

The small GTPase, Rap 1, mediates CD31-induced integrin adhesion

Reedquist, Kris; Ross, Ewan; Koop, EA; Wolthuis, RM; Zwartkuis, FJ; Van Kooyk, Y; Salmon, Michael; Buckley, Christopher; Bos, JL

DOI:

[10.1083/jcb.148.6.1151](https://doi.org/10.1083/jcb.148.6.1151)

Document Version

Publisher's PDF, also known as Version of record

Citation for published version (Harvard):

Reedquist, K, Ross, E, Koop, EA, Wolthuis, RM, Zwartkuis, FJ, Van Kooyk, Y, Salmon, M, Buckley, C & Bos, JL 2000, 'The small GTPase, Rap 1, mediates CD31-induced integrin adhesion', *Journal of Cell Biology*, vol. 148, no. 6, pp. 1151-8. <https://doi.org/10.1083/jcb.148.6.1151>

[Link to publication on Research at Birmingham portal](#)

Publisher Rights Statement:

© 2000 The Rockefeller University Press

Available online at: <http://dx.doi.org/10.1083/jcb.148.6.1151>

Eligibility Checked October 2015

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

The Small GTPase, Rap1, Mediates CD31-induced Integrin Adhesion

Kris A. Reedquist,* Ewan Ross,[‡] Elianne A. Koop,* Rob M.F. Wolthuis,* Fried J.T. Zwartkruis,* Yvette van Kooyk,[§] Mike Salmon,[‡] Christopher D. Buckley,[‡] and Johannes L. Bos*

*Laboratory for Physiological Chemistry and Centre for Biomedical Genetics, Utrecht University Medical Center, Universiteitsweg 100, 3584 CG, Utrecht, The Netherlands; [‡]Division of Immunity and Infection, and MRC Center for Immune Regulation, University of Birmingham, Birmingham B15 2TT, United Kingdom; and [§]Department of Tumor Immunology, University Hospital Nijmegen, Nijmegen, The Netherlands

Abstract. Integrin-mediated leukocyte adhesion is a critical aspect of leukocyte function that is tightly regulated by diverse stimuli, including chemokines, antigen receptors, and adhesion receptors. How cellular signals from CD31 and other adhesion amplifiers are integrated with those from classical mitogenic stimuli to regulate leukocyte function remains poorly understood. Here, we show that the cytoplasmic tail of CD31, an important integrin adhesion amplifier, propagates signals that induce T cell adhesion via $\beta 1$ (VLA-4) and $\beta 2$ (LFA-1) integrins. We identify the small GTPase, Rap1, as a critical mediator of this effect. Importantly, CD31 selectively activated the small Ras-related GTPase, Rap1, but not Ras, R-Ras, or Rap2. An activated Rap1 mutant stimulated T lymphocyte adhesion to intercellular adhesion molecule (ICAM) and vascular cell adhe-

sion molecule (VCAM), as did the Rap1 guanine nucleotide exchange factor C3G and a catalytically inactive mutant of RapGAP. Conversely, negative regulators of Rap1 signaling blocked CD31-dependent adhesion. These findings identify a novel important role for Rap1 in regulating ligand-induced cell adhesion and suggest that Rap1 may play a more general role in coordinating adhesion-dependent signals during leukocyte migration and extravasation. Our findings also suggest an alternative mechanism, distinct from interference with Ras-proximal signaling, by which Rap1 might mediate transformation reversion.

Key words: guanine nucleotide exchange factor • extravasation • leukocyte function-associated antigen 1 • integrin-mediated adhesion • lymphocyte

Introduction

In addition to soluble mitogenic stimuli, a number of leukocyte cell surface molecules have been shown to regulate integrin-mediated leukocyte adhesion. One particular receptor, CD31, a platelet endothelial cell adhesion molecule (PECAM-1)¹ (Newman, 1997), stimulates integrin-dependent adhesion in a wide variety of vascular cells, including leukocytes and platelets, via a process termed

adhesion amplification (Tanaka et al., 1992; Berman and Muller, 1995; Berman et al., 1996; Ranger et al., 1997). Numerous studies have also demonstrated a critical role for CD31 in mediating integrin-dependent leukocyte chemotaxis and extravasation in vitro and in vivo (Berman et al., 1996; Newman, 1997; Duncan et al., 1999). The molecular basis for this has remained obscure, but recent studies have demonstrated that, in addition to mediating homophilic cell adhesion (Newton et al., 1997), CD31 acts as an adhesion-dependent signaling receptor (Newman, 1997; Newton et al., 1997; Pumphrey et al., 1999). CD31 ligation stimulates tyrosine phosphorylation of CD31, leading to its association with a number of cytoplasmic signaling proteins, including SHP-1, SHP-2, SHIP, and PLC- γ 1, suggesting that CD31 functions as a dynamic modulator of vascular cell behavior (Newman, 1997; Newton et al., 1997; Pumphrey et al., 1999).

Address correspondence to J.L. Bos, Laboratory for Physiological Chemistry and Centre for Biomedical Genetics, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands. Tel.: 31 30 2538989. Fax: 31 30 2539035. E-mail: j.l.bos@med.uu.nl

¹*Abbreviations used in this paper:* GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; GPI, glycosylphosphatidylinositol; GST, glutathione-S-transferase; HA, hemagglutinin; ICAM, intercellular cell adhesion molecule; PECAM, platelet endothelial cell adhesion molecule; RBD, Ras-binding domain; VCAM, vascular cell adhesion molecule; WT, wild-type.

Several reports suggest a role for Ras family GTPases in the control of integrin-mediated adhesion. Ras superfamily GTPases cycle between inactive GDP-bound forms and active GTP-bound forms, and exchange for GTP and hydrolysis of GTP to GDP are catalyzed by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), respectively (Bos, 1997, 1998). Use of active and dominant-negative mutants of H-Ras and R-Ras has revealed that these enzymes can regulate coupling of $\beta 1$ and $\beta 2$ integrin-dependent adhesion to T cell receptor (TCR/CD3), interleukin-3, and chemokine receptor signaling in T lymphocytes and pro-B cell leukemic cell lines (Zhang et al., 1996; O'Rourke et al., 1998; Liu et al., 1999; Shibayama et al., 1999; Tanaka et al., 1999). We have examined whether CD31 stimulates integrin-dependent adhesion via an intracellular signaling pathway, and if so, whether members of the Ras family were involved in this process. We find that the cytoplasmic tail of CD31 is essential for CD31-induced adhesion, and that Rap1 is specifically and selectively involved in this process.

Materials and Methods

Hemagglutinin (HA)-tagged Rap1 (Rap), RapV12, and H-RasV12 in the mammalian expression vector pMT2HA, pRSV, and pRSV-RapN17 have been described previously (Zwartkruis et al., 1998). pMT2HA-R-RasV38 was generated by subcloning a full-length NcoI (Klenow-filled)/XhoI fragment of R-RasV38 into NotI (mung bean nuclease-digested)/XhoI-digested pMT2HA. A Sall/NotI fragment encoding amino acids 200–297 of human RalGDS was amplified by PCR to generate pMT2HA-RalGDS-RBD. pMT2-HA-RapGAP was generated by subcloning full-length RapGAP (provided by Dr. Paul Polakis, Onyx Pharmaceuticals, Richmond, CA) as a Sall/BglII fragment into pMT2HA. Catalytically inactive RapGAP LIG was generated by mutating RapGAP amino acids 284–286 (RKR) to LIG using the Stratagene Quick site-directed mutagenesis kit. All PCR-generated sequences were checked by DNA sequencing. pCAGGS-C3G and pSR-His-tagged Spa1 were provided by Drs. Michiyuki Matsuda (National Institutes of Health, Tokyo, Japan) and Masakazu Hattori (Kyoto University, Kyoto, Japan), respectively.

Jurkat T cell lines were maintained as described previously (Reedquist and Bos, 1998). The Jurkat cell line JHM1 2.2 was provided by Dr. Doreen Cantrell (Imperial Cancer Research Fund, London, UK), with kind permission of Dr. Art Weiss (University of California at San Francisco, San Francisco, CA). Jurkat cells used to generate CD31 wild-type (WT), CD31 glycosylphosphatidylinositol (GPI), and Y663/686F stable transfectants were provided by Dr. D. Samson (University of Bath, Bath, UK). CD31⁺ and CD31⁻ variants were established by six rounds of MACs-sorting of parental Jurkat cells with anti-CD31 antibody 10B9. The negative variant was transfected with CD31 WT, CD31 GPI, or CD31 Y663/686F cDNA in pCDNA3 (Jackson et al., 1997; Newton et al., 1997), and stable polyclonal lines established by selection in G418 (1 mg/ml) and FACS sorting. Jurkat cells were transiently transfected by electroporation with 35 μ g plasmid DNA. Jurkat cells (1.2×10^7 cells/ml in 0.4 ml complete media) were pulsed at 250 V and 960 μ F with 5 μ g TK-luciferase plasmid DNA, construct plasmids as indicated in figure legends, and added vector plasmid to keep DNA amounts constant. 24 h after transfection, cells were transferred to serum-free media and used 42–48 h after transfection for adhesion assays. Subconfluent A14 and COS-7 cells were transfected by calcium phosphate precipitation as described previously (Zwartkruis et al., 1998).

Anti-CD3 antibody T3b was kindly provided by Dr. Hergen Spits (Netherlands Cancer Institute, Amsterdam, The Netherlands). Anti-integrin $\beta 2$ antibody L15, activating anti-integrin $\beta 1$ antibody TS2/16, and activating anti-integrin $\beta 2$ KIM185 have been described previously (van de Wiel-van Kemenade et al., 1992; Andrew et al., 1993). mAb 24, recognizing a ligand-induced epitope of αL , was kindly provided by Dr. Nancy Hogg (Imperial Cancer Research Fund, London, UK). Anti-CD31 antibodies, PECAM 1.2 and PECAM 1.3, were generous gifts from Dr. Peter Newman (Blood Center of Southeastern Wisconsin, Milwaukee, WI). Anti-CD31 antibodies 2H8, WM59, GI18, and 9G11 have been described previously (Yan et al., 1995; Newton et al., 1997). Other mAbs used in these

studies were anti-Rap1, RalA, and Ras antibodies (Transduction Laboratories). Rabbit polyclonal anti-Rap2 and R-Ras antibodies were from Santa Cruz.

Purification of GST-RBD fusion proteins and their use in detecting activated Rap1, Rap2, Ras, and R-Ras by precipitation, SDS-PAGE, and immunoblotting have been described previously (de Rooij and Bos, 1997; Franke et al., 1997; Reedquist and Bos, 1998). Detection of bound radiolabeled GDP and GTP to Rap1 was also described previously (Zwartkruis et al., 1998).

For adhesion assays, transiently transfected Jurkat cells were harvested, washed, and resuspended in TSM media (20 mM Tris, pH 8, 150 mM NaCl, 1 mM CaCl₂, 2 mM MgCl₂). 96-well Costar Maxisorp plates were coated overnight at 4°C with goat anti-human IgG antibodies (Jackson ImmunoResearch; 4 μ g/ml in TSM), washed, blocked for 30 min at 37°C with 1% BSA/TSM, followed by incubation 1 h at 37°C with 50 ng/ml recombinant intercellular adhesion molecule (ICAM)-1 or vascular cell adhesion molecule (VCAM)-1 human IgG Fc fusion proteins. The 50- μ l cell suspension was mixed with 50 μ l TSM or TSM with stimuli indicated. Cells were allowed to adhere for 30–60 min, and nonadherent cells removed with warm 0.5% BSA/TSM. Adherent cells were lysed and subjected to luciferase assays as described previously (Medema et al., 1992). Expression of transfected constructs was confirmed by immunoblotting of total cell lysates. Cells bound were calculated and were corrected for transfection efficiency and nonspecific effects of constructs by measuring luciferase activity of total input cells. Comparison of effects of cDNA construct overexpression on adhesion were made by paired *t* test or unpaired *t* test as appropriate. Fluorescent labeling of stably transfected cells with 2'-7'-bis-(2-carboxyethyl)-5-(and-6)-carboxy fluorescein acetoxymethyl ester (Molecular Probes) and measurement of adherent cells with a Fluorescan Ascent fluorescent plate reader (Lab Systems) has been described previously (Newton et al., 1997).

Flow cytometric analysis of transfected cells was performed by cotransfecting Jurkat JHM1 cells with 1 μ g pCMV-EGFP C1 plasmid (Promega) and the indicated cDNA constructs. After overnight serum starvation, cells were equilibrated in 0.5% BSA/1 mM CaCl₂/PBS (FACS buffer) and left unstimulated, or stimulated for 30 min with plate-immobilized anti-CD31 antibody 2H8 (10 μ g/ml). Cells were harvested, resuspended in FACS buffer containing primary antibodies (10 μ g/ml), and incubated for 30 min on ice, or 37°C for mAb 24 epitope expression. For mAb 24 staining, additional sets of transfected cells were coincubated with 400 μ M MnCl₂ during primary antibody staining. Cells were washed with FACS buffer and stained with secondary rabbit anti-mouse RPE-Cy5-conjugated antibodies (Dako). Fluorescence intensity of EGFP-transfected cells was determined using a FACs Caliber flow cytometer and CellQuest software (both from Becton Dickinson).

Results and Discussion

Previously, CD31 has been demonstrated to stimulate T lymphocyte adhesion to ICAM and VCAM via T cell LFA-1 ($\alpha L\beta 2$ integrin) and VLA-4 ($\alpha 4\beta 1$ integrin), respectively (Tanaka et al., 1992). To examine whether CD31-stimulated integrin-dependent adhesion in T cells was regulated by signals generated through the CD31 cytoplasmic tail or via interactions using the extracellular domain of CD31 (Hemler, 1998), we made use of Jurkat cells stably expressing full-length CD31 (CD31 WT), a GPI-anchored CD31 construct, previously shown to mediate CD31 homophilic binding (Newton et al., 1997), but lacking the CD31 cytoplasmic tail, or full-length CD31 containing two tyrosine-to-phenylalanine mutations (Y663/686F) in the major tyrosine-phosphorylation sites of the CD31 cytoplasmic tail (Pumphrey et al., 1999). CD31 expression of parental, CD31⁺- and CD31⁻-selected variants, and stable transfectants is shown in Fig. 1 A, and was equivalent in CD31 WT, CD31 GPI, and Y663/686F lines. All cell lines also expressed similar levels of αL , $\alpha 4$, $\alpha 5$, $\beta 1$, and $\beta 2$ integrins (data not shown). Although all cell lines adhered to ICAM-1 and VCAM-1 when stimulated by PMA (data not shown), only CD31 WT transfectants, but not CD31

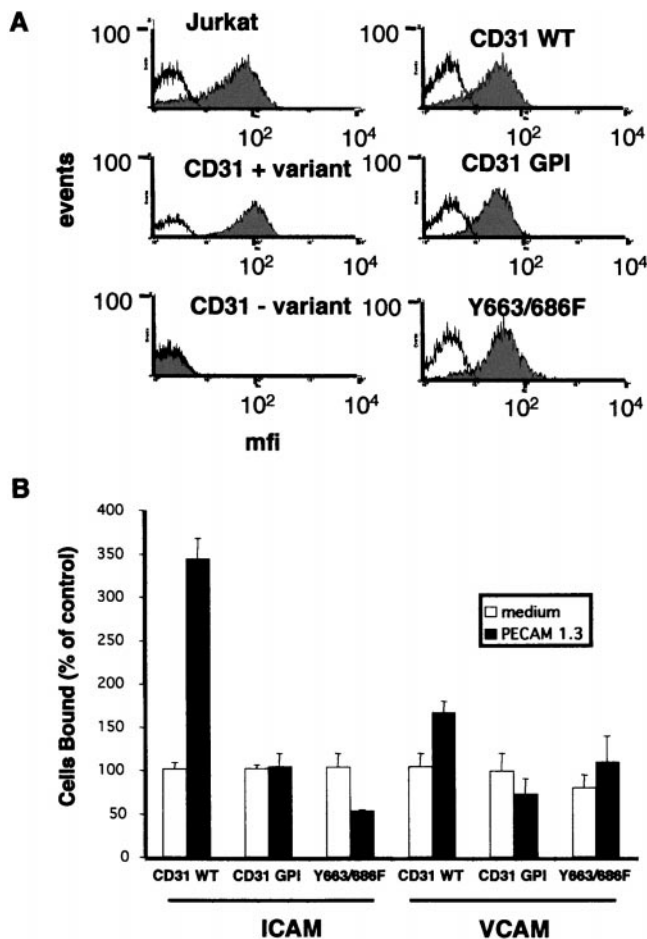


Figure 1. CD31-dependent adhesion to ICAM and VCAM requires the cytoplasmic tail of CD31. **A**, CD31 expression on parental, CD31 positive and negative variants and CD31 WT, CD31 GPI, and Y663/686F Jurkat cell transfectants. Cells were stained with control antibody (open histogram) or anti-CD31 antibody 10B9 (shaded histogram) and mean fluorescent intensity (mfi) analyzed by FACS analysis. **B**, Adhesion of CD31 WT, CD31 GPI, and Y663/686F Jurkat cell lines to ICAM and VCAM. Fluorescently labeled cells were allowed to adhere to purified ICAM or VCAM (2 μ g/ml) coated on 96-well plates (Nunc Maxisorp) in the absence (medium) or presence of 10 μ g/ml each of anti-CD31 antibody PECAM 1.3 and goat anti-mouse antibody. Total input and bound fluorescent cells was measured. Bars represent the average mean and error of the percent of cells bound (cells bound/total input cells \times 100, normalized to 100% for unstimulated cells) of two representative experiments performed in triplicate.

GPI and Y663/686F cells, adhered to ICAM-1 or VCAM-1 after stimulation with anti-CD31 antibodies (Fig. 1 B). This suggested that CD31-induced, integrin-mediated adhesion requires intracellular signaling pathways generated by the cytoplasmic tail of CD31, specifically tyrosine phosphorylation of tyrosine residues 663 and/or 686, but not signaling pathways mediated by cis-interactions of the CD31 extracellular or transmembrane domains.

To address which Ras family members, if any, might participate in CD31 signaling, we precipitated endogenous GTPases from lysates of CD31-stimulated Jurkat cells with GST fusion proteins of Ras family-binding domains (RBDs) of Raf and RalGDS, which bind with high selec-

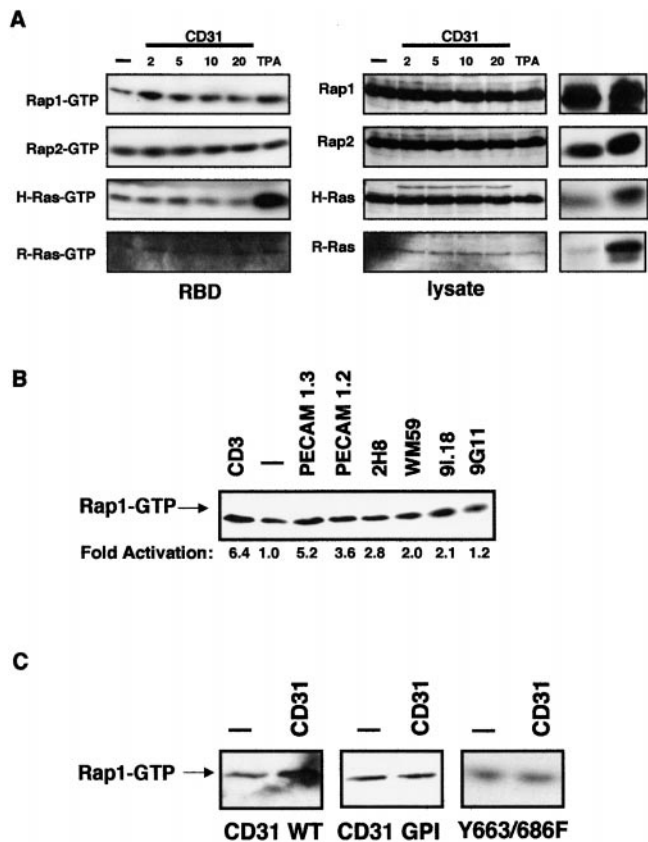


Figure 2. CD31 selectively activates Rap1, but not other Ras family GTPases. **A**, Jurkat JHMI 2.2 cells were unstimulated (-) or stimulated with anti-CD31 antibody PECAM 1.3 (10 μ g/ml) for the indicated times or with TPA (100 ng/ml, 5 min), lysed, and GTP-bound GTPases were precipitated with immobilized GST fusion proteins of RalGDS-RBD (for Rap1 and Rap2) or Raf-RBD (for Ras and R-Ras). Bound GTPases were resolved on SDS-PAGE, transferred to PVDF membrane, and detected by immunoblotting with antibodies against the indicated proteins, followed by enhanced chemiluminescence. **B**, Antibodies against CD31 extracellular domains regulating cell adhesion activate Rap1. JHMI cells were activated for 2 min with medium (-), anti-CD3 antibody T3b (1:200) or the indicated anti-CD31 antibodies (10 μ g/ml), and Rap1 activation detected as in **A**. Fold activation of Rap1 compared with untreated cells was obtained by quantitating pixels from representative blots using Adobe Photoshop software. **C**, CD31-dependent activation of Rap1 requires CD31 cytoplasmic tail. CD31 WT, CD31 GPI, and Y663/686F Jurkat cell lines were stimulated for 2 min with medium (-) or 10 μ g/ml PECAM 1.3 antibody and activated Rap1 detected as in **A**. Results shown in each panel are representative of three independent experiments.

tivity and specificity to activated Ras and Rap GTPases, respectively (de Rooij and Bos, 1997; Franke et al., 1997). Immunoblotting of precipitated GTPases provides a qualitative representation of GTPase activation status after cell stimulation, corresponding to quantitative changes detected using classical GDP/GTP-binding ratio techniques. Time course analyses of Jurkat cells treated with anti-CD31 antibodies or TPA (Fig. 2 A) surprisingly revealed a rapid activation of the Ras-related GTPase, Rap1, by CD31, which was maximal 2–5 min after stimulation and decreased slowly toward basal levels after 20 min. In con-

trast, whereas both Rap1 and Ras were activated by TPA, CD31-dependent stimulation of Rap1 was highly selective and no activation of Rap2, Ras, or R-Ras was noted at any point during this time course. Both Rap1 and Ras, but not the other GTPases tested, were also activated by TCR stimulation (data not shown). GTP-bound R-Ras was detected in Raf-RBD pull-downs only after longer exposures of films, reflecting the relatively low levels of R-Ras protein expression in Jurkat cells. R-Ras protein expression was at least tenfold lower in Jurkat cells as compared with A431 fibroblasts, while Rap1, Rap2, and Ras levels were relatively equivalent (Fig. 2 A, right, last two lanes).

Functional anti-CD31 antibodies against CD31 extracellular domains 1 and 6 (PECAM 1.2, PECAM 1.3, and 2H8), activated Rap1 more potently than nonfunctional antibodies (WM59, 9G11, and GI18; Fig. 2 B), correlating with the ability of these antibodies to induce CD31-dependent adhesion (Newton et al., 1997). Additionally, activation of Rap1 by anti-CD31 antibodies was observed in CD31 WT Jurkat cell transfectants, but not CD31 GPI or Y663/686F transfectants (Fig. 2 C), demonstrating a correlative requirement for the CD31 cytoplasmic tail in the activation of Rap1 and induction of adhesion.

As CD31 stimulation selectively activated Rap1, and Rap1 activation correlated with CD31-dependent adhesion to ICAM and VCAM, we next addressed whether constitutive activation of Rap1 was sufficient to induce adhesion. Jurkat T cells transiently transfected with a luciferase reporter plasmid and indicated cDNA constructs were allowed to bind to immobilized recombinant ICAM and adhering transfected cells detected by measurement of luciferase activity. T cells expressing constitutively active Rap1 (RapV12) or the Rap1 GEF C3G (Tanaka et al., 1994) displayed ~3.5- and 3-fold increases in adhesion to ICAM, respectively (Fig. 3 A). C3G-induced spreading and adhesion on fibronectin has been observed previously in 32-D cells, although involvement of Rap1 was not examined and Ras, R-Ras, or other GTPases were implicated (Arai et al., 1999). Expression of either the active form of Ras (RasV12) or R-Ras (R-RasV38) resulted in a

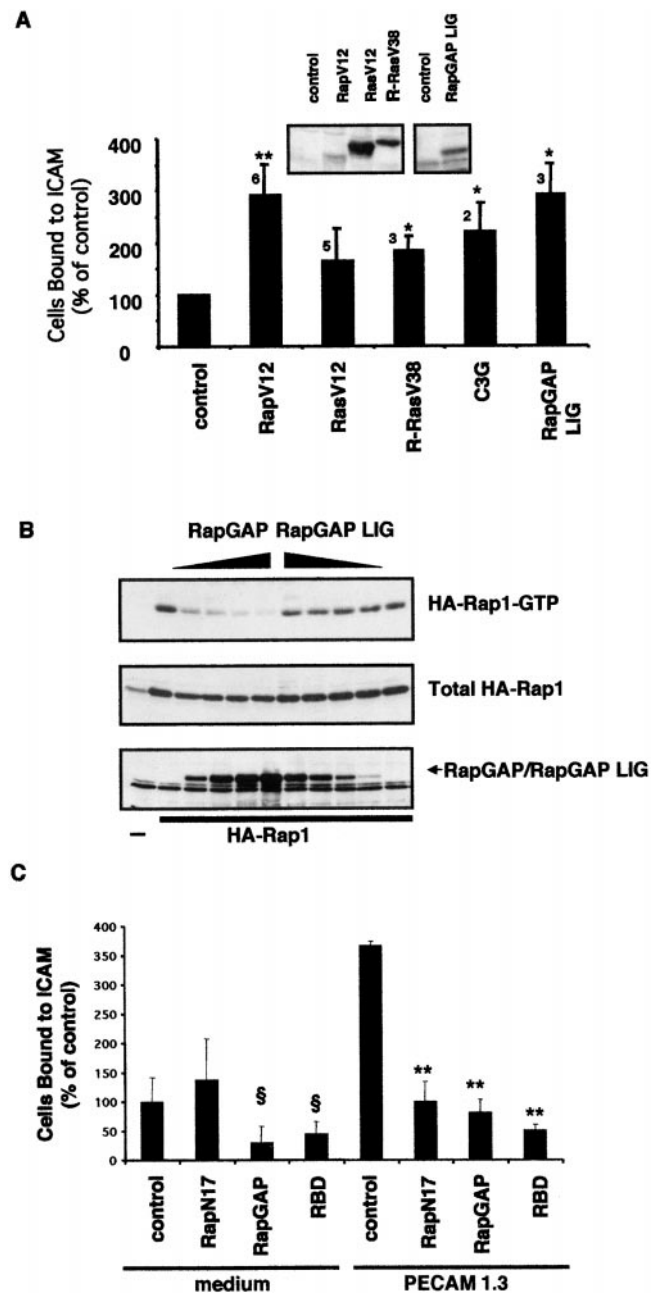


Figure 3. Rap1 regulates Jurkat cell adhesion via LFA-1 ($\beta 2$) integrin. **A**, Activation of Rap1 induces Jurkat cell adhesion to ICAM. Jurkat JHMI cells were transiently transfected with 5 μ g PG3-TK luciferase reporter plasmid and empty vector (control) or pMT2-HA-RapV12, H-RasV12, R-RasV38 (each 10 μ g), RapGAP-LIG (20 μ g), or pCAGGS-C3G (10 μ g). Cells were allowed to adhere for 1 h on immobilized ICAM, washed, lysed, and adherent cells were quantitated by luciferase assay. Bars represent the average mean binding and standard error of the percent of cells bound from two to six independent experiments (indicated above bars) performed in triplicate or quadruplicate, as calculated in Fig. 1. Typically, 10–20% of control unstimulated cells adhered to ICAM. ** $P < 0.01$; * $P < 0.05$; and § $P < 0.1$, compared with control cells by paired t test. Expression levels of transfected GTPases and RapGAP-LIG from a representative adhesion assay were examined by immunoblotting lysates with anti-HA epitope antibody 12CA5 as in Fig. 2 A (insets). Blots shown in insets are cut from a single film exposure of one immunoblot. **B**, RapGAP-LIG mutant fails to stimulate GTP-hydrolysis on Rap1. A14 cells were transfected with vector alone, HA-Rap1 alone, or in combination with increasing amounts (0.3, 1,

2.5, and 5 μ g) of HA-tagged RapGAP or RapGAP-LIG, as indicated above top panel. Active HA-Rap1 was precipitated from cell lysates with RalGDS-RBD and detected by 12CA5 immunoblotting as in Fig. 2 (top). Transfection of HA-Rap1 (middle) and RapGAP constructs (bottom) was monitored by immunoblotting of total cell lysates with anti-HA antibodies. **C**, Rap1 signaling is required for CD31-dependent adhesion to ICAM. WT CD31 cells transfected with pG3-TK luciferase reporter plasmid and 30 μ g of empty vector (control) or the indicated DNA construct were allowed to adhere to immobilized ICAM as in **A** after 30-min stimulation with medium or 10 μ g/ml anti-CD31 antibody PECAM 1.3 cross-linked with goat anti-mouse antibodies. Cells bound were quantitated using luciferase assay and the percent of cells bound was calculated as in Fig. 1. Shown is a representative experiment performed in quadruplicate. P values are indicated as in **A** and represent transfected cell populations compared with control unstimulated cells (medium) or CD31-stimulated control cells.

lower, but detectable induction of adhesion (1.5–2-fold). However, RasV12-stimulated adhesion was not statistically significant, RasV12 stimulated adhesion in only two of five experiments, and RapV12-induced adhesion was always higher than that induced by RasV12 and R-RasV38, even though the latter two constructs were expressed at significantly higher levels than RapV12 (Fig. 3 A, left inset). Introduction of a RapGAP mutant (RapGAP-LIG; Rubinfeld et al., 1991), containing a substitution of amino acids 284–286 in the RapGAP arginine finger critical for RapGAP catalytic activity (RKR, mutated to LIG), which, unlike wild-type RapGAP, displays no GAP activity toward Rap1 (Fig. 3 B), increased the number of ICAM-bound cells to a similar level as observed with RapV12 and C3G. RapGAP-LIG may perturb Rap1 signaling by displacing endogenous RapGAP from a subcellular localization required for downregulating Rap1 or may constitutively associate with basally activated Rap1 and serve as an effector protein linking Rap1 to an as yet unidentified target.

We next examined if inhibition of Rap1 signaling would abolish CD31-dependent adhesion to ICAM. Therefore, we introduced a putative dominant-negative mutant of Rap1, RapN17, into cells. RapN17 did not affect basal adhesion of Jurkat cells to ICAM, but completely abolished CD31-dependent increases in adhesion (Fig. 3 C). Although the RapN17 mutation was modeled on the corresponding dominant-negative mutation in H-Ras (Medema et al., 1991), RapN17 is not considered to function as a strict dominant-negative protein. Whereas RasN17 blocks Ras signaling by sequestering Ras GEFs, RapN17 fails to bind to Rap1 GEFs *in vitro* (van den Berghe et al., 1997), and fails to inhibit EGF-dependent activation of cotransfected wild-type Rap1 in COS-7 cells (Wolthuis, R.M.F., and J.L. Bos, unpublished observation).

Because of uncertainties in the mechanism by which RapN17 might block CD31-stimulated adhesion, we sought independent confirmation that Rap1 was required for CD31-induced adhesion of Jurkat cells to ICAM. Transient expression of the Rap1-specific GAP, RapGAP, resulted in a strong reduction of basal adhesion to ICAM, and significantly reduced CD31-dependent adhesion (Fig. 3 C). This result is compatible with the notion that a Rap1GAP should decrease levels of GTP-bound Rap1 (see Fig. 3 B), but should still allow ligand-induced activation of Rap1. Finally, we used the Rap1 binding domain of RalGDS, RBD, which binds with high affinity and specificity to GTP-bound Rap1 *in vitro* (Franke et al., 1997). This fragment is hypothesized to block effector binding to Rap1. Expression of RBD significantly abolished both basal and CD31-induced adhesion to ICAM (Fig. 3 C). Thus, using three independent strategies for interfering with Rap1 signaling, overexpression of inactive Rap1, Rap1-specific RapGAPs, and an isolated RBD of a Rap-binding protein, these experiments demonstrate a critical role for Rap1 in mediating CD31-induced adhesion to ICAM.

To address whether Rap1 selectively regulated β 2 integrins (LFA-1) on Jurkat cells, or if it was also coupled to the regulation of β 1 integrins (VLA-4), we examined the effects of activating and inactivating mutants of Rap signaling on Jurkat cell adhesion to VCAM. Stimulation of

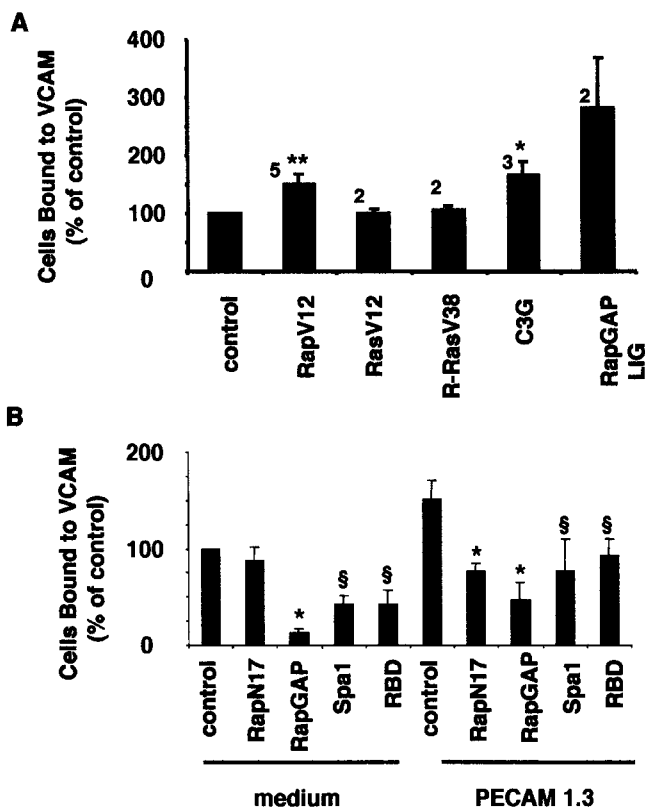


Figure 4. Rap1 mediates CD31-dependent adhesion via β 1 (VLA-4) integrins. **A**, Activation of Rap1 is sufficient to induce adhesion to VCAM. CD31 positive variant Jurkat cells were transfected with 5 μ g pG3-TK luciferase reporter plasmid and empty vector (control), HA-tagged RapV12, H-RasV12, R-RasV38 (10 μ g each), C3G (10 μ g each), or RapGAP-LIG (20 μ g), and were allowed to adhere to immobilized VCAM (50 ng/ml). Adherent cells and the percent of cells bound from five (RapV12), three (C3G), or two (RasV12, R-RasV38, and RapGAP-LIG) independent experiments performed in triplicate or quadruplicate were quantitated and calculated as in Fig. 1. 30–50% specific adhesion of control cells to VCAM was observed in the presence of medium alone. Cells transfected with RapV12 (** $P < 0.01$) and C3G (* $P < 0.05$), but not RasV12 or R-RasV38, bound at significantly higher levels than control cells. RapGAP-LIG, $P < 0.1003$. **B**, Rap1 signaling is required for CD31-dependent adhesion to VCAM. CD31 WT cells were transfected with 5 μ g pG3-TK-luciferase and 30 μ g empty vector (control), HA-tagged RapN17, RapGAP, RalGDS-RBD, or His-tagged Spa1 were allowed to adhere to VCAM as in **A** in the absence or presence of cross-linked anti-CD31 antibody PECAM 1.3 (top). Paired *t* tests of transfected cells revealed that cells transfected with RapGAP (* $P < 0.05$), Spa1, and RBD ($\$P < 0.1$) had significantly lower adhesion than control unstimulated cells, and that RapN17, RapGAP ($P < 0.05$), Spa1, and RBD ($P < 0.1$) significantly blocked CD31-dependent adhesion.

CD31 resulted in a smaller induction of adhesion to VCAM (1.5–2-fold increase; Fig. 4 A) than observed to ICAM, in part due to the higher basal level of adhesion observed on VCAM (typically 30–50%, compared with 10–20% on ICAM). A similar increase was also observed in cells expressing RapV12, C3G, and mutant RapGAP-LIG, but not RasV12 or R-RasV38. Conversely, RapN17,

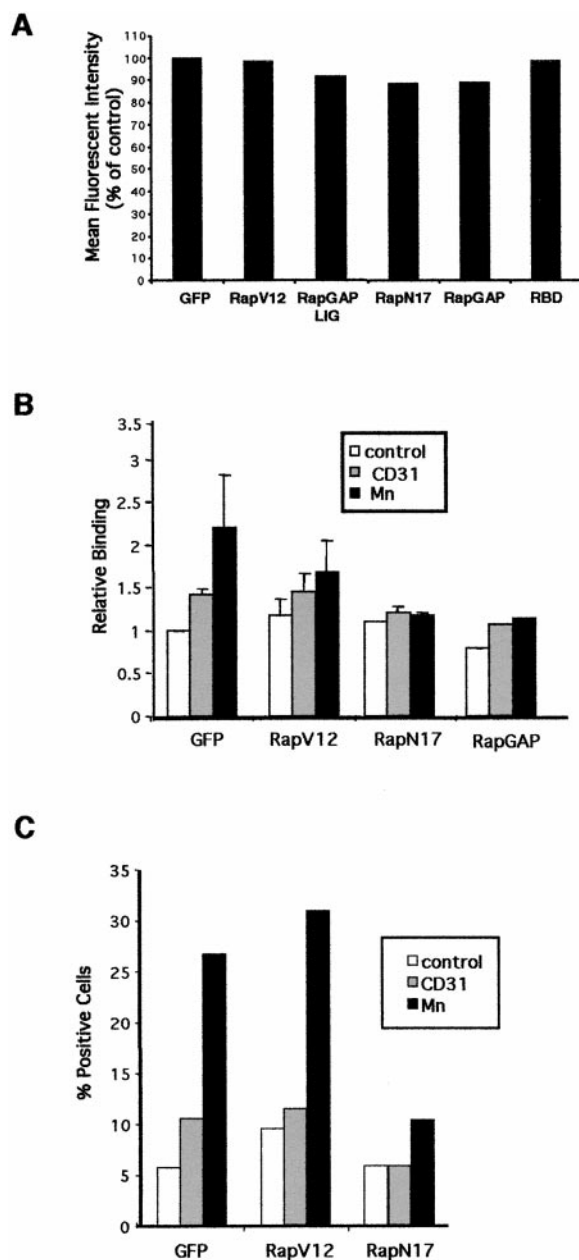
RapGAP, and a second GAP for Rap1, Spa1 (Kurachi et al., 1997), significantly reduced basal Jurkat cell adhesion to VCAM, and strongly blocked CD31-dependent adhesion (Fig. 4 B).

It has been reported previously that Ras regulates TCR-stimulated lymphocyte adhesion to ICAM (O'Rourke et al., 1998; Tanaka et al., 1999). As we failed to detect Ras activation by CD31, and RasV12 did not induce a clear increase in adhesion to ICAM or VCAM, it is unlikely that Ras mediates the main signaling pathway from CD31 stimulation to integrin-mediated adhesion. However, overexpression of RasN17 did inhibit CD31-dependent adhesion to VCAM (data not shown), suggesting that Ras might also contribute to adhesion responses.

Integrin-dependent adhesion can be regulated by changes in integrin surface expression, integrin surface distribution (avidity), or induction of conformational changes that increase integrin ligand affinity (Stewart and Hogg, 1996). To investigate at which level Rap1 might influence cell adhesion, we first examined if Rap signaling affected LFA-1 expression levels. Jurkat cells were cotransfected with EGFP as a reporter marker, along with indicated Rap1 signaling pathway cDNA constructs. Staining of transfected cells with anti- β 2 integrin antibody revealed that overexpression of RapV12, RapN17, RBD, RapGAP, or RapGAP LIG failed to influence surface expression levels of LFA-1 (Fig. 5 A). CD31 expression levels were also unaffected (data not shown). Similarly, RapV12, RapGAP LIG, RapGAP, and RapN17 failed to induce LFA-1 surface clustering, as detected with the NKI-L16 antibody (data not shown), although active RasV12 induced clustering of LFA-1, as previously reported (Tanaka et al., 1999).

The anti- α L integrin antibody mAb 24 recognizes a conformation-dependent ligand-induced epitope on LFA-1, and induction of the mAb 24 epitope correlates with increased LFA-1 affinity for ICAM (Dransfield and Hogg, 1989). TCR and TPA-induced mAb 24 epitope expression previously have been found to be dependent on the presence of ICAM, suggesting that signals generated by TCR and TPA facilitate weak basal interactions between LFA-1 and ICAM, stabilizing LFA-1 in a high-affinity conformation (Cabanas and Hogg, 1993). This conformational change can also be induced by LFA-1 exposure to Mn^{2+} , independently of "inside-out" signaling. We found that anti-CD31 stimulation of Jurkat cells, or Jurkat cells trans-

Figure 5. Rap signaling regulates ligand-induced conformational changes in LFA-1. A, Expression levels of LFA-1 β 2 integrin on transfected cells. Jurkat JHMI cells were transiently transfected with 1 μ g pCMV-EGFP C1 reporter plasmid along with empty vector (GFP) or the indicated cDNA constructs. Cells were sequentially stained with anti- β 2 integrin antibody L15 and rabbit anti-mouse Ig-RPE-Cy5 conjugate, and GFP-expressing cells were analysed by flow cytometry. Values are presented as mean fluorescent intensity (arbitrary units) normalized to 100% for cells expressing GFP alone. B, Rap signaling is required for CD31 and Mn^{2+} -induced conformational changes in LFA-1. JHMI cells were transfected as in A and left unstimulated (control) or were stimulated for 30 min with anti-CD31 2H8 antibody (CD31), followed by staining with the ligand-induced binding anti-LFA-1 antibody mAb 24, as in Materials and Methods. Al-



ternatively, unstimulated cells were coincubated with mAb 24 and 400 μ M $MnCl_2$ (Mn). mAb 24 expression was assessed by flow cytometry, and expression levels were normalized to 1 for unstimulated GFP-alone cells in each experiment. Data represent the means and standard errors of the representative experiments (GFP, $n = 4$; RapV12 and RapN17, $n = 3$; RapGAP LIG and RBD, $n = 2$). Statistically significant differences between control and stimulated cells, and between stimulated transfected cells are noted ($*P < 0.01$; $**P < 0.05$; $^{\S}P < 0.1$). C, RapN17 blocks mAb 24 induction. The experiment was performed as in B, but data represents the percent of mAb 24-expressing cells. D, Rap signaling regulates Mn^{2+} -induced adhesion to ICAM. Jurkat cells were transfected with luciferase reporter plasmid and indicated constructs as in Fig. 3, and were allowed to adhere to ICAM in absence or presence of 4 mM $MnCl_2$, and the percent of the specific adhesion was calculated as in Fig. 3, and normalized to 100 for mock-transfected stimulated cells. Data represent the average mean and error of two to four experiments, with each construct performed in quadruplicate. Statistically significant differences are indicated ($*P < 0.01$; $**P < 0.05$).

ected with GFP alone, resulted in an ~1.5-fold increase in mAb 24 expression (Fig. 5 B and data not shown), whereas a 2.25–2.5-fold increase was observed in Mn²⁺-treated cells. Incubation of cells with mAb 24 at 4°C abolished CD31 and Mn²⁺-induced mAb 24 expression to background levels (data not shown), consistent with the temperature sensitivity of the mAb 24 epitope (Dransfield and Hogg, 1989). Although RapV12 expression had no statistically significant effect on basal or induced mAb 24 epitope expression, RapN17 and RBD abolished induction of the mAb 24 epitope by CD31 and Mn²⁺. Conversely, RapGAP LIG enhanced CD31- and Mn²⁺-induced expression of the mAb 24 epitope. RapN17 also inhibited the increase in the percentage of mAb 24-reactive cells observed after CD31 or Mn²⁺ treatment (Fig. 5 C). Thus, our studies suggest that whereas Rap does not directly stimulate activation of LFA-1, signaling via Rap1, or basal activation of Rap1, appears to be a requisite factor for stabilization of the active conformation of LFA-1. Moreover, our finding that RapN17 blocks direct conformational changes induced by extracellular Mn²⁺ may suggest that inactive Rap may act to lock LFA-1 in an inactive conformation. Consistent with this model, overexpression of RapN17, RapGAP, or RalGDS RBD significantly blocked Mn²⁺-induced adhesion of Jurkat cells to immobilized ICAM, whereas RapV12 augmented the induced cell adhesion (Fig. 5 D).

Our report is the first example of requisite involvement of Rap1 in coupling cell surface receptor stimulation to integrin-mediated adhesion. Previously, it was shown that Rap1 is activated after stimulation of a large variety of cell surface receptors, including B and T cell antigen receptors, chemokine and cytokine receptors (including fMLP, PAF, and GM-CSF), and receptors for platelet agonists (thrombin, thromboxane A₂, and ADP; reviewed in Bos, 1997). Many of these receptors have been implicated in the control of integrin-mediated adhesion. Furthermore, the involvement of Rap1 in the control of cell adhesion recently has been suggested by studies in which Spa1 was found to inhibit G-CSF-induced adhesion of 32-D cells to culture dishes (Tsukamoto et al., 1999). Also, FcεRI-mediated adhesion of RBL cells to culture dishes is inhibited by expression of RapGAP (M'Rabet, 1999). Therefore, it is plausible to propose that one of the functions of Rap1 is to control integrin-mediated adhesion.

The mechanism by which Rap1 signaling modulates integrin function is unknown, although our data suggest that Rap1 may regulate the ability of LFA-1 to undergo ligand-induced conformational changes. This effect could be direct, by binding of Rap1 to integrins or integrin-associated complexes. Interestingly, Rap2, a very close relative of Rap1, has been found in complex with the α_{IIB}β₃ integrin (Torti et al., 1999), although no extracellular stimuli coupling to Rap2 activation have been identified. Alternatively, Rap1 may stimulate effector pathways that regulate integrins. Putative downstream signaling pathways of Rap1 include B-Raf/MAP kinase cascade and Ral GTPase GEFs (Bos, 1997, 1998), although we have not detected CD31-dependent activation of MAP kinase or Ral in Jurkat cells (data not shown). Novel binding partners of activated Rap1 recently identified *in vitro*, Krit1 (Serebriiskii et al., 1997) and AF6 (Linnemann et al., 1999), are pro-

teins lacking any obvious enzymatic activity and are thought to associate with cytoskeletal components. Our identification of integrin-adhesion as a physiological target of Rap1 should facilitate identification of Rap1 effectors. Finally, the unique coupling of CD31, which is also expressed on vascular cells, to Rap1 suggests a critical role for Rap1 in mediating not only leukocyte adhesion, but other integrin-dependent events mediated by CD31 that require platelet and leukocyte interactions with vascular endothelial cells, including leukocyte transmigration and extravasation. Interestingly, regulation of integrin adhesion might also explain the flat revertant phenotype of Ras-transformed cells expressing Rap1 (Kitayama et al., 1989).

We thank Drs. Peter Newman for the generous gifts of PECAM 1.2 and 1.3 antibodies and CD31 cDNA constructs, Nancy Hogg (Imperial Cancer Research Fund, London) for mAb 24 antibody, Doreen Cantrell (Imperial Cancer Research Fund, London) for Jurkat JHMI cells, and Hergen Spits (The Netherlands Cancer Institute, Amsterdam) for anti-CD3 T3b antibody. We are grateful to Veronique Smits and Dr. Rene Medema (Utrecht University) for assistance with FACS analysis, and Miranda van Triest (our laboratory) for excellent technical assistance.

K.A. Reedquist was supported by a postdoctoral fellowship from the Netherlands Royal Academy of Sciences (KNAW). F.J.T. Zwartkruis and R.M.F. Wolthuis were funded by the Dutch Cancer Society. E. Ross and C.D. Buckley were supported by grants from the Nuffield Foundation (RHE/97/273) and the Wellcome Trust, respectively.

Submitted: 6 December 1999

Revised: 7 February 2000

Accepted: 8 February 2000

References

- Andrew, D., A. Shock, E. Ball, S. Ortlepp, J. Bell, and M. Robinson. 1993. KIM185, a monoclonal antibody to CD18 which induces a change in the conformation of CD18 and promotes both LFA-1- and CR3-dependent adhesion. *Eur. J. Immunol.* 9:2217–2222.
- Arai, A., Y. Nosaka, H. Kohsaka, N. Miyasaka, and O. Miura. 1999. CrkL activates integrin-mediated hematopoietic cell adhesion through the guanine nucleotide exchange factor C3G. *Blood.* 93:3713–3722.
- Berman, M.E., and W.A. Muller. 1995. Ligation of platelet/endothelial cell adhesion molecule 1 (PECAM-1/CD31) on monocytes and neutrophils increases binding capacity of leukocyte CR3 (CD11b/CD18). *J. Immunol.* 154: 299–307.
- Berman, M.E., Y. Xie, and W. Muller. 1996. Roles of platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31) in natural killer cell transendothelial migration and β₂ integrin activation. *J. Immunol.* 156:1515–1524.
- Bos, J.L. 1997. Ras-like GTPases. *Biochim. Biophys. Acta.* 1333:19–31.
- Bos, J.L. 1998. All in the family? New insights and questions regarding interconnectivity of Ras, Rap1 and Ral. *EMBO (Eur. Mol. Biol. Organ.) J.* 17: 6776–6782.
- Cabanas, C., and N. Hogg. 1993. Ligand intracellular adhesion molecule 1 has a necessary role in activation of integrin lymphocyte function-associated molecule 1. *Proc. Natl. Acad. Sci. USA.* 90:5838–5842.
- de Rooij, J., and J.L. Bos. 1997. Minimal Ras-binding domain of Raf1 can be used as an activation-specific probe for Ras. *Oncogene.* 14:623–625.
- Dransfield, I., and N. Hogg. 1989. Regulated expression of Mg²⁺ binding epitope on leukocyte integrin α subunits. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3759–3765.
- Duncan, G.S., D.P. Andrew, H. Takimoto, S.A. Kaufman, H. Yoshida, J. Spellberg, J.L. de la Pompa, A. Elia, A. Wakeham, B. Karan-Tamier, et al. 1999. Genetic evidence for functional redundancy of platelet/endothelial cell adhesion molecule-1 (PECAM-1): CD31-deficient mice reveal PECAM-1-dependent and PECAM-1-independent functions. *J. Immunol.* 162:3022–3030.
- Franke, B., J.-W.N. Akkerman, and J.L. Bos. 1997. Rapid Ca²⁺-mediated activation of Rap1 in human platelets. *EMBO (Eur. Mol. Biol. Organ.) J.* 16: 252–259.
- Hemler, M.E. 1998. Integrin associated proteins. *Curr. Opin. Cell Biol.* 10:578–585.
- Jackson, D.E., K.R. Kupcho, and P.J. Newman. 1997. Characterization of phosphotyrosine binding motifs in the cytoplasmic domain of platelet/endothelial cell adhesion molecule-1 (PECAM-1) that are required for the cellular association and activation of the protein-tyrosine phosphatase, SHP-2. *J. Biol. Chem.* 272:24868–24875.

- Kitayama, H., Y. Sugimoto, T. Matsuzaki, Y. Ikawa, and M. Noda. 1989. A ras-related gene with transformation suppressor activity. *Cell* 56:77–84.
- Kurachi, H., Y. Wada, N. Tsukamoto, M. Maeda, H. Kubota, M. Hattori, K. Iwai, and N. Minato. 1997. Human SPA-1 gene product selectively expressed in lymphoid tissues is a specific GTPase-activating protein for Rap1 and Rap2. *J. Biol. Chem.* 272:28081–28088.
- Linnemann, T., M. Geyer, B.K. Jaitner, C. Block, H.R. Kalbitzer, A. Wittinghofer, and C. Herrmann. 1999. Thermodynamic and kinetic characterization of the interaction between the Ras binding domain of AF6 and members of the Ras subfamily. *J. Biol. Chem.* 274:13556–13562.
- Liu, Z.-J., Y. Tanaka, H. Fujimoto, S. Mine, A. Morinobu, H. Yagita, K. Okumura, I. Oishi, J. Udagawa, H. Yamamura, and Y. Minami. 1999. A novel role for H-Ras in the regulation of very late antigen-4 integrin and VCAM-1 via c-Myc-dependent and -independent mechanisms. *J. Immunol.* 163:4901–4908.
- M'Rabet, L. 1999. Regulation and function of Ras, Rap1 and Ral in granulocytes. Ph.D. thesis. Utrecht University, Utrecht, The Netherlands. 120 pp.
- Medema, R.H., R. Wubbolts, and J.L. Bos. 1991. Two dominant inhibitory mutants of p21ras interfere with insulin-induced gene expression. *Mol. Cell Biol.* 11:5963–5967.
- Medema, R.H., W.L. de Laat, G.A. Martin, F. McCormick, and J.L. Bos. 1992. GTPase-activating protein SH2-SH3 domains induce gene expression in a Ras-dependent fashion. *Mol. Cell Biol.* 12:3425–3430.
- Newman, P.J. 1997. The biology of PECAM-1. *J. Clin. Invest.* 100:S25–S29.
- Newton, J.P., C.D. Buckley, E.Y. Jones, and D.L. Simmons. 1997. Residues on both faces of the first immunoglobulin fold contribute to homophilic binding sites of PECAM-1/CD31. *J. Biol. Chem.* 272:20555–20563.
- O'Rourke, A.M., H. Shao, and J. Kaye. 1998. A role for p21ras/MAP kinase in TCR-mediated activation of LFA-1. *J. Immunol.* 161:5800–5803.
- Pumphrey, N.J., V. Taylor, S. Freeman, M.R. Douglas, P.F. Bradfield, S.P. Young, J.M. Lord, M.J.O. Wakelman, I.N. Bird, M. Salmon, and C.D. Buckley. 1999. Differential association of cytoplasmic signalling molecules SHP-1, SHP-2, SHIP, and phospholipase C- γ 1 with PECAM-1/CD31. *FEBS Lett.* 450:77–83.
- Ranger, G.E., C. Buckley, D.L. Simmons, and G.B. Nash. 1997. Cross-talk between cell adhesion molecules regulates the migration velocity of neutrophils. *Curr. Biol.* 7:316–325.
- Reedquist, K.A., and J.L. Bos. 1998. Costimulation through CD28 suppresses T cell receptor-dependent activation of the Ras-like small GTPase Rap1 in human T lymphocytes. *J. Biol. Chem.* 273:4944–4949.
- Rubinfeld, B., S. Munemitsu, R. Clark, L. Controy, K. Watt, W.J. Crosier, F. McCormick, and P. Polakis. 1991. Molecular cloning of a GTPase activating protein specific for the Krev-1 protein p21rap1. *Cell* 65:1033–1042.
- Serebriiskii, I., J. Estojak, G. Sonoda, J.R. Testa, and E.A. Golemis. 1997. Association of Krev-1/rap1A with Krit1, a novel ankyrin repeat-containing protein encoded by a gene mapping to 7q21-22. *Oncogene* 15:1043–1049.
- Shibayama, H., N. Anzai, S.E. Braun, S. Fukuda, C. Mantel, and H.E. Broxmeyer. 1999. H-Ras is involved in the inside-out signaling pathway of interleukin 3-induced integrin activation. *Blood* 93:1540–1548.
- Stewart, M., and N. Hogg. 1996. Regulation of leukocyte integrin function: affinity vs avidity. *J. Cell. Biochem.* 61:554–561.
- Tanaka, S., T. Morishita, Y. Hashimoto, S. Hattori, S. Nakamura, T. Takenawa, K. Matuoka, M. Shibuya, T. Kurata, K. Nagashima, and M. Matsuda. 1994. C3G, a guanine nucleotide releasing protein, binds to the SH3 domains of CRK and ASH/GRB2. *Proc. Natl. Acad. Sci. USA* 91:3443–3447.
- Tanaka, Y., S.M. Albelda, K.J. Horgan, G.A. van Seventer, Y. Shimizu, W. Newman, J. Hallam, P.J. Newman, C.A. Buck, and S. Shaw. 1992. CD31 expressed on distinctive T cell subsets is a preferential amplifier of β 1 integrin-mediated adhesion. *J. Exp. Med.* 176:245–253.
- Tanaka, Y., Y. Minami, S. Mine, H. Hirano, C.-D. Hu, H. Fujimoto, K. Fujii, K. Saito, J. Tsukada, Y. van Kooyk, et al. 1999. H-Ras signals to cytoskeletal machinery in induction of integrin-mediated adhesion of T cells. *J. Immunol.* 163:6209–6216.
- Torti, M., A. Bertoni, I. Canobbio, F. Sinigaglia, E.G. Lapetina, and C. Balduini. 1999. Interaction of the low-molecular weight GTP-binding protein Rap2 with the platelet cytoskeleton is mediated by direct binding to the actin filaments. *J. Cell. Biochem.* 75:675–685.
- Tsukamoto, N., M. Hattori, H. Yang, J.L. Bos, and N. Minato. 1999. Rap1 GTPase-activating protein SPA-1 negatively regulates cell adhesion. *J. Biol. Chem.* 274:18463–18469.
- van de Wiel-van Kemenade, E., Y. van Kooyk, A.J. de Boer, R.J. Huijbens, P. Weder, W. van de Kastele, C.J. Melief, and C.G. Figdor. 1992. Adhesion of T and B lymphocytes to extracellular matrix and endothelial cells can be regulated through the beta subunit of VLA. *J. Cell Biol.* 17:461–470.
- van den Berghe, N., R.H. Cool, G. Horn, and A. Wittinghofer. 1997. Biochemical characterization of C3G: an exchange factor that discriminates between Rap1 and Rap2 and is not inhibited by Rap1A(S17N). *Oncogene* 15:845–850.
- Yan, H.C., J.M. Pilewski, Q. Zhang, H.M. DeLisser, L. Romer, and S.M. Albelda. 1995. Localization of multiple functional domains on human PECAM-1 (CD31) by monoclonal antibody epitope mapping. *Cell. Adhes. Commun.* 3:45–66.
- Zhang, A., K. Vuori, H.G. Wang, J.C. Reed, and E. Ruoslahti. 1996. Integrin activation by R-Ras. *Cell* 85:61–69.
- Zwartkruis, F.J.T., R.M.F. Wolthuis, N.M.J.M. Nabben, B. Franke, and J.L. Bos. 1998. Extracellular signal-regulated activation of Rap1 fails to interfere in Ras effector signalling. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:5905–5912.