(^0\)C for either 10 minutes or as indicated otherwise. Subsequently, non-adherent cells were removed by washing gently but extensively with wash buffer (PBS with 1.8 mM CaCl\(_2\) and 2 mM MgCl\(_2\)) five times. Cells were then lysed in luciferase lysis buffer (15% glycerol, 25 mM Tris-phosphate pH 7.8, Triton X-100 1%, 8 mM MgCl\(_2\), 1 mM DTT), and luciferase activity (light units) was quantified with addition of an equal volume of luciferase assay buffer (25 mM Tris-phosphate, pH 7.8, 8 mM MgCl\(_2\), 1 mM DTT, 1 mM ATP, pH 7.1 mM luciferin) using a luminometer. Unseeded cells were also measured to determine luciferase activity in the total input of cells. Expression of transfected cDNAs was confirmed by Western blotting. Specific adhesion (%) was determined (light units in cells bound/light units in total input x 100) and plotted relative to the basal adhesion of untreated, HA vector–transfected cells. Alternatively, cell adhesion was measured as follows. After washing the tissue culture plates with PBS, the cells were fixed in 4% formaldehde in PBS for 30 minutes at 4\(^0\)C. Cells were then permeabilized and blocked in ice-cold TBST (Tris 50 mM pH 7.5, NaCl 150 mM, Triton X-100 0.5%, glycine 100 mM, and fatty acid-free BSA 1%) for 30 minutes. Subsequently, cells were incubated
with phalloidin-TRITC overnight at 4°C, washed with TBST, mounted in fluorescence mounting medium (DAKO), and photographed at 100x magnification using a CCD camera mounted on an inverted fluorescence microscope (Olympus IX 81, fitted for tissue culture dishes), after which cells were counted directly.

Cell spreading assay – As indicated, cells were not transfected or transfected with cDNAs for Rap1GAPI or Rap1V12. GFP was co-transfected in order to visualize transfected cells. After 24 hours, cells were trypsinized and recovered in serum-free DMEM containing Hepes 20 mM pH 7.4 and 1% fatty acid-free BSA for 2 hours at 37°C while rocking gently, to allow re-expression of cell surface markers. In studies with H-89 (10 µM) or function-blocking antibodies, cells were pre-incubated during the final 40 minutes of the recovery period, whereas stimuli like forskolin, 8CPT-2Me-cAMP, and isoproterenol were added during the final 10 minutes of the recovery period. Cells (2.5 x 10^4 per well in case of 24-well plates) were then directly transferred to plates and allowed to adhere at 37°C on Ln-5 (15 minutes), or fibronectin (30 minutes), and experiments were carried out in triplicates. After washing the tissue plates with PBS, the cells were fixed in 4% formaldehyde in PBS for 30 minutes at 4°C. Cells were then permeabilized and blocked in ice-cold TBST (Tris 50 mM pH 7.5, NaCl 150 mM, Triton X-100 0.5%, glycine 100 mM, and fatty acid-free BSA 1%) for 30 minutes. Subsequently, cells were incubated with phalloidin-TRITC overnight at 4°C, washed with TBST, mounted in fluorescence mounting medium (DAKO), and imaged at 400x magnification using a cooled CD camera mounted on an inverted fluorescence microscope (Olympus IX 81) fitted for tissue culture dishes. The total surface covered by cells (at least 33 cells per

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well, and a total of 3 wells per transfection) was plotted relative to the surface covered by untreated cells. Error bars represent average deviation among experiments. Alternatively, and as indicated in the figure legends, the percentage of spread cells (i.e. cells that had become flattened with their total diameter more than twice the diameter of the nucleus) per microscopic field (4 different fields per treatment) was determined rather than the total cell surface.

**FACS analysis**

Ovcar-3 cells were grown to ~70% confluency, trypsinized, and recovered in serum-free DMEM containing Hepes 20 mM pH 7.4 and 1% fatty acid-free BSA for 2 hours at 37°C while rocking gently. During the last 30 min cells were treated with 100 μM 8CPT-2Me-cAMP or left untreated. Subsequently, cells were washed with PBS, fixed in 4% formaldehyde in PBS for 30 minutes at 4°C, washed with PBS, and incubated with 10 μl FITC-conjugated antibodies against β3 integrin (CD49c, Chemicon).

**Rap1 activation assay and phosphorylation of CREB**- Rap1 activation assays were performed as described previously (25,26). Briefly, adherent cells were stimulated as indicated 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, 5 mM NaF, 1 mM NaVO3, and 1 mM PMSF). Lysates were clarified by centrifugation, and 500 μl of lysate was incubated with GST-tagged RBD of RalGDS pre-coupled 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

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insulin syringe. Proteins were eluted with Laemmlie sample buffer and analyzed by SDS-PAGE and Western blotting using Rap1 antibodies. To 100 μl of clarified lysate 30 μl 4x Laemmli sample buffer was added, and phosphorylation of CREB was analyzed by Western blotting using a phospho-specific antibody directed against phosphorylated Ser\textsuperscript{133}. 

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Results

Rap1 induces adhesion to Ln-5

To investigate whether Rap1 is able to induce adhesion to Ln-5, we used 804G rat bladder carcinoma cells, which are known to adhere to Ln-5 (27). Cells were transfected with luciferase cDNA under control of a CMV promoter in combination with either Rap1V12, an activated mutant of Rap1 that is preferentially GTP-bound, or with Rap1GAPI, an inhibitor of Rap1. After detachment with trypsin-EDTA, the cells were plated on a Ln-5 matrix and adhesion was quantified. As shown in Fig. 1A, Rap1V12 promoted adhesion of cells to Ln-5, whereas transfection with Rap1GAPI had no stimulatory effect. Expression of Rap1V12 and RapGAPI is shown in Fig. 1B. To test whether activation of endogenous Rap1 also induces adhesion of cells to Ln-5, we transiently transfected HaCaT keratinocyte cells, which also express β3β1 and β6β4, and 804G cells with the exchange factor Epac1, a specific activator of the family of Rap small GTPases which does not activate any other small GTPase (12,13). Transient transfection of cells with Epac1 cDNA has been shown previously to activate endogenous Rap1, even in absence of stimuli that raise intracellular cAMP levels (17,28). As shown in Fig. 1C, Epac1-expressing cells adhered better to a Ln-5 matrix than mock transfected cells. Activation of endogenous Rap1 and expression of Epac1 is shown in Fig. 1D. PDZ-GEFI is another exchange factor that does not activate any other small GTPase than the GTPases of the Rap family (20,29). Overexpression of PDZ-GEF in 804G cells also increased adhesion to Ln-5, whereas Rap1GAPI had a slight inhibitory effect (Fig. 1E). Activation of endogenous Rap1 and expression of the transgenes is shown in Fig. 1F. Interestingly, HEK293T cells, which only express β3β1 and not β6β4, also adhered

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better to Ln-5 upon overexpression of Epac1 (Fig. 1G), which was inhibited by co-expression of the inhibitor of Rap1, Rap1GAPI. Rap1-GTP levels are shown in Fig. 1H. Taken together, these results show that activation of endogenous Rap1 results in stimulation of cell adhesion to Ln-5 in a variety of cell lines, and indicate that Rap1 may specifically regulate β3β1.

**cAMP promotes cell adhesion to Ln-5 through Epac**

Cyclic AMP is a versatile second messenger implicated in numerous cellular processes, many of which are believed to be carried out by PKA. However, some of the effects of cAMP have been shown to be mediated by the Epac1 signaling pathway rather than the PKA pathway, like calcium release, insulin secretion, and secretion of sAPPalpha (30-32). In addition, cAMP has recently been reported to induce adhesion of Ovcar-3 cells to fibronectin (FN) through the Epac-Rap1 pathway (17). Ovcar-3 cells originate from a human ovarian carcinoma, and have been reported to express both β3β1 and β6β4 and to adhere to laminins (33). All this makes the Ovcar-3 cell line is an ideal cell line to study the role of cAMP in adhesion of cells to Ln-5. As shown in Fig. 2A, treatment of Ovcar-3 cells with the cAMP-elevating agent forskolin, a direct activator of adenylate cyclase, resulted in increased adhesion to Ln-5, which was resistant to the PKA inhibitor H-89. Importantly, treatment with isoproterenol, an agonist of the Gβγ-coupled β2-adrenergic receptor, also resulted in increased cell adhesion (Fig. 2A), indicating that physiologically relevant stimuli also regulate adhesion to Ln-5. The finding that cAMP-induced cell adhesion was resistant to H-89 indicates that the effect of cAMP on cell adhesion is mediated by the Epac pathway rather than the PKA pathway. Therefore, we

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made use of a recently described Epac-specific cAMP analog, 8CPT-2Me-cAMP, which does not activate PKA even at high concentrations or long incubation times (21). 8CPT-2Me-cAMP has previously been used to rapidly and very specifically ‘switch on’ the endogenous Epac-Rap1 pathway, and has been established as a tool for studying Epac signaling in a wide range of cellular processes (17,30,34-36). As shown in Fig. 2B (upper panel), 8CPT-2Me-cAMP rapidly activates Rap1 in Ovcar-3 cells, which is not affected by H-89, confirming that cAMP-induced Rap1 activation is mediated by the Epac pathway. The same results were obtained with forskolin and isoproterenol (Fig. 2B, upper panel). As a control, phosphorylation of CREB, a direct target of PKA (37), was not affected by 8CPT-2Me-cAMP, whereas forskolin and isoproterenol clearly induced PKA-dependent phosphorylation of CREB (Fig. 2B, lower panel). Equal loading was confirmed by probing total cell lysates with Rap1 antibodies (Fig. 2B, middle panel). Importantly, as observed for forskolin and isoproterenol, stimulation of Ovcar-3 cells with 8CPT-2Me-cAMP resulted in increased adhesion to Ln-5 (Fig. 2C), indicating the involvement of the Epac-Rap1 pathway. To confirm whether cAMP-induced cell adhesion to Ln-5 is indeed mediated by Rap1, we transfected cells with Rap1GAPI and performed an adhesion assay. As shown in Fig. 2D, over-expression of Rap1GAPI completely blocks forskolin-induced cell adhesion. Finally, we observed that Ovcar-3 cells undergo extensive cytoskeletal rearrangements upon treatment with forskolin, resulting in increased cell spreading (see below, Fig. 4). In order to test whether the increase in cell spreading is actually required for augmented cell adhesion, we performed an adhesion assay in which cells were only allowed to adhere for 5 min to an Ln-5 matrix. At this short time interval, forskolin-treated Ovcar-3 cells already adhered better
to a Ln-5 matrix than untreated cells (Fig. 2E, left). The forskolin-treated cells were still round at this timepoint, without having undergone the large cytoskeletal changes that results in cell spreading (Fig. 2E, right). The fact that the rapid forskolin-induced increase in cell adhesion precedes the increase in cell spreading suggests that extensive cytoskeletal rearrangements are not required for Rap1-induced augmented cell adhesion. This view is in line with previous reports that Rap1 can regulate integrin affinity and avidity (16). Taken together, these results show that cAMP induces rapid, Epac-Rap1-dependent cell adhesion of Ovcar-3 cells to Ln-5.

cAMP-induced cell adhesion to Ln-5 is mediated by β3β1

Since over-expression of Epac1 resulted in increased adhesion to Ln-5 of HEK293T cells, which lack β6β4, we hypothesized that Rap1 may regulate the β3β1 integrin rather than β6β4. Therefore, we performed an adhesion assay with Ovcar-3 cells (which, as mentioned above, express both β3β1 and β6β4) in the presence of function-blocking antibodies against β3 or β6 integrins. As shown in Fig. 3A, anti-β6 antibodies failed to influence forskolin-induced adhesion of Ovcar-3 cells to Ln-5, indicating that β6β4 is not required. In contrast, anti-β3 antibodies greatly impaired adhesion of Ovcar-3 cells to Ln-5 (Fig. 3A), whereas as a control adhesion to FN was not affected by antibodies against either β3 or β6 (data not shown). These results show that although both β3β1 and β6β4 integrins may mediate adhesion of Ovcar-3 cells to Ln-5, cAMP-induced cell adhesion to Ln-5 is specifically mediated by the β3β1 integrin. To further substantiate the finding that Rap1 regulates β1 integrins and not β4 integrins, we made use of human erythroleukemic K562 cells stably transfected with β6β4 (38). Importantly, Rap1 regulates β3β1 and not β6β4
these cells do not express β3β1, and therefore adhesion to Ln-5 is strictly mediated by β6β4 (23). First, we tested whether activation of Rap1 results in increased adhesion to FN, which is mediated by β1 integrins. Indeed, upon stimulation with the phorbol ester PMA, which is a strong stimulus for Rap1 (39), these cells rapidly adhered to FN. This was dependent on Rap1, since over-expression of Rap1GAPI strongly inhibited adhesion (Fig. 3B). As expected, this PMA-induced, Rap1-mediated induction of cell adhesion was almost completely blocked by function-blocking antibodies against β1 integrins, whereas as a control antibodies against β6 had no effect. In contrast, PMA did not enhance cell adhesion to Ln-5, and over-expression of Rap1GAPI had no effect on cell adhesion, showing that Rap1 does not regulate β6β4 (Fig. 3C). Rap1 has previously been shown to regulate the avidity and affinity, but not cell-surface expression of various integrins. As shown in Fig. 3D, Rap1 also does not increase the cell-surface expression of β3β1 integrins, indicating that the cAMP-induced increase in cell adhesion to Ln-5 is mediated by increased β3β1 avidity or affinity, or both, but not the number of integrins on the cell surface. Altogether, we conclude that Rap1-dependent cell adhesion to Ln-5 is mediated by β3β1 and is independent of the number of β3β1 integrins on the cell surface. Importantly, these data indicate that Rap1 specifically regulates β1 integrins and not β4 integrins.

cAMP potentiates cell spreading on Ln-5 through Epac

Rap1 has not only been implicated in cell adhesion, but also in cell spreading of a number of cell lines on FN or collagen I, including HEK293, B lymphocytes, and MEFs (40-42).

Cell spreading involves reorganization of the actin cytoskeleton, and the resulting Rap1 regulates β3β1 and not β6β4
flattening of the cell increases the contact region with the extracellular matrix, allowing stronger adhesion. To test whether Rap1 promotes spreading on a Ln-5 matrix, 804G cells were transiently transfected with either Rap1V12 or Rap1GAPI. GFP was cotransfected in order to visualize transfected cells. 24 hours after transfection cells were trypsinized and plated on a Ln-5 matrix for 15 minutes, fixed, and photographed. As shown in Fig. 4A, Rap1V12 potentiates cell spreading, whereas Rap1GAPI had no effect. Quantitative analysis showed that cells expressing Rap1V12 have a cell surface approximately twice the size of mock transfected cells (Fig. 4B). Previously, cAMP has been shown to induce cell spreading through PKA-dependent activation of Rac1 (18). We wanted to test whether cAMP might also influence cell spreading through the Epac-Rap1 pathway. Indeed, when left untreated, Ovcar-3 cells spreaded on a fibronectin matrix, but treatment with forskolin resulted in more rapid and more extensive cell spreading (Fig. 4C). This was not blocked by the PKA-inhibitor H-89, and it was mimicked by the Epac-specific cAMP analogue 8CPT-2Me-cAMP. Furthermore, treatment of Ovcar-3 cells with either forskolin or isoproterenol also promoted cell spreading on a Ln-5 matrix, which was not inhibited by H-89 (Fig. 4D), indicating that this also may be mediated by the Epac-Rap1 pathway. Indeed, treatment of cells with 8CPT-2Me-cAMP mimicked forskolin- and isoproterenol-enhanced cell spreading (Fig. 4E), showing that specific activation of the Epac pathway is sufficient to promote cell spreading. Since Rap1 mediates cell adhesion to Ln-5 specifically through β3β1, we wanted to test whether cell spreading on Ln-5 is also mediated by β3β1 rather than β6β4. Therefore, we pretreated cells with antibodies against either β3 or β6 integrins before performing a cell spreading assay. As shown in Fig. 4F, cAMP-induced cell spreading on Ln-5 was not altered by

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anti-β6 antibodies, whereas anti-β3 antibodies almost completely blocked cell spreading. Together, these results show that cAMP, through Epac and Rap1, promotes cell spreading on a variety of substrates. Importantly, cell spreading on Ln-5 is β3β1-dependent, indicating that Rap1 specifically regulates actin cytoskeleton-coupled β1 integrins and not β4 integrins.

Rap1 regulates β3β1 and not β6β4
Discussion

Our results reveal a role for the cAMP-Epac-Rap1 signaling pathway in cell adhesion on various substrates, including Ln-5, which is independent of PKA. Furthermore, we show for the first time that cAMP-induced cell spreading can also be regulated by the Epac-Rap1 pathway, which thus far was thought to be mediated by PKA, through activation of Rac (43). Finally, and in our eyes most importantly, our results indicate that Rap1 specifically regulates β3 integrins and not β4 integrins.

Various integrin functions on Ln-5 have previously been shown to be dependent on cAMP, PKA and the small GTPases RhoA and Rac (44,45). It is possible that cAMP utilizes both pathways to fine-tune its effects on cell adhesion and migration. Indeed, in addition to PKA, the Epac-Rap1 pathway has recently been shown to regulate Rac (31). Furthermore, PKA and Epac have opposing roles in activation of protein kinase B (PKB) in HEK293 cells (46). These findings indicate that PKA and Epac may share at least some effector proteins involved in cell adhesion and migration. It is not likely, however, that the effects of cAMP and Epac-Rap1 on cell adhesion and cell spreading described in this report are mediated by either Rac or PKB, since these proteins are not activated by cAMP in Ovcar-3 cells (JME, unpublished observations).

Rap1 regulates cell adhesion to a series of substrates, including fibronectin, fibrinogen, collagen, ICAM, and VCAM, through a wide variety of integrins, including β1β3, β4β1, β5β1, β6β2, and βMβ2 (16). Here we show that Rap1 also controls adhesion to Ln-5, and indicate that the β3β1 integrin can be added to this still growing list. Ovcar-3 cells have previously been shown to express both β3β1 and β6β4 and to adhere to laminins (33). The fact that cAMP-induced adhesion of Ovcar-3 cells to Ln-5 is Rap1 regulates β3β1 and not β6β4
blocked by antibodies against β3β1 integrins, whereas antibodies against β6β4 have no effect suggests that Epac and Rap1 specifically regulate the β3β1 integrin and not β6β4. Indeed, Rap1 fails to stimulate adhesion to Ln-5 in β6β4-expressing K562 cells, which lack β3β1. In contrast, β1-dependent adhesion to FN was clearly potentiated by Rap1. Therefore, our results show that Rap1 specifically regulates β3β1 and not β6β4.

β3β1 couples to the actin cytoskeleton, whereas β6β4 couples to the intermediate filament cytoskeleton and thus recruits different proteins than β3β1 (8). Regulation of β6β4 may therefore differ from other integrins. β1 integrins recruit proteins like talin, which in turn recruits vinculin and the Arp2/3 complex, which mediates actin polymerization. Reorganization of actin filaments in stress fibers increases integrin clustering and avidity (47). In contrast, β4 integrins interact with different proteins, like bullous pemphigoid antigen-1 and -2, and plectin (48). Therefore, we speculate that Rap1 recruits or increases the activity of one of the β1-binding proteins, thereby promoting actin polymerization, as has been reported previously for the β4 integrin (41), whereas it does not influence the activity of proteins recruited by the β4 integrin.

Rap1 has been shown to increase integrin avidity and affinity, but not cell surface expression of integrins (16). In line with these reports, we find that the cAMP-Epac-Rap1 pathway does not increase the cell surface expression of integrin β3β1, indicating that Rap1 probably influences integrin avidity or affinity, or both. Furthermore, our data show that the rapid cAMP-induced increase in cell adhesion precedes the increase in cell spreading, suggesting that extensive cytoskeletal rearrangements are not required for Rap1-induced cell adhesion. Indeed, in Jurkat T cells stably transfected with Epac1

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cDNA, treatment with cAMP results in increased cell adhesion, which is not blocked by inhibitors of the actin cytoskeleton (L.S.P., unpublished results). Therefore, although Rap1 stimulates cell spreading, the Rap1-mediated increase in cell adhesion seems to be independent of the actin cytoskeleton. Finally, Rap1 has recently been shown to regulate E-cadherins, and thereby cell-cell adhesion (49). Altogether, this suggests that Rap1 may act as a global regulator of cell adhesion, functioning relatively high in the hierarchy of a number of specific signalling pathways that control several aspects of cell adhesion. Unfortunately, the direct effectors of Rap1 remain unclear (15). Rap1 has been shown to bind to and activate B-Raf, resulting in activation of ERK, although this is still a matter of debate (21). Rap1 may also regulate the cell-cell adhesion molecule AF-6 (50), which is found in adherens junctions and which may be involved in regulation of cell adhesion (51). Furthermore, in thyroid cells Rap1 regulates PI3K signaling pathways (46,52,53), which may affect cell adhesion. Interestingly, in haematopoietic cells Rap1 has been shown to bind to RAPL, which in turn would bind directly to and activate LFA-1 integrins (54). However, it is unlikely that this protein also functions in Rap1-induced cell adhesion in fibroblastic and epidermal cells, as its expression pattern is almost exclusively restricted to haematopoietic cells and spleen (54). Furthermore, Rap1 has been shown to interact with the B4.1 domain of the cytoskeleton-associated protein Krit1 (55). Many integrin-regulating proteins contain such a B4.1 domain, including talin and FAK. Rap1 may directly interact with these proteins and thereby affect their activity. Indeed, in B cells, Rap activation results in increased phosphorylation of the FAK family member PYK2 (41). The possibility that Rap1 may directly interact with such B4.1 containing proteins is currently under investigation. Clearly, identification of the

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components of the Rap1 effector pathway that mediates cell adhesion remains a major challenge.

In summary, we find that cAMP regulates adhesion and cell spreading of a number of cell types to various substrates, including Ln-5. The fact that adhesion and spreading to Ln-5 is mediated by Epac and Rap1 rather than PKA provides a novel ‘inside-out’ regulatory mechanism for β3β1 adhesive properties. These results may have important consequences for our current understanding of the effect of a wide variety of cAMP-controlling hormones as for instance isoproterenol, SDF-1, serotonin, carbachol, or PGE2 on many integrin-mediated cellular functions, like wound healing, migration, axon guidance, and signaling.

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Abbreviations

cAMP, cyclic adenosine monophosphate; PKA, cAMP-dependent kinase; PKB, protein kinase B; Epac, exchange protein directly activated by cAMP; Ln-5, laminin-5; FN, fibronectin; 8CPT-2Me-cAMP, 8-(4-chloro-phenylthio)-2-O-methyladenosine-3,5 cyclic monophosphate

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Figure 1. **The small GTPase Rap1 stimulates adhesion to Ln-5.**

*Figure Legends*

*Figure 1.* The small GTPase Rap1 stimulates adhesion to Ln-5. 

*A*, Overexpression of a constitutively active mutant of Rap1 induces adhesion to Ln-5. 804G cells were transfected with CMV-luciferase in combination with mock DNA, cDNA for Rap1V12 or cDNA for Rap1GAPI, respectively, and plated on a Ln-5 matrix for the indicated times. Non-adherent cells were washed away, and adherent cells were lysed and luciferase units were measured. 

*Figure 1.* The small GTPase Rap1 stimulates adhesion to Ln-5. 

*B*, Expression of Rap1GAPI and Rap1V12 was confirmed by Western blotting using HA antibodies. 

*C*, Overexpression of Epac1 increases cell adhesion to Ln-5. HaCaT and 804G cells were transfected with CMV-luciferase in combination with either mock DNA or Epac1, and an adhesion assay was performed as described under *(A)*. 

*D*, Overexpression of Epac1 results in activation of endogenous Rap1. HaCaT and 804G cells were transfected with Epac cDNA as indicated, and cell lysates were incubated with immobilized GST-RalGDS-RBD to specifically pull down the GTP-bound form of Rap1 *(upper panel)*. To confirm equal loading, total Rap1 levels in whole cell lysates were determined by Western blotting with Rap1 antibodies *(middle panel)*, and expression of Epac is shown using HA antibodies *(lower panel)*. 

*E*, Expression of PDZ-GEF increases adhesion of 804G cells to Ln-5. Cells were transfected with CMV-luciferase in combination with either mock DNA or PDZ-GEF, and an adhesion assay was carried as described under *(A)*. 

*F*, Regulation of Rap1 by PDZ-GEF and Rap1GAPI. 804G cells were transfected as indicated, and cell lysates were incubated with immobilized GST-RalGDS-RBD to specifically pull down the GTP-bound form of Rap1 *(upper panel)*. Total Rap1 in whole cell lysates is shown *(middle panel)*, and

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expression of transfected cDNAs was confirmed by blotting with HA antibodies (lower panel). G, Expression of Rap1GAPI inhibits Epac-induced cell adhesion. HEK293T cells were transfected with CMV-luciferase in combination with indicated cDNAs, and an adhesion assay was performed as described under (A). H, Activation of endogenous Rap1 in transfected HEK293 cells. HEK293 cells were transfected as indicated, and cell lysates were incubated with immobilized GST-RalGDS-RBD to specifically pull down the GTP-bound form of Rap1 (upper panel). Total Rap1 in whole cell lysates is shown (middle panel), and expression of transfected cDNAs was confirmed by blotting with HA antibodies (lower panel).

Figure 2. cAMP promotes cell adhesion to Ln-5 through Epac. A, cAMP-induced cell adhesion is independent of PKA. As indicated, Ovcar-3 cells were pretreated with H-89 for 30 minutes and incubated with either forskolin (‘Forsk’) or isoproterenol (‘Iso’) for 15 minutes before being plated on Ln-5 for 10 minutes. Non-adherent cells were washed away, adherent cells were fixed with formaldehyde, stained with phalloidin-TRITC, and imaged. Subsequently, cells in three different microscopic fields were counted. The experiment was performed in triplicates. B, cAMP activates Rap1 through Epac. Ovcar-3 cells were pre-treated with H-89 or left untreated before incubation with 8CPT-2Me-cAMP, forskolin, or isoproterenol, respectively. Subsequently, cells were lysed and incubated with immobilized GST-RalGDS-RBD, to specifically pull down the GTP-bound form of Rap1 (upper panel). Total lysates were probed with Rap1 antibodies to show equal input (middle panel), or with phospho-specific CREB antibodies to monitor PKA activity (lower panel). C, 8CPT-2Me-cAMP increases cell adhesion to Ln-5. Ovcar-
3 cells were treated with 8CPT-2Me-cAMP, plated on Ln-5, and an adhesion assay was carried out as in (A). D, over-expression of Rap1GAPI blocks cAMP-induced cell adhesion. Ovcar-3 cells were transfected with mock cDNA or with Rap1GAPI, treated with forskolin, plated on Ln-5, and an adhesion assay was performed as in Fig. 1A. Inset shows expression of Rap1GAPI. E, Short-term forskolin-induced adhesion of Ovcar-3 cells occurs without extensive cytoskeletal rearrangements. Ovcar-3 cells were treated with forskolin, plated on Ln-5 for 5 min, and an adhesion assay was performed as in Fig. 2A (left). The forskolin-treated cells were photographed at 100x magnification using phase-contrast microscopy to show morphology (right), bar: 20 µm.

Figure 3. **Rap1 specifically regulates β3β1 and not β6β4.** A, cAMP-induced cell adhesion to Ln-5 is mediated by β3β1 integrins. Ovcar-3 cells were incubated with goat- anti-mouse IgG or antibodies against β3, β6, or a combination of β3 and β6 integrins before receiving treatment with forskolin for 15 minutes. Cells were then plated on Ln-5 for 10 minutes, and non-adherent cells were washed away. Adherent cells were fixed with formaldehyde, stained with phalloidin-TRITC, and imaged, and cells in three different microscopic fields were counted. The experiment was performed in triplicates. B, Rap1 regulates β1 integrins. K562 cells stably transfected with β6β4 were transfected with mock DNA or with Rap1GAPI. Cells were treated with function-blocking antibodies against β6 or β1 before stimulation with PMA. Cells were then plated on FN and an adhesion assay was performed as described in Fig.1A. C, Rap1 does not regulate β6β4. β6β4-expressing K562 cells were transfected with mock DNA or with Rap1GAPI as

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indicated. Cells were pre-treated with function-blocking antibodies against β6 or β1, stimulated with PMA, plated on Ln-5 and an adhesion assay was performed as described in Fig.1A. D, Rap1 does not change cell-surface expression of β3β1 integrins. Ovcar-3 cells were trypsinized, recovered, and left untreated or treated with 100 μM 8CPT-2Me-cAMP for 30 min. Subsequently, cells were fixed, incubated with FITC-conjugated antibodies against β3 (CD49c), and analyzed by flow cytometry. The FACS profile is representative for three independent experiments.

Figure 4. cAMP promotes cell spreading through Epac and Rap1. A, 804G cells were transiently transfected with mock DNA, Rap1V12, or Rap1GAPI. GFP was co-transfected in order to visualize transfected cells. Cells were detached, allowed to recover, and plated on Ln-5 for 15 minutes at 37°C. Subsequently, cells were fixed and cells positive for GFP were photographed at 400x magnification. Bar, 15 μm. B, quantification of cell spreading. Data are shown as fold induction of total cell surface relative to mock transfected cells. Error bars represent standard error of three independent experiments, with n>33. C, cAMP promotes Epac-Rap1-mediated cell spreading on fibronectin. Ovcar-3 cells were detached, allowed to recover, and plated on fibronectin (1 μg/ml) for 30 minutes at 37°C. Subsequently, cells were fixed, permeabilized, and stained with phalloidin-TRITC to visualize the actin cytoskeleton. Cells were photographed at 400x magnification. Representative images are shown. Bar, 20 μm. D, cAMP induces Ovcar-3 cell spreading on Ln-5. Ovcar-3 cells were detached and allowed to recover. During the last 40 minutes of recovery either DMSO or H-89 was added, and cells were treated with forskolin (‘Forsk’) or isoproterenol (‘Iso’) for the final 10 minutes. Cells

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were then plated on Ln-5 for 15 minutes at 37°C, fixed and photographed at 400x. The percentage of spread cells (i.e. cells that had become flattened with their total diameter more than twice the diameter of the nucleus) in four microscopic fields was determined. Representative data of one experiment are shown, and experiment was performed four times with similar results. E, 8CPT-2Me-cAMP promotes cell spreading on Ln-5. Ovcar-3 cells were detached and allowed to recover, and 8CPT-2Me-cAMP was added during the final 10 minutes. Subsequently, a cell spreading assay was carried out as described under (D). F, Ovcar-3 cell spreading is blocked by antibodies against β3 integrins. Ovcar-3 cells were detached and allowed to recover. During the last 40 minutes cells were incubated with goat-anti-mouse IgG, or antibodies against β3, β6, or a combination of β3 and β6 integrins. Forskolin was added during the final 15 minutes of recovery, and a cell spreading assay was carried out as described under (D).
Figure 1

A

B

C

D

E

F

G

H
Figure 2

A

Fold induction

Untreated  H-89  Forsk  Forsk + H-89  Iso  Iso + H-89

B

8CPT-2Me-cAMP  Forskolin  Isoproterenol  H-89

Rap1-GTP

Total Rap1

P-CREB

C

Fold induction

Untreated  8-CPT-2Me-cAMP

D

Adhesion (%)

Mock  Rap1GAPI

Forsk

E

Fold induction

Untreated  Forskolin

WB: HA

Rap1GAPI

Mock  Rap1GAPI

Adhesion (%)
Figure 3

A

Fold induction

B

% adhesion to FN

C

% adhesion to Ln-5

D

Cell count

CD49c

untreated Forskolin

Mock

Rap1GAPI

untreated PMA

PMA + anti-α6

PMA + anti-β1

untreated PMA

untreated PMA

untreated PMA

untreated PMA

untreated PMA

untreated PMA

untreated PMA

untreated PMA

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