Platelet-monocyte complexes in touch with the endothelium

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Epac1-Rap1 signaling regulates monocyte adhesion and chemotaxis

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Abstract
Extravasation of leukocytes is a crucial process in the immunological defence. In response to a local concentration of chemokines, circulating leukocytes adhere to and migrate across the vascular endothelium towards the inflamed tissue. The small GTPase Rap1 plays an important role in the regulation of leukocyte adhesion, polarization and chemotaxis. We investigated the role of Epac1 (a guanine nucleotide exchange factor for Rap1, directly activated by cAMP) in adhesion and chemotaxis in a pro-monocytic cell line and in primary monocytes. We found that Epac1 is expressed in primary leukocytes, platelets, CD34-positive hematopoietic cells and in the leukemic cell lines U937 and HL60. Epac activation with an Epac-specific cAMP analogue induced Rap1 activation, β1-integrin-dependent cell adhesion and cell polarization. In addition, activated Epac1 enhanced chemotaxis of U937 cells and primary monocytes. Similar to activation of Epac1, stimulation of cells with serotonin to induce cAMP production resulted in Rap1 activation, increased cell adhesion and polarization and enhanced chemotaxis. The effects of serotonin on U937 cell adhesion were dependent on cAMP production but could not be blocked by a PKA inhibitor, implicating Epac in the regulation of serotonin-induced adhesion. In summary, our work reveals the existence of previously unrecognized cAMP-dependent signaling in leukocytes regulating cell adhesion and chemotaxis through the activation of Epac1.

Introduction
Leukocyte extravasation from the blood stream is of key importance in physiological processes, such as immunosurveillance and acute inflammation. This process is tightly regulated by cytokines that activate the adhesive and migratory capacities of leukocytes. In pathological conditions, an excess of pro-inflammatory cytokines leads to excessive leukocyte extravasation. This is the main determinant in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis, asthma and atherosclerosis. Chemokine-induced leukocyte migration involves integrin-mediated adhesion of circulating leukocytes to the endothelium, polarization of leukocytes toward the source of chemokine and leukocyte migration across the endothelium (diapedesis) 1.

In lymphocytes, the small GTPase Rap1 plays a crucial role in the stimulation of integrin-mediated adhesion, cell polarization and cell motility 2. Rap1 is activated upon GTP binding, which is induced by specific guanine nucleotide exchange factors (GEFs). Several GEFs for Rap1 have been identified, including the recently found Epac (exchange protein directly activated by cAMP) 3. Two Epac isoforms, Epac1 and 2, have been described. Epac1 was found to be expressed in most tissues, while Epac2 is expressed in the adrenal gland and in the brain 4. Attempts to detect Epac expression in leukocytes showed the presence of Epac1 mRNA only in B cells and U937 cells, while Epac1 protein was detected in macrophages. Epac2 was undetectable in all cell types 5.

The second messenger cAMP is crucially involved in multiple cellular processes. Until Epac was identified, cAMP-dependent signaling was thought to be carried out by protein kinase A (PKA). Currently, a growing number of studies implicate Epac1 in the regulation of several cAMP-dependent effects, including substrate adhesion and cell-cell adhesion in adherent cells 6-10, Ca++-induced exocytosis 11,12, neurite extension 13 and Fcγ receptor-mediated phagocytosis in
macrophages. However, the role of Epac in leukocyte adhesion and chemotaxis has not been established.

Epac becomes activated by stimuli that bind to receptors signaling via the heterotrimeric Gs proteins, which induce a rise in cAMP levels through the activation of adenylate cyclase. These stimuli include serotonin, prostaglandins and β2 adrenergic agonists. In neurons, the Epac-Rap pathway regulates serotonin-induced secretion of amyloid precursor protein as well as ERK activation. Interestingly, in addition to being a neurotransmitter, serotonin plays an important role in inflammation. It is secreted by mast cells and platelets and induces chemotaxis of eosinophils, lymphocytes and macrophages.

In this study, we demonstrate that Epac1 is expressed in leukocytes, platelets and hematopoietic cells and we investigate its functionality in monocytic U937 cells and in primary monocytes. Our results show that activation of Epac1 promotes cell adhesion and polarization and enhances chemokine-induced migration.

Materials and methods

Reagents

8CPT-2Me-cAMP and Rp-8-CPT-cAMPS were purchased from Biolog LSI (Germany). SQ22536 and 2’,5’-Dideoxyadenosine were from Calbiochem (Darmstad, Germany). Serotonin (5-hydroxytryptamine, 5-HT), PMA and H89 were from Sigma. Stromal cell-derived factor 1 (SDF-1, CXCL12) and Monocyte chemoattractant protein-1 (MCP-1, CCL2) were from Strathman Biotech (Hannover, Germany). Recombinant tumor necrosis factor (TNF)-α was purchased from Boehringer Mannheim (Germany).

Cell culture

All cell lines were purchased from ATCC (Manassas, VA, USA) and were cultured at 37°C and 5% CO2. U937 cells (monocytic cell line) were maintained in RPMI 1640 medium (GIBCO) containing 10% (v/v) heat-inactivated FCS (GIBCO), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. HL-60 cells (leukemic cell line) and CHO (Chinese hamster ovary) cells were cultured in IMDM medium (BioWhittaker, Brussels, Belgium) containing 10% (v/v) heat-inactivated FCS (GIBCO), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cord veins as previously described. Cells were cultured to confluency in M199 medium (GIBCO) containing 20% (v/v) heat-inactivated FCS (GIBCO), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml heparin and 50 μg/ml endothelial mitogen (Sanbio BV). Endothelial cells of second or third passage were used. HUVEC monolayers were stimulated for 16 h with 100 U/ml TNF-α prior to the perfusion experiments.

Cell isolation

Blood was obtained from healthy volunteers. Granulocytes and peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of 500 ml of blood by density gradient centrifugation over isotonic Percoll (Pharmacia, Uppsala, Sweden) with a specific gravity of 1.076 g/ml. For further purification of monocytes and lymphocytes, the PBMC fraction was incubated with magnetic beads coated with anti-CD14 antibodies and monocytes were purified with a MACS separation system according to the manufacturer’s instructions (Miltenyi Biotec...
GmgH, Bergisch Gladbach, Germany). Previous experiments performed in our lab have shown that this isolation protocol does not induce monocyte activation. The remaining monocyte-depleted fraction of PBMCs was labelled with anti-CD3 and anti-CD19 antibodies and B and T cells were subsequently sorted with a Mo Flo sorter (Dako Cytomation; Denver, CO, USA).

After lysis of the erythrocytes with ice-cold lysis buffer containing 155 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA (pH 7.4), the granulocyte fraction was incubated with an anti-CD16 antibody. Subsequently, neutrophils were sorted with a Mo Flo sorter. Eosinophils were isolated by means of the fMLP method. In brief, granulocytes were incubated for 30 minutes at 37°C to restore initial cell density. Cells were then washed and resuspended in PBS containing 0.5% (w/v) HSA (human serum albumin, Sanquin, Amsterdam, The Netherlands) and 13 mM trisodium citrate and incubated for 10 minutes at 37°C after the addition of 10 nM fMLP to the cell suspension. Eosinophil and neutrophil fractions were separated by centrifugation (20 minutes, 1000 ×g) over isotonic Percoll (1.082 g/ml, pH 7.4). Platelets were isolated as previously described.

CD34⁺ hematopoietic progenitor cells were isolated from cord blood by density gradient centrifugation over Ficoll-paque (1.077 g/ml) (Pharmacia Biotech, Upsala, Sweden) as previously described.

The purity of all the isolated cell populations was greater than 95%.

RT-PCR
RNA was isolated from purified leukocyte fractions, platelets and CD34⁺ cells and the cell lines U937 and HL-60 by lysis of the cells in a solution containing guanidine isothiocyanate, followed by centrifugation over a layer of CsCl (5.7 M) in a Beckman OptimaTM L-100 XP Ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA, USA) with an SW41 rotor. cDNA was synthesized using 2.5 μM oligo-dT primers and 10 U/ml Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) (5 minutes, 65°C). Quantitative PCR was performed with the FastStart DNA Master Plus SYBR Green I kit in the LightCycler Instrument (Hoffman-La Roche, Basel, Switzerland). Epac1 RNA was amplified by PCR with the following protocol: 10 minutes at 95°C, followed by 50 cycles of 5 seconds at 95°C, 30 seconds at 65°C and 15 seconds at 72°C. The following Epac1-specific primers were used: forward primer-5'TTGGAGAATGGCTGTGGGAATGCATC3' (exon 14); reverse primer 5'CCGAGTTGCTGAGGCCAAACATGAC3' (exon 19). The mRNA of the house-keeping gene -glucuronidase was amplified as internal control using specific primers. Amplified Epac1-specific cDNA was compared to the standard -glucuronidase. The specificity of the PCR products was checked by DNA sequencing with the Big-dye Terminator Sequencing kit v1.1 and analyzed on a Genetic Analyzer 3100 Platform (both from Applied Biosystems, Foster City, CA, USA).

Western blot analysis
Isolated primary leukocytes, platelets and cell lines were lysed in Laemmli sample buffer containing a protease inhibitor cocktail (Roche) and incubated for 15 minutes at 95°C. Cell lysates were separated on 10% SDS-PAGE gels and transferred onto PVDF membranes (BioRad Laboratories, Hercules, CA, USA). Epac1 was detected with a rabbit polyclonal antibody against Epac1 antibody (Upstate). Actin was detected with a mouse monoclonal antibody against actin (Ab1, Oncogene, Darmstadt, Germany).
**Integrin activation**

U937 cells were resuspended in IMDM medium containing 0.25% (w/v) BSA and stimulated with 8CPT-2Me-cAMP for various time periods at 37°C. Stimulation was stopped by the addition of ice-cold PBS containing 0.5% (w/v) BSA, and cells were pelleted by centrifugation (490 xg, 5 minutes, 4°C). Cells were then resuspended in ice-cold PBS containing 0.5% BSA and incubated with a mouse monoclonal antibody against the activated conformation of β1-integrins (12G10, IMGEN, The Netherlands) or β2-integrins (CBRM1/5, a kind gift from Dr. Kevin L. Moore; University of Oklahoma, USA) for 30 minutes at 4°C. Total surface expression of β1- and β2-integrins was detected with mouse monoclonal antibodies specific for β1- or β2-integrins (Sanquin, Amsterdam, The Netherlands). After washing with ice-cold PBS-0.5% (w/v) BSA, bound monoclonal antibodies were detected with PE-labeled goat-anti-mouse-Ig (DakoCytomation, Denmark) for 30 minutes at 4°C. The fluorescence intensity of labelled cells was measured with a FACScan flow cytometer (Becton and Dickinson, San Jose, CA, USA). Integrin activation was calculated by correcting for the amount of total integrins in every sample and was expressed as fold increase over control-unstimulated cells.

**Adhesion assay**

Flat-bottom 96-well plates (Maxi Sorp Nunc; Denmark) were coated with 20 μg/ml of human fibronectin (Sigma) for 16 hours at 4°C and blocked with 0.5% BSA (Sigma) for at least 1 hour at 37°C. U937 cells were washed in IMDM medium containing 0.25% BSA, labeled with Calcein-AM, according to the manufacturer’s instructions (Molecular Probes, Leiden, The Netherlands) for 30 minutes at 37°C, and washed twice with IMDM-BSA. Labeled U937 cells were pre-incubated or not with 8CPT-2Me-cAMP for 15 minutes and subsequently added to the fibronectin-coated 96-well plates (2×10^5 cells per well). Plates were centrifuged at 40 xg for 1 minute and stimuli were immediately added. After a further incubation for 30 minutes at 37°C, non-adhered cells were removed by washing three times with warm PBS. Adherent cells were lysed with 0.5% (w/v) Triton X-100 and fluorescence was measured on a GENios Plus plate reader (TECAN; Salzburg, Austria). The percentage of adhesion was calculated by dividing the measured fluorescence intensity by the fluorescence intensity of the input cells (set to 100%). VLA-5 and CD11b blocking experiments were performed by pre-incubating cells for 30 minutes with the monoclonal antibodies SAM-1 (Sanquin, Amsterdam, The Netherlands) and 44a (ATCC, Rockville, MD, USA), respectively. In order to block undesirable Fc receptor activation by the blocking VLA-4 antibody HP 2/1, cells were incubated for 10 minutes with the anti-Fc receptor antibodies anti-CD32 (Medarex, Annandale, NJ, USA) and anti-CD16b (Sanquin, Amsterdam, The Netherlands), prior to the incubation with a mixture of HP 2/1 and anti-Fc receptor antibodies for additional 30 minutes.

**Perfusion assay**

Perfusion experiments were performed as previously described. In brief, monocyte suspension (2×10^6 cell/ml in incubation buffer) was aspirated from a reservoir through plastic tubing and perfused through a chamber (containing the HUVEC monolayer) with a Harvard syringe pump (Harvard Apparatus, USA) at flow rate of at 0.8 dyn/cm^2. During perfusions the flow chamber was mounted on a microscope stage (Axiovert 25, Zeiss, Germany), equipped with a B/W CCD video camera (Sanyo, Osaka, Japan) and coupled to a VHS video recorder. Video images were evaluated for the number of adherent monocytes and the rolling velocity per
cell, with dedicated routines made in the image analysis software Optimas 6.1 (Media Cybernetics Systems, USA). The number of surface-adherent monocytes was measured after 5 minutes of perfusion at a minimum of 25 randomized high-power fields. To automatically determine the velocity of rolling cells, custom-made software was developed in Optimas 6.1. A sequence of 50 frames representing an adjustable time interval (δt, with a minimal interval of 80 milliseconds) was digitally captured. The position of every cell was detected in each frame, and for all subsequent frames the distance traveled by each cell and the number of images in which a cell appears in focus were measured. The cut-off value to distinguish between rolling and static adherent cells was set at 1μm/s. With this method, static adherent cells, rolling and free-flowing cells could be clearly distinguished and quantified.

**Transmigration assay**

Migration assays were performed as previously described 25. In brief, Transwells of 6.5 mm diameter, with 5-μm pore size filters (Costar, Cambridge, MA, USA) were coated with 20 μg/ml fibronectin (Sigma). Before use, cells were washed once with migration medium (IMDM containing 0.25% BSA). At the start of the assay, 10^5 cells were placed in the upper compartment of the Transwells and allowed to migrate for 1 hour at 37 ºC to chemokine-containing medium added to the lower compartment. Migrated cells were collected from the lower compartment and quantified by flow cytometric analysis in the presence of a fixed amount of control cells labeled with Calcein-AM (Molecular Probes, Leiden, The Netherlands). The percentage of migrated cells was calculated as a fraction of the total cell input as follows: % of migrated cells = [(number of transmigrated cells/number of input labeled cells)/number of not labeled input cells/number of labeled input cells)] × 100%.

For primary monocyte migration, transmigrated cells were collected from the lower compartment of the Transwell as well as from the bottom of the Transwell filter (since a fraction of transmigrated monocytes remains adhered to the bottom of the filter). Cells adhering to the bottom side of the filters were counted under a fluorescent microscope (three random fields) after removing cells on the top side of the filters with a cotton swab, followed by fixation and staining with Hoechst (Molecular Probes). The percentage of transmigrated monocytes that adhered to the filter was calculated as follows: % of migrated cells adhered to the filter = [(number of transmigrated cells counted per field/surface of the field)]/[number of input cells/surface of the filter)] × 100%. The calculated percentages of the two fractions were added to give the total percentage of transmigrated monocytes.

**Rap1 activation assays**

Rap1 pull-down experiments were performed as previously described 30. In brief, following stimulation U937 cells were lysed in ice-cold lysis buffer containing 10 mM Tris-HCl, 150 mM NaCl, 1% (w/v) NP-40, 0.5% (w/v) deoxycholic acid, 0.1% (w/v) SDS, 1 mM NaF, 2 mM NaVO_3_ and protease inhibitor cocktail (Roche) for 10 minutes on ice. Lysates were clarified by centrifugation at 10,000 ×g for 10 minutes at 4°C. GST-RalGDS-RBD coupled to glutathione-Sepharose beads (Amersham Biosciences) was added to the supernatants and incubated for 1 hour at 4°C. Beads were washed three times in lysis buffer and bound proteins were eluted with Laemmli sample buffer. Rap1 in total cell lysates and precipitates was detected by Western blotting with a mouse monoclonal anti-Rap1 antibody (Santa Cruz Biotechnology).
Densitometric analysis was performed with a CanoScan LiDE20 scanner (Canon) and Gene Tools Analysis Software version 3.03.03 (SynGene, Cambridge, UK).

**Electroporation and Immunofluorescence**

A plasmid containing HA-Epac1 (pMT2SM-HA-Epac1, 30 μg) was added to U937 cells (12 x 10^6 cells) resuspended in RPMI medium. Cells were subsequently electroporated with a BioRad Gene Pulser II electroporator (950 μF, 250 V) and cultured in RPMI medium containing 20% FCS for forty-eight hours. Subsequently, cells were collected by centrifugation, washed and resuspended in IMDM medium containing 0.25% (w/v) BSA. Transfected cells were allowed to adhere to fibronectin-coated coverslips for 10 minutes at 37°C followed by a 20 minute incubation in the presence of stimuli. Cells were then washed with PBS containing 0.5% (w/v) BSA, fixed with 3.7% (w/v) formaldehyde for 10 minutes at RT and permeabilized with 0.1% (w/v) Triton X-100 for 5 minutes. For immunofluorescence staining of HA-Epac-1, cells were incubated with a mouse monoclonal antibody to HA (12CA5; Boehringer Mannheim Corp., Indianapolis, IN) followed by a goat-anti-mouse-Ig antibody labeled with Alexa 488 (Molecular Probes, Leiden, The Netherlands). F-actin was visualized with TexasRed-labelled phalloidin (Molecular Probes, Leiden, The Netherlands). Images were recorded with a Zeiss LSM 510 confocal laser scanning microscope. Fluorescence distribution profiles were created with Zeiss LSM 510 confocal laser scanning microscope software.

**Statistical analysis**

All results were expressed as a mean ± SEM of at least three independent experiments. Where applicable, values were compared with paired two-tailed Student t-test. Multiple comparisons were analyzed with a two-way ANOVA test. A p value lower than 0.05 was considered significant. All statistical analyses were performed with GraphPad Prism version 3.0 software.

**Figure 2.1: Expression of Epac1 in primary leukocytes and leukocytic cell lines.** (A) RT-PCR for Epac1 of cDNA derived from purified human primary neutrophils, monocytes, lymphocytes, CD34+ cells, eosinophils, platelets and the myelocytic cell lines U937 and HL60. The size of the amplified PCR fragment was 735 bp. As a control, cDNA for β-glucuronidase was amplified. Representative results of 3 independent experiments are shown. (B) Western blot detection of Epac1 in cell lysates from purified human primary neutrophils, monocytes, B cells, T cells, CD34+ cells, eosinophils, platelets and the myelocytic cell lines U937 and HL60. CHO cells were used as a positive control for Epac1 protein expression. Bands corresponding to Epac1 are ~110 kDa. β-actin was used as a control for equal protein loading. Representative results of four independent experiments are shown.
Results

Expression of Epac1 in human primary leukocytes and leukocytic cell lines

We examined Epac1 mRNA and protein expression in peripheral blood leukocytes, platelets, cord blood-derived CD34-positive hematopoietic cells and two human myelocytic cell lines (HL60 and U937) by RT-PCR and Western blot analysis. RT-PCR was performed under optimized conditions for maximal sensitivity to enable the analysis of low copy number transcripts. In contrast to previous reports, we detected Epac1 mRNA in all cell populations examined, with CT values in the range between 30-34 (Figure 2.1A and Table 2.1). The analysis of the expression of Epac2 transcript resulted in very high CT values (CT higher than 38; 31), indicating that Epac2 is not expressed in leukocytes (data not shown). Western blotting experiments showed expression of Epac1 protein in all leukocyte populations examined with the exception of neutrophils, despite the presence of Epac1 mRNA in these cells (Figure 2.1B). Similarly, eosinophils and platelets showed low Epac1 protein expression levels, while CT values for Epac1 transcript in these cells were comparable to CT values of the other cell populations analyzed. These data suggest that Epac1 expression is differentially regulated among leukocytes at the posttranslational level.

Table 2.1: CT values of Epac1 and β-glucuronidase.

<table>
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<th>Cell type</th>
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<th>β-Glucuronidase CT</th>
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Epac1 enhances monocyte adhesion through integrin activation

To study Epac signaling in leukocytes, we made use of the Epac-specific cAMP analogue 8CPT-2Me-cAMP, which does not activate PKA. This analogue has been extensively used as a tool to study the role of Epac as a regulator of a variety of cellular processes. We verified the functionality of Epac1 in the monocytic cell line U937 by assaying Rap1 activation upon cell incubation with 8CPT-2Me-cAMP. U937 cells stimulated with 8CPT-2Me-cAMP for 0.5, 1 and 5 minutes were lysed, and GTP-bound Rap1 was precipitated in a GST-RalGDS-RBD pull-down assay. Thirty seconds stimulation of U937 cells with 8CPT-2Me-cAMP increased Rap1 activation. This induction was transient and declined to basal levels after 5 minutes of stimulation (Figure 2.2A). Notably, the activation kinetics of Rap1 by Epac in U937 cells is similar to the previously described kinetics in adherent cells. These data show that U937 cells express functional Epac1.
Figure 2.2: Epac1 activates Rap1 in U937 cells and promotes cell adhesion to fibronectin and endothelial cells. (A) U937 cells were treated with 100 μM 8CPT-2Me-cAMP for the indicated time periods, and GTP-bound Rap1 was precipitated with GST-Ral-GDS-RBD, followed by western blotting and Rap1 detection by immunoblotting (upper panel). Total levels of Rap1 in whole cell lysates are shown (lower panel). Representative results of three independent experiments are shown. Bar graph represents densitometric analysis of Rap1 activation. Data are mean of three independent experiments (± SEM). (B) U937 cells were pre-treated or not with 100 μM 8CPT-2Me-cAMP for 15 minutes and placed in a fibronectin-coated plate for 30 minutes. As a positive control, PMA (100 ng/ml) was added at the time of plating. The percentage of adhesion was determined as described in Materials and methods. Data are mean (± SEM) of five independent experiments performed in triplicate. * p<0.05; ** p<0.005 (C) Primary monocytes were pre-treated or not with 100 μM 8CPT-2Me-cAMP for 30 minutes and perfused over TNF-α-stimulated monolayers of human umbilical vein endothelial cells for 5 minutes at 0.8 dyn/cm². Video images were evaluated for the number of adherent monocytes and cell rolling as described in Materials and methods. Data are mean (± SEM) of five independent experiments performed in duplicate. * p<0.05.

Epac was recently shown to regulate integrin-mediated adhesion in ovarian carcinoma cells ⁶,⁷. To investigate whether Epac1 has a similar function in U937 cells, we analyzed cell adhesion to fibronectin after thirty minutes incubation with 8CPT-2Me-cAMP. In addition, stimulation with PMA was included as a positive control. Epac1 activation induced a 60% increase in adhesion.
when compared to control untreated cells (Figure 2.2B). This indicates that Epac1 activation promotes adhesion of U937 cells to fibronectin. To further substantiate the role of Epac1-Rap1 signaling in monocyte adhesion we studied the effect of 8CPT-2Me-cAMP in the adhesion of freshly isolated monocytes to cultured human umbilical vein endothelial cells under flow. Epac1 activation resulted in a clear induction of monocyte firm adhesion to endothelial cells, without affecting rolling, which is mainly mediated by selectins (Figure 2.2C) 34-36. This provides evidence for a role of Epac1-Rap1 signaling in the regulation of primary monocyte adhesion to endothelial cells under flow, and thus suggests that this pathway may also function in vivo.

To gain insight into the mechanism by which Epac1 enhances U937 cell adhesion to fibronectin we analyzed the surface expression of total and activated β1- and β2-integrins upon treatment with 8CPT-2Me-cAMP. Epac1 activation resulted in a rapid and significant increase of activated β1-integrins on the cell surface followed by their down-modulation (Figure 2.3A). In contrast, no changes were observed for β2-integrin activation (data not shown). Our results show a rapid but transient activation of integrins by Epac, while the effects on cell adhesion are prolonged for at least 30 minutes. This may be explained by the fact that integrin activation was measured in cells in suspension, where no integrin engagement takes place. Upon seeding, stable engagement of the activated integrins by fibronectin may induce a prolonged effect on adhesion.

Figure 2.3: Epac1 induces β1-integrin activation and β1-integrin-mediated adhesion. (A) U937 cells were treated with 100 μM 8CPT-2Me-cAMP for the indicated time periods. Surface expression of activated β1-integrins was determined by flow cytometry using a mouse monoclonal antibody that recognizes the activated state of β1-integrins (12G10). The activation of β1-integrins is expressed as fold increase over control untreated cells and is corrected for total β1-integrin surface expression. Data are mean (± SEM) of three independent experiments. * p<0.05. (B-D) U937 cells were pre-incubated with integrin-blocking monoclonal antibodies to VLA-5 (B), VLA-4 (C) or Mac-1 (D) for 30 minutes before addition of the cells to fibronectin-coated plates. Data are mean (± SEM) of three independent experiments performed in triplicate. * p<0.05.
To confirm the role of β1-integrins in Epac1-mediated U937 cell adhesion to fibronectin, cells were incubated with blocking anti-integrin antibodies before their addition to fibronectin-coated wells. A blocking anti-VLA-5 (α5β1) antibody completely abrogated adhesion of both control and 8CPT-2Me-cAMP-treated cells (Figure 2.3B), indicating that VLA-5 is the main integrin involved in U937 cell attachment to fibronectin. However, an anti-VLA-4 (α4β1) antibody specifically reduced 8CPT-2Me-cAMP-induced increase of adhesion, which returned to control levels (Figure 2.3C), whereas an anti-Mac1 (αMβ2) antibody showed no inhibitory effect (Figure 2.3D). From these experiments we conclude that Epac1 activation triggers inside-out signaling resulting in the activation of β1-integrins, which leads to VLA-4, and likely VLA-5-, mediated adhesion of U937 cells to fibronectin.

**Epac1 induces cell polarization and localizes to the uropod**

Cell polarization plays a crucial role in directional cell movement. Polarized cells develop a leading edge where membrane extension and lamellipodia formation occur, and a retracting rear (uropod). Since Rap1 is proposed to play a central role in lymphocyte polarization 37, we analyzed whether Epac1 activation triggers polarization of U937 cells. Fibronectin-adherent U937 cells were treated with 8CPT-2Me-cAMP, fixed and stained for F-actin. The percentage of polarized cells was quantified by microscopy according to morphological criteria: non-polarized cells are round in shape whereas polarized cells have a morphologically-defined leading edge and a uropod (cell images in Figure 2.4A). Quantitative analysis indicated that 8CPT-2Me-cAMP induced a two-fold increase in the number of polarized cells (Figure 2.4A). We next investigated the intracellular distribution of Epac1 in polarized versus non-polarized cells. U937 cells were transfected with HA-tagged Epac1, seeded on fibronectin and stained with HA antibodies for microscopy analysis. Epac1 localized at the cell periphery in non-polarized cells, whereas it concentrated at the uropod of polarized cells (Figure 2.4B). These observations suggest that Epac1 activates Rap1 at a perinuclear location.

**Epac1 promotes U937 cell and primary monocyte chemotaxis**

Rap1 plays a role in the regulation of chemokine-induced lymphocyte migration 38,39. To assess the role of Epac1 in monocyte chemotaxis, we analyzed the effect of 8CPT-2Me-cAMP on chemokine-induced migration of monocytic U937 cells and of freshly isolated monocytes. 8CPT-2Me-cAMP was not chemotactic by itself (data not shown). However, treatment with 8CPT-2Me-cAMP resulted in an 80% increase in migration of U937 cells to CXCL12 (stromal cell-derived factor 1, SDF-1) (Figure 2.5). To exclude the possibility that the Epac1-induced migration was due to the up-regulation of chemokine receptors, we analyzed whether 8CPT-2Me-cAMP modulated the cell surface levels of CXCR4 in U937 cells. No differences in CXCR4 surface levels were detected between untreated or 8CPT-2Me-cAMP-treated cells (not shown). We next examined the effect of 8CPT-2Me-cAMP on the chemotaxis of freshly isolated human monocytes toward CCL2 (monocyte chemoattractant protein 1, MCP-1). Similar to the findings in U937 cells, Epac activation resulted in an 80% increase in CCL2-induced chemotaxis. These data show that, although Epac1 activation does induce cell migration by itself, it significantly promotes chemokine-induced monocyte migration.
Serotonin activates Rap1 and promotes CXCL12-induced adhesion and chemotaxis of U937 cells

We next investigated whether stimulation of leukocytes with cAMP-raising receptor agonists would induce similar effects as direct Epac1 activation. To test this, we used serotonin (5-hydroxytryptamine, 5-HT), a neurotransmitter that has been shown to be an inflammatory mediator. Serotonin is released by activated platelets and can be found at micromolar concentrations within the inflammatory site. Notably, Epac1 has been implicated in signaling from serotonin receptors in neurons. We hypothesized that serotonin could enhance leukocyte chemotaxis through the Epac1-Rap1 pathway. To test this hypothesis we analyzed the effect of serotonin on U937 cell chemotaxis towards CXCL12. Serotonin was not chemotactic by itself, but significantly enhanced CXCL12-induced migration of U937 cells (Figure 2.6A). Furthermore, serotonin induced a small but significant increase in cell adhesion to fibronectin and cooperated with CXCL12 to enhance cell adhesion (Figure 2.6B).

We next investigated whether serotonin was able to trigger cell polarization, similar to Epac activation. U937 cells seeded on fibronectin-coated coverslips were stimulated with serotonin for 30 minutes. Cells were fixed and stained for F-actin for confocal microscopy analysis. The number of polarized versus non-polarized cells was determined as described above. The results showed that serotonin induced a significant increase in the number of polarized cells (Figure 2.6C). Thus, serotonin is able to induce a similar phenotype as activation of Epac. This suggested that serotonin signals through Epac to promote cell adhesion, polarization and chemotaxis. To test this hypothesis we first investigated whether serotonin is able to induce Rap1 activation. Treatment of U937 cells with serotonin resulted in the rapid activation of Rap1.
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Rap1, observed already after 30 seconds of stimulation. This activation was sustained for at least 5 minutes, decreasing to basal levels after 10 minutes stimulation (Figure 2.6D). Interestingly, the kinetics of Rap1 activation by serotonin is similar to the kinetics observed upon Epac activation with 8CPT-2Me-cAMP (Figure 2.2A). In contrast, stimulation of cells with CXCL12 resulted in a strong but short activation of Rap1. The kinetics of Rap1 activation upon stimulation with serotonin and CXCL12 together resembled that induced by serotonin alone (Figure 2.6D). Thus, the enhancing effects of serotonin on CXCL12-induced adhesion and migration might be due to the ability of serotonin to induce a more sustained Rap1 activation than CXCL12 alone, and therefore improved integrin-mediated adhesion.

To unequivocally implicate Epac in the mediation of serotonin effects on adhesion and chemotaxis, we transfected U937 cells with a dominant negative Epac mutant. However, no quantitative data could be obtained due to very low transfection efficiencies. We then followed an indirect approach by demonstrating that serotonin-induced adhesion requires cAMP production but is PKA-independent. U937 cells were treated with two different adenylyl cyclase inhibitors, SQ22536 and 2’-5’-deoxyadenosine, to block cAMP generation upon stimulation with serotonin. Treatment with either inhibitor abrogated the differences in adhesion between serotonin-stimulated and unstimulated cells and between CXCL12-stimulated and CXCL12/serotonin-stimulated cells (Figure 2.7A). These data suggest that cAMP production is required for the enhancing effects that serotonin has on adhesion. Since cAMP can activate both Epac1 and PKA, we excluded the possibility that serotonin-induced Rap1 activation and cell adhesion were mediated by PKA by using the specific PKA inhibitor H89. Pre-treatment of cells with H89 did not inhibit serotonin-induced Rap1 activation (Figure 2.7B) or prevent the effects of serotonin on cell adhesion (Figure 2.7C). Similar results were obtained with the competitive PKA inhibitor Rp-cAMPS (data not shown). These data indicate that PKA is not involved in serotonin-induced Rap1 activation and adhesion of U937 cells to fibronectin.

Together, these results suggest that serotonin enhances U937 cell adhesion and chemotaxis through the activation of the Epac1-Rap1 pathway.

Figure 2.5: Epac1 promotes chemotaxis of U937 cells and primary monocytes. U937 cells (left panel) or primary monocytes (right panel) were allowed to migrate for 1 hour to 100 ng/ml CXCL12 (SDF-1) or 10 ng/ml CCL2 (MCP-1), respectively, in the presence or absence of 100 μM 8CPT-2Me-cAMP added to the cell suspension in the upper compartment of Transwell system. When no chemoattractant was present 1-2% U937 cells or primary monocytes migrated regardless of the presence or absence of 8CPT-2Me-cAMP. In the presence of chemoattractant, 30-40 percent of untreated U937 cells and 6-7% of untreated monocytes migrated. Data represent the percentage of cell migration compared to the untreated cells (set at 100%). Data are mean (± SEM) of three to five independent experiments. * p<0.05; ** p<0.005
Figure 2.6: Serotonin activates Rap1, induces U937 cell adhesion and increases CXCL12-induced chemotaxis. (A) U937 cells were allowed to migrate across fibronectin-coated filters towards CXCL12 (10 ng/ml), serotonin (5-HT; 1 μM) or a combination of both for the indicated time periods. The percentage of migration was determined as described in Materials and methods. Data are mean (± SEM) of three independent experiments. The percentage of migration toward CXCL12 was compared with the percentage of migration toward the combination of 5-HT and CXCL12 by a two-way ANOVA test (p<0.05). (B) U937 cells were assayed for adhesion to fibronectin in the presence of CXCL12 (100 ng/ml), 5-HT (10 μM) or a combination of both. The percentage of adhesion was determined as described in Materials and methods. Data are mean (± SEM) of five independent experiments performed in triplicate. * p<0.05; ** p<0.005. (C) U937 cells were allowed to adhere to fibronectin-coated coverslips, and were stimulated or not with 10 μM serotonin for 30 minutes, fixed, stained for F-actin and analyzed by confocal microscopy. Data represent the percentage of polarized cells, scored on the basis of morphology, of a total of 100 to 120 cells per condition. (D) U937 cells were stimulated with 5-HT (10 μM), CXCL12 (100 ng/ml) or combination of both stimuli for the indicated time periods and Rap1 GTP-loading was assayed (upper panel). Total levels of Rap1 in whole cell lysates are shown (lower panel). Representative results of three independent experiments are shown. Bar graph represents densitometric analysis of Rap1 activation. Data are mean of three independent experiments (± SEM).
Figure 2.7: Serotonin increases adhesion in a cAMP-dependent, PKA-independent manner. (A) U937 cells were pre-incubated with the adenylate cyclase inhibitors SQ22536 or 2′,5′-deoxyadenosine (2′,5′-dd-Ado) for 1 hour before addition of the cells to fibronectin-coated plates. The percentage of adhesion was determined as described in Materials and methods. Data are mean (± SEM) of three independent experiments performed in triplicate. (B) U937 cells were pre-treated or not with 10 μM H89 for 30 minutes, stimulated with 5-HT (10 μM) and Rap1 GTP-loading was assayed (upper panel). Total levels of Rap1 in whole cell lysates are shown (lower panel). Representative results of three independent experiments are shown. Bar graph represents densitometric analysis of Rap1 activation. Data are mean of three independent experiments (± SEM). (C) U937 cells pretreated or not with H89 (10 μM) for 30 minutes were assayed for adhesion to fibronectin in the presence of CXCL12 (100 ng/ml) and 5-HT (10 μM). The percentage of adhesion was determined as described in Materials and methods. Data are mean (± SEM) of five independent experiments performed in triplicate. * p<0.05; ** p<0.005; *** p<0.0005
**Discussion**

Our report provides the first comprehensive analysis of Epac expression in leukocytes and hematopoietic cells. We show that Epac1 is functional in primary monocytes and in monocytic U937 cells where it regulates β1-integrin dependent cell adhesion, cell polarization and chemotaxis.

The small GTPase Rap1 is activated by almost all receptor types and regulates adhesion-related functions such as cell-cell contact and integrin-mediated adhesion \(^{42,43}\). In lymphocytes, Rap1 plays a crucial role in integrin-mediated adhesion, polarization and transendothelial migration downstream of chemokine receptors \(^{38,39}\). Recently, Epac1 was identified as a Rap1 exchange factor directly activated by cAMP and shown to be involved in integrin-mediated adhesion in ovarian carcinoma cells through Rap1 activation \(^{6}\). Although Epac1 is expressed in most tissues, previous studies failed to show Epac1 expression in primary leukocytes with the exception of B cells and macrophages \(^{5}\). Here, we have used optimized RT-PCR conditions to detect low copy number transcripts and found Epac1 mRNA in circulating leukocytes (monocytes, eosinophils, neutrophils and B and T cells), platelets, CD34-positive hematopoietic cells and the myelocytic cell lines U937 and HL60. Importantly, we found Epac1 protein expression in all leukocytes with the exception of neutrophils. Epac1 protein levels did not always correlate directly with Epac1 mRNA levels, e.g. in eosinophils and platelets. This might be due to differential posttranslational regulation of Epac1 expression in different types of leukocytes and may have functional consequences during the response of different leukocyte types to cAMP-elevating agents.

In this study, we have investigated the function of the Epac1-Rap1 pathway in the pro-monocytic cell line U937 and in primary monocytes. We found that a cAMP analogue (8CPT-2Me-cAMP) that specifically activates Epac1, and not PKA, was able to induce cell adhesion, polarization and chemotaxis. In U937 cells, Epac1 activation induced β1-integrin activation and VLA-4-mediated adhesion to fibronectin. Thus, Epac1 appears to have a similar role in leukocytes as in adherent cells, namely the activation of Rap1 and the consequent ‘inside-out’ signaling toward β1-integrins \(^{6,7}\).

In line with previous studies showing that Rap1 activation induces T cell polarization \(^{37}\), we show that activation of the Epac1-Rap1 pathway induces polarization of U937 cells. Additionally, ectopically expressed Epac1 redistributes from the cell periphery to the perinuclear area upon cell polarization. This suggests that Epac1 activates Rap1 at a perinuclear location and that activated Rap1 subsequently translocates to the plasma membrane. Accordingly, wild type (active and inactive) Rap1 was shown to localize to a perinuclear vesicular compartment and to the plasma membrane in T cells, whereas activated GTP-bound Rap1 was found exclusively at the plasma membrane \(^{44}\).

We have used monocytic U937 cells for most of our studies; however, we have demonstrated that the Epac pathway is functional in primary human monocytes. We show that Epac1 activation upregulates adhesion of freshly isolated monocytes to endothelial cells under flow as well as monocyte migration towards CCL2 (MCP-1). This chemokine is a potent monocyte chemoattractant that has a key role in the recruitment of monocytes to atherosclerotic lesions. Based on these data we postulate that Epac1 activation by cAMP-raising agonists plays a role in the pathophysiology of atherosclerosis. Supporting this notion, we have shown that Epac1 activation induces β1-integrin-mediated adhesion to fibronectin. β1-integrins mediate the arrest and initial adhesion of monocytes to the endothelium \(^{35}\). In addition, β1-integrins can bind to
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the alternatively spliced connecting segment-1 (CS-1) domain of fibronectin, which contributes to monocyte-endothelium interactions 45,46. Both endothelial β1-integrins and CS-1-containing fibronectin have been suggested to play a crucial role in atherogenesis through the recruitment of circulating monocytes 46-48. Additionally, minimally modified LDL (MM-LDL) induces the deposition of CS-1 on the endothelial surface and the induction of β1-integrin-mediated monocyte binding to this integrin fragment 46. Interestingly, treatment of endothelial cells with MM-LDL has been demonstrated to cause a rapid increase in cAMP that is necessary for the induction of monocyte binding by MM-LDL 49. The same study showed that other cAMP-elevating agents were also inducing monocyte, but not neutrophil, binding to endothelium. This is interesting, since we show here that neutrophils do not contain Epac1 protein. Together, these data suggest a model in which local concentrations of cAMP-elevating agonists in atherosclerotic lesions induce endothelial activation and monocyte recruitment, which is mediated by Epac1-induced activation of monocyte β1-integrin-mediated adhesion to fibronectin.

Serotonin is a cAMP-elevating agonist secreted by activated platelets and mast cells, and increased plasma levels of serotonin are associated with the pathophysiology of atherosclerosis and asthma 19,50. Interestingly, serotonin was recently shown to be a chemotactic factor for eosinophils 18 and to modulate cytokine and chemokine release by monocytes 51. We show here that serotonin is able to induce adhesion, polarization and chemokinesis of U937 cells similarly to Epac activation. Although we could not directly implicate Epac1 in these effects, we show that serotonin-induced adhesion requires cAMP but is PKA independent, which suggests that Epac1 activation by cAMP mediates serotonin-induced adhesion of monocytes to fibronectin. Accordingly, Epac1 has previously been shown to be activated by serotonin receptors in neuronal cells 15,16. In the concentrations used in our study, serotonin did not show chemotactic properties for U937 cells, similar to the Epac activator 8CPT-2Me-cAMP. However, both agents increased CXCL12-induced chemotaxis, indicating that other signaling pathways engaged by chemokines are likely to be required for cell movement 53-55. The enhancing effects of serotonin on migration could be due to its ability to induce a more sustained Rap1 activation than does CXCL12. This may result in the improvement of Rap1-mediated functions, such as polarization and adhesion, which are pre-requisites for directional migration. In conclusion, our data support a pro-inflammatory role for serotonin as an enhancer of monocyte adhesion and chemotaxis.

Previous reports have shown that agents that increase cAMP such as forskolin, IBMX or prostaglandin E2 inhibit chemokine-induced monocyte adhesion and migration 56,57. However, other studies demonstrated that u-PA and relaxin stimulate monocyte adhesion and migration through cAMP-dependent pathways 58,59. An explanation for these contradictory observations may be the compartmentalization of cAMP signaling in cells 60. Different signaling receptors activate differentially located members of the adenylate cyclase family and specific phosphodiesterases degrade cAMP to prevent its diffusion. This results in the formation of cAMP ‘clouds’ at discrete sites within the cell, which activate only nearby located effectors. Thus, it may be that cAMP more potently activates either PKA or Epac1, depending on the stimulus, resulting in different outcomes for adhesion and migration. Interestingly, cAMP is known to consistently inhibit neutrophil migration, which may be explained by the fact that they do not express Epac1 protein, as shown here.
In conclusion, our work reveals a previously unrecognized cAMP-dependent signaling pathway in monocytes, regulating cell adhesion, polarization and chemotaxis through the activation of Epac1. Finally, our data suggest that cAMP-elevating receptor agonists may regulate inflammatory processes through the activation of Epac1-Rap1 signaling in monocytes.

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References
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Chapter 2