

The Small GTPase Rap1 Is Required for Mn^{2+} - and Antibody-induced LFA-1- and VLA-4-mediated Cell Adhesion*

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In T-lymphocytes the Ras-like small GTPase Rap1 plays an essential role in stimulus-induced inside-out activation of integrin LFA-1 ($\alpha_1\beta_2$) and VLA-4 ($\alpha_4\beta_1$). Here we show that Rap1 is also involved in the direct activation of these integrins by divalent cations or activating antibodies. Inhibition of Rap1 either by Rap GTPase-activating protein (RapGAP) or the Rap1 binding domain of RalGDS abolished both Mn^{2+} - and KIM185 (anti-LFA-1)-induced LFA-1-mediated cell adhesion to intercellular adhesion molecule 1. Mn^{2+} - and TS2/16 (anti-VLA-4)-induced VLA-4-mediated adhesion were inhibited as well. Interestingly, both Mn^{2+} , KIM185 and TS2/16 failed to induce elevated levels of Rap1GTP. These findings indicate that available levels of GTP-bound Rap1 are required for the direct activation of LFA-1 and VLA-4. Pharmacological inhibition studies demonstrated that both Mn^{2+} - and KIM185-induced adhesion as well as Rap1-induced adhesion require intracellular calcium but not signaling activity of the MEK-ERK pathway. Moreover, functional calmodulin signaling was shown to be a prerequisite for Rap1-induced adhesion. From these results we conclude that in addition to stimulus-induced inside-out activation of integrins, active Rap1 is required for cell adhesion induced by direct activation of integrins LFA-1 and VLA-4. We suggest that Rap1 determines the functional availability of integrins for productive binding to integrin ligands.

Circulating lymphocytes require a dynamic and flexible regulation of their integrin-dependent adhesive properties. Although these cells circulate in blood and lymph or migrate through tissues, rapid transitions between adherent and non-adherent states are required. The leukocyte function-associated antigen 1 (LFA-1)¹ (integrin $\alpha_1\beta_2$) is a transmembrane

heterodimer composed of a unique α subunit (α_L or CD11a) and a β_2 subunit (CD18) that is common to a subset of leukocyte integrins. Expressed at the cell surface of resting leukocytes, LFA-1 is inactive. However, rapid conversion into an active state allows the integrin to bind to its ligands, intercellular adhesion molecules 1, 2, and 3 (ICAM-1, -2, and -3) (1–3). Relevant examples of the LFA-1/ICAM-1-mediated adhesion include leukocyte-endothelial cell interaction to direct lymphocyte homing or extravasation (4) and the establishment and strengthening of contacts between T cells and antigen-presenting cells (APC) (5, 6). In addition, lymphocytes express VLA-4 (integrin $\alpha_4\beta_1$), which binds to vascular cell adhesion molecule 1 (VCAM-1) (7).

The binding of ligand by LFA-1 and VLA-4 is activated through inside-out signaling (8, 9). Cytokines, chemokines, or other T cell surface receptors such as the antigen-specific T cell receptor or CD2 lead to the production of second messengers and subsequent regulation of these integrins. For LFA-1 these signals are also generated by stimuli that increase the intracellular Ca^{2+} concentration or by protein kinase C upon phorbol ester treatment. Models explaining the regulation of LFA-1 activity incorporate two distinct mechanisms. The first postulates modulation of the intrinsic affinity of the integrin for its ligand, whereas the second suggests a critical role for increased integrin receptor clustering or integrin redistribution at the cell surface (avidity regulation) (8). In addition to inside-out signaling, integrins can be activated directly from the outside by divalent cations like Mn^{2+} or Mg^{2+} (in the presence of EGTA), which bind to the ectodomain (10, 11), or after stimulation with activating monoclonal antibodies, like the β_2 -specific antibody KIM185 (12) and the β_1 -specific antibody TS2/16 (13).

Recently, the small GTPase Rap1 has been demonstrated to play a role in T cell receptor-, CD31-, and cytokine-induced adhesion mediated by LFA-1, VLA-4, and VLA-5 (14–16). Overexpression of the active mutant RapV12 induced adhesion to immobilized ICAM-1 and VCAM-1. T cell receptor- and CD31-induced activation of integrins was abolished by the introduction of constructs that inhibit Rap1 signaling, *i.e.* Rap GTPase-activating protein (RapGAP), which lowers the level of Rap1GTP, the Rap1 binding domain (RBD) of RalGDS, which presumably inactivates Rap1 by binding to it, and the putative dominant negative mutant RapN17 (15). Interestingly, transgenic mice constitutively expressing Rap1aV12 in their T cell lineage have been generated (17). Expression of active Rap1A in primary T cells from these mice is sufficient to induce inside-out signaling leading to β_1 and β_2 integrin-mediated adhesion.

very late antigen 4; MEK-ERK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; TK, thymidine kinase.

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¹ The abbreviations used are: LFA-1, leukocyte function-associated antigen 1; RapGAP, Rap GTPase activating protein; GEF, guanine-nucleotide exchange factor; HA, hemagglutinin; ICAM-1, intercellular cell adhesion molecule-1; RBD, Ras binding domain; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; VCAM-1, vascular cell adhesion molecule 1; VLA-4,

In these cells, active Rap1A did not modulate integrin affinity, as was measured by soluble ICAM-1 binding. However, thymocytes expressing Rap1AV12 showed integrin clustering at the surface, pointing to a role for Rap1 in integrin avidity modulation.

In the present study we show that overexpression of Rap1-inhibitory signaling proteins, RapGAPs and RBD, interferes with the ability of Mn^{2+} and KIM185 to induce LFA-1-mediated adhesion to ICAM-1 and with the ability of Mn^{2+} and TS2/16 to induce VLA-4-mediated adhesion to VCAM-1. Both divalent cations and the activating antibodies do not induce accumulation of active Rap1GTP, consistent with the notion that they are thought to bypass the requirement for intracellular signaling events (18, 19). This indicates that direct activation of integrins does not depend on further Rap1 activation. Although TPA-induced adhesion (inside-out integrin activation) may similarly not require activation of Rap1 (14), we demonstrate that it is blocked by inhibition of Rap1 signaling. Apparently, both stimulus-induced inside-out regulation of integrins and activation of integrin-mediated cell adhesion by cations and integrin-activating antibodies require Rap1. Use of pharmacological inhibitors reveals that Mn^{2+} , KIM185, but also RapV12-induced LFA-1-mediated adhesion, is critically dependent upon intracellular Ca^{2+} levels but independent of protein kinase C or MEK-ERK signaling. Furthermore, RapV12-induced LFA-1-mediated adhesion requires calmodulin. We propose that Rap1 activity, either basal or induced, regulates the functional availability of integrins for the adhesion process by or in combination with a calcium/calmodulin-dependent mechanism.

MATERIALS AND METHODS

Plasmids and Constructs—Heamagglutinin (HA)-tagged Rap1, Rap1V12 (HA-RapV12), Rap1GAP (HA-RapGAP I), RalGDS-RBD, and PDZ-GEF as well as pCAGGS-C3G and pSR-His-tagged *SpaI* have previously been described (15, 20, 21). HA-RapGAP II was generated by PCR amplification of a 450-bp *SalI/XhoI* fragment including the 30 additional N-terminal amino acids of RapGAP II from pCA-RapGAP II (provided by Dr. Michiyuki Matsuda, (Department of Tumor Virology, Institute for Microbial Diseases, Osaka University, Osaka, Japan). Subsequently, this fragment was subcloned into *SalI/XhoI*-digested pMT2-SM-HA-RapGAP I to generate pMT2-SM-HA-RapGAP II, and integrity of the construct was confirmed by DNA sequencing.

Cell Culture, Cell Line, and Transfection—The Jurkat T cell line JHM1 2.2 was provided by Dr. D. Cantrell (Imperial Cancer Research Fund, London, UK) with the kind permission of Dr. A. Weiss (University of California at San Francisco). Jurkat T cells were grown at 37 °C in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated (30 min at 56 °C) fetal bovine serum and 0.05% glutamine in the presence of penicillin and streptomycin. The erythroleukemic K562 cells, either wild type or stably expressing LFA-1, were grown in 75% RPMI 1640 and 25% Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum and 0.05% glutamine in the presence of penicillin and streptomycin. The stable transfectants were cultured in the presence of 2 mg/ml Geneticin as has been described (22). Cells were transiently transfected by electroporation using 35 μ g of plasmid DNA. Cells (1.2×10^7 cells/ml in 0.4 ml of complete medium) were pulsed at 250 V and 960 microfarads with 5 μ g of TK-luciferase plasmid DNA, construct plasmid as indicated in the figure legends, and added vector plasmid to keep DNA amounts constant. Subsequently, 24 h after transfection, cells were transferred to serum-free medium and used 42–48 h after transfection.

Adhesion Assay—For adhesion assays, transiently transfected Jurkat cells were harvested, washed, and resuspended in TSM buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) at a concentration of 5×10^5 cells/ml. 96-Well Nunc Maxisorp plates were coated overnight at 4 °C with goat anti-human IgG antibodies (Jackson ImmunoResearch, West Grove, PA; 4 μ g/ml) in sodium bicarbonate buffer (Sigma), washed, and blocked for 30 min at 37 °C with 1% bovine serum albumin, TSM followed by incubation for 1 h at 37 °C with 50 ng/ml recombinant ICAM-1 or 2 μ g/ml VCAM-1 human IgG Fc fusion proteins, depending on the integrin studied. For studies on cell adhesion to fibronectin (Sigma), 96-well Nunc Maxisorp plates were coated

overnight at 4 °C with 1–5 μ g/ml fibronectin in sodium bicarbonate buffer, washed with TSM, and blocked for 30 min at 37 °C with 2% bovine serum albumin, TSM. Poly-L-Lysine (Sigma) was coated on plates for 1 h at room temperature as a 0.1% w/v solution in water. After washing, 50 μ l of TSM was added per well with or without the indicated stimuli. Subsequently, 50 μ l cell suspension was added per well, after which the cells were spun down for 1 min at 200 rpm in a Heraeus Sepatech Megafuge 1.0. Cells were allowed to adhere for 30 min at 37 °C, and non-adherent cells were removed with warmed 0.5% bovine serum albumin, TSM. Adherent cells were lysed and subjected to a luciferase assay as described previously (23). Expression of transfected constructs was confirmed by immunoblotting of total cell lysates. Cells bound were calculated, and numbers were corrected for transfection efficiency and nonspecific effects of constructs by measuring luciferase activity of total input cells ((counts in cells bound/counts in total input cells) \times 100%). Activating anti-integrin β_1 and β_2 antibodies TS2/16 and KIM185, respectively, have been described previously (12, 13) and were used at 10 μ g/ml. Mn^{2+} was used at 4 mM, and TPA (Sigma) was used at 100 ng/ml. Preincubations with the following pharmacological inhibitors were performed for 30 min at 37 °C: Roche 31-8220 (Calbiochem; 5 μ M), GF 109203X (Biomol, Plymouth, PA; 5 μ M), BAPTA-AM (Molecular Probes, Eugene, OR; 20 μ M), PD 98059 (Sigma; 10 μ M), U0126 (Promega, Madison, WI; 10 μ M), LY294002 (Biomol; 10 μ M), wortmannin (Sigma; 100 nM), U73122 (1 μ M), SK&F96365 (concentration described in the legend to Fig. 7), calmidazolium chloride (10 μ M), E6 berbamine (10 μ M), and W-7 (100 μ M) (all five from Biomol), calpeptin (Sigma; 100 μ M), KN-93 (Calbiochem; 10 μ M), and cyclosporin A (Biomol, 200 ng/ml).

Analysis of Rap1 Activation in Vivo—Jurkat cells were serum-starved overnight and resuspended at 25×10^6 cells/ml in RPMI without serum. 200 μ l of this suspension was used per sample. After transfer to Eppendorf tubes, cells were left untreated for 15 min at 37 °C. Next Mn^{2+} (4 mM), KIM185 (10 μ g/ml), TS2/16 (10 μ g/ml), or TPA (100 ng/ml) was added for indicated periods of time. Subsequently cells were lysed for 15 min at 4 °C by the addition of ice-cold lysis buffer (10% glycerol, 1% Nonidet P40, 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 2.5 mM $MgCl_2$, 1 μ M leupeptin, 0.1 μ M aprotinin), and lysates were cleared by centrifugation at maximal speed in an Eppendorf centrifuge for 15 min at 4 °C. The GTP-bound form of Rap1 was isolated using RalGDS-RBD as an activation-specific probe and subsequently quantified by Western blotting using anti-Rap1 antibody, as has been previously described (24, 25).

Western Blotting—Western blotting of all protein samples was carried out using polyvinylidene difluoride membranes. The antibodies used for protein detection are the monoclonal anti-HA (12CA5), polyclonal anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-Rap1 (Transduction Laboratories, Lexington, KY), polyclonal anti-Rap1 (Santa Cruz Biotechnology), and anti-phosphomito-gen-activated protein kinase (Cell Signaling Technology, New England Biolabs, Inc., Beverly, MA).

RESULTS

Inhibition of Rap1 Abolishes Divalent Cation-induced Adhesion to ICAM-1 and VCAM-1—To determine whether Rap1 is involved in Mn^{2+} -dependent activation of LFA-1 we measured the effect of Rap1 inhibitory proteins on Mn^{2+} -induced adhesion of Jurkat cells to ICAM-1, the counter receptor of LFA-1. Cells were transfected with RapGAP I, RapGAP II (which both lower the level of Rap1GTP), or RalGDS-RBD (which can form an inactive complex with Rap1GTP) together with a luciferase construct to detect transfected cells. These cells were subsequently plated on ICAM-1 in the presence or absence of Mn^{2+} , and after 30 min nonadherent cells were washed away and the percentage of luciferase-positive cells attached was measured. In control cells transfected with empty vector, Mn^{2+} treatment strongly induced adhesion to ICAM-1 (Fig. 1a). In contrast, adhesion induced by Mn^{2+} was blocked in cells transfected with RapGAP I, RapGAP II (Fig. 1a), or RalGDS-RBD (Fig. 1b). The ability of Rap1 inhibitory proteins to block divalent cation-induced integrin adhesion was not limited to LFA-1, as RapGAP I and RalGDS-RBD also blocked Mn^{2+} -induced adhesion of VLA-4 to VCAM-1 (Fig. 1c). Furthermore, Mg^{2+} /EGTA-induced adhesion to ICAM-1 was also blocked by RapGAP and RalGDS-RBD (data not shown).

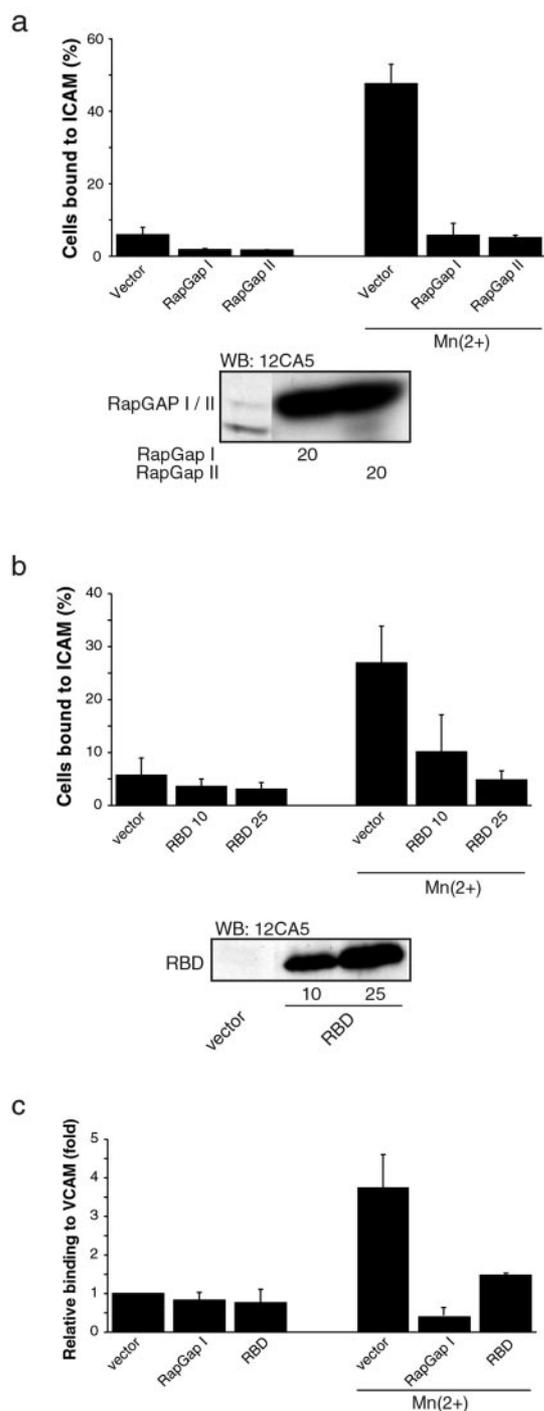


FIG. 1. Inhibition of Rap1 activity abolishes Mn²⁺-induced LFA-1- and VLA-4-mediated cell adhesion. Inhibition of Rap1 activity by transiently overexpressing Rap1-specific GAPs (data represent the average mean and S.E. of four independent experiments, with each experiment performed in quadruplicate) (*a*) or RalGDS-RBD (representative experiment in quadruplicate) (*b*) resulted in decreased basal adhesion and inhibited cells in their Mn²⁺-induced LFA-mediated adhesion response. Jurkat cells were cotransfected with 5 μ g of pG3-TK luciferase reporter plasmid and either empty pMT2-SM-HA vector (*vector*), HA-RapGAP I (*RapGAP I*) (20 μ g), HA-RapGAP II (*RapGAP II*) (20 μ g), or HA-RalGDS-RBD (*RBD*) (10 or 25 μ g), and after 42 h the cells were allowed to adhere to immobilized ICAM-1 for 30 min at 37 $^{\circ}$ C. Fraction of cells bound was determined by fraction of luciferase activity bound. In the *bottom panels* expression levels of the transfected proteins are shown. *c*, inhibition of Rap1 activity abolished Mn²⁺-induced VLA-4-mediated adhesion. Jurkat cells were cotransfected with constructs as indicated in *a*, and the cells were allowed to adhere to immobilized VCAM-1. Results are the average from two independent experiments in quadruplicate and indicated as the relative binding to VCAM-1 as compared with vector control. WB, Western blot.

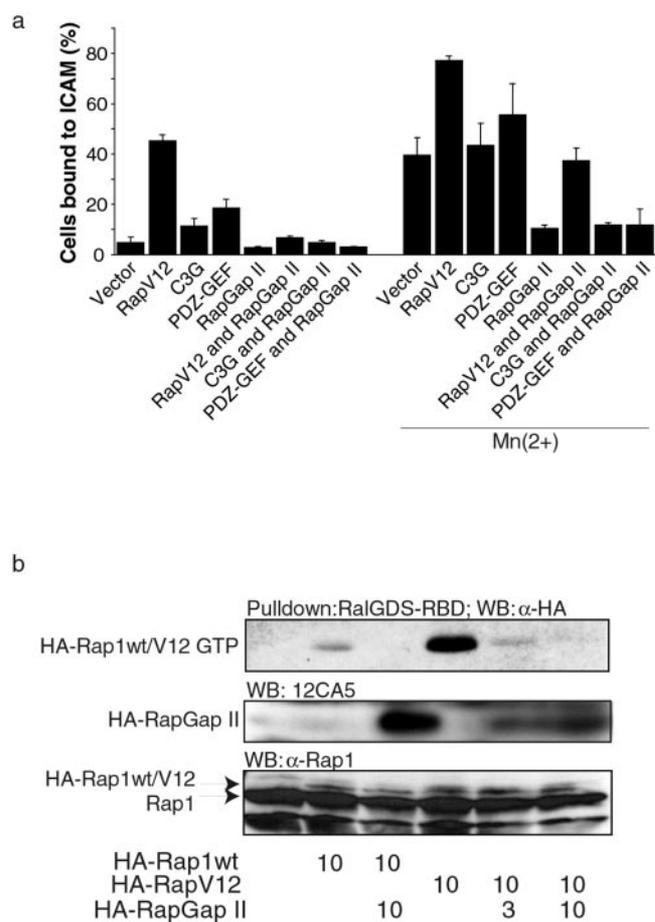


FIG. 2. Inhibition of active Rap1-induced adhesion and decrease of RapGTP levels by RapGap proteins. *a*, Rap1-specific GEFs induced LFA-mediated adhesion. Both RapV12- and Rap1GEF-induced LFA-1-mediated adhesion could be blocked by cotransfection of RapGAP II (representative experiment in quadruplicate). Jurkat cells were cotransfected with 5 μ g of pG3-TK luciferase reporter plasmid and either empty vector, HA-RapV12 (10 μ g), C3G (10 μ g), HA-PDZ-GEF (10 μ g), HA-RapGAP II (10 μ g), or indicated combinations of them (10 μ g of each construct). *b*, RapGAP II efficiently decreased GTP levels of both cotransfected Rap1wt and RapV12. Jurkat cells were transfected with empty vector, HA-Rap1, or HA-RapV12 alone or in combination with HA-RapGAP II (amounts of DNA (μ g) as indicated in the figure). The *upper panel* shows the GTP levels of the Rap proteins determined using the pull-down assay ("Materials and Methods"), the *middle panel* demonstrates the expression of HA-RapGAP II in the total lysates, and the *lower panel* shows the presence of HA-Rap1wt and HA-RapV12 in the total lysates. WB, Western blot.

RapGAP Proteins Act on Active Rap1-induced Adhesion—To confirm the specificity of RapGAP proteins in blocking Rap1-dependent adhesion, Jurkat cells were transfected with active RapV12 or the Rap1 guanine-nucleotide exchange factors (GEFs) C3G or PDZ-GEF to activate endogenous Rap1 either alone or in combination with RapGAP II (Fig. 2*a*). As in our previous studies, RapV12 strongly induced adhesion to ICAM-1, whereas overexpression of C3G or PDZ-GEF resulted in a more modest but significant induction of adhesion. Cotransfection of cells with RapGAP II, however, completely blocked adhesion induced by RapV12, C3G, or PDZ-GEF, indicating that RapGAP II inhibited Rap1-dependent signaling. Precipitation of GTP-bound transfected Rap1 with GST-RalGDS-RBD fusion protein (Fig. 2*b*) confirmed that transfected RapGAP II catalyzed hydrolysis of GTP of both wild-type Rap1 and RapV12. Together these results show that RapGAP overexpression blocks Rap1-induced adhesion by decreasing the Rap1GTP amount. We conclude that Rap1GTP is required for

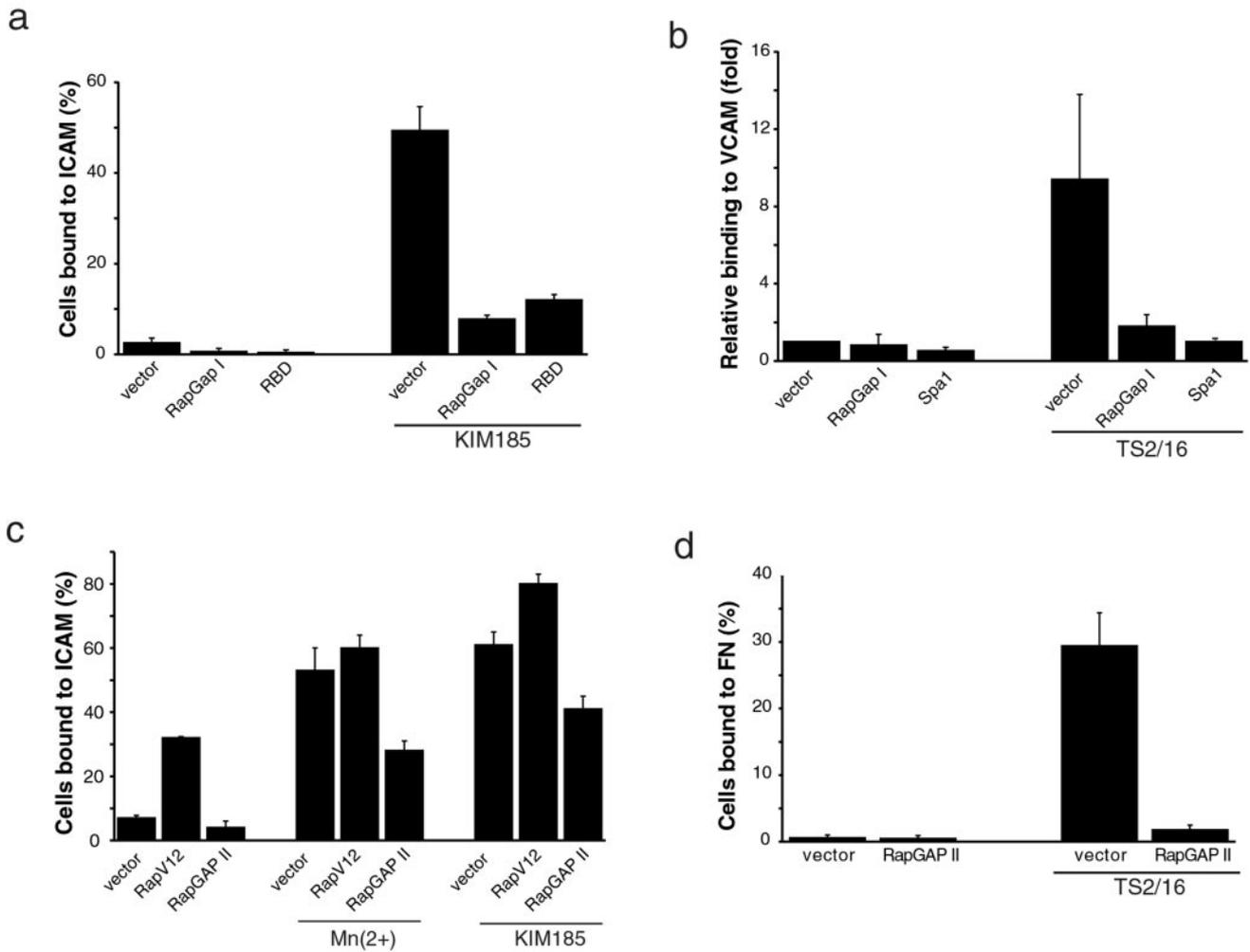


FIG. 3. Inhibition of Rap1 activity abolishes anti-integrin antibody-induced LFA-1- and VLA-4-mediated cell adhesion. *a*, inhibition of Rap1 activity by transient expression of either HA-RapGAP I or HA-RalGDS-RBD inhibited KIM185-induced LFA-mediated adhesion (representative experiment performed in quadruplicate). *b*, transient expression of either HA-RapGAP I or *SpaI* inhibited TS2/16-induced VLA-4-mediated adhesion (average of two independent experiments performed in quadruplicate, normalized to 1 for untreated vector control). *c*, transient expression of RapGAP II inhibited Mn²⁺- or KIM185-induced adhesion to ICAM-1 of K562 cells stably expressing LFA-1. *d*, RapGAP abolished TS2/16-induced adhesion of wild-type K562 cells to fibronectin (coated with 1 μg/ml concentration). Jurkat and K562 cells with and without stably expressed LFA-1 were transfected with 5 μg pG3-TK luciferase reporter plasmid and empty pMT2-SM-HA vector (*vector*), HA-RapV12 (*RapV12*) (10 μg), HA-RapGAP I (*RapGAP I*) (10 μg), HA-RalGDS-RBD (*RBD*) (10 μg), or *SpaI* (10 μg). After 42 h cells were allowed to bind to immobilized ICAM-1 (*a* and *c*), VCAM-1 (*b*), or fibronectin (FN) (*d*) in the presence and absence of anti-integrin antibody KIM185 (*a* and *c*), TS2/16 (*b* and *d*), or Mn²⁺ (*c*).

induction of LFA-1 and VLA-4 integrin-dependent adhesion induced by divalent cations.

Inhibition of Rap1 Blocks Adhesion Induced by β₁ and β₂ Integrin-activating Antibodies—Integrin-mediated cell adhesion induced by Mn²⁺ is proposed to occur by direct interactions of these cations with the extracellular domains of integrins rather than via intracellular signaling pathways. However, Mn²⁺ might also influence other cell surface or intracellular signaling proteins. Therefore, we tested the effect of Rap1 on cell adhesion induced by the monoclonal antibody KIM185. This antibody directly and specifically activates LFA-1 integrin in its ligand binding capacity (12). Treatment of Jurkat cells with KIM185 strongly induced adhesion to ICAM-1 (Fig. 3*a*). Similar to Mn²⁺, cell adhesion induced by KIM185 was completely blocked by transfection of either RapGAP I or RalGDS-RBD. VLA-4-mediated adhesion to VCAM-1 induced by the activating anti-β₁ integrin antibody TS2/16 was also completely blocked by transfection of cells with either of two RapGAPs, RapGAP I or *SpaI* (Fig. 3*b*). The inhibitory effect of RapGAP on Mn²⁺- and KIM185-induced adhesion to ICAM-1 was also observed in the erythroleukemic cell line K562 stably

expressing LFA-1 (22) (Fig. 3*c*). In wild-type K562 cells, which only express VLA-5 (α₅β₁) as a β₁ class of integrin (26), RapGAP II abolished TS2/16-induced adhesion to fibronectin (Fig. 3*d*). Our findings demonstrate that Rap1 is required for adhesion induced by integrin-activating antibodies.

Blocking Rap1 Specifically Affects Integrin/Ligand-mediated Adhesion—To show that the Rap1 requirement is specific for integrin-ligand binding, we tested cell adhesion to poly-L-lysine. No difference in cell attachment was observed upon expression of Rap1V12 or RapGAP either in the absence or presence of TS2/16 (Fig. 4*a*). We also used another approach to demonstrate that RapGAP inhibits integrin-ligand-based cell binding. Increasing the amount of immobilized fibronectin concomitantly increased basal cell binding, which could be abolished by expression of RapGAP (Fig. 4*b*). Interestingly, we observed that higher fibronectin densities rescued the inhibitory effect of RapGAP on TS2/16-induced adhesion both with K562 and Jurkat cells. Mn²⁺-induced adhesion could still be inhibited by RapGAP II at the highest fibronectin concentration used (5 μg/ml; data not shown). From these results we conclude that blocking Rap1 specifically inhibits adhesion me-

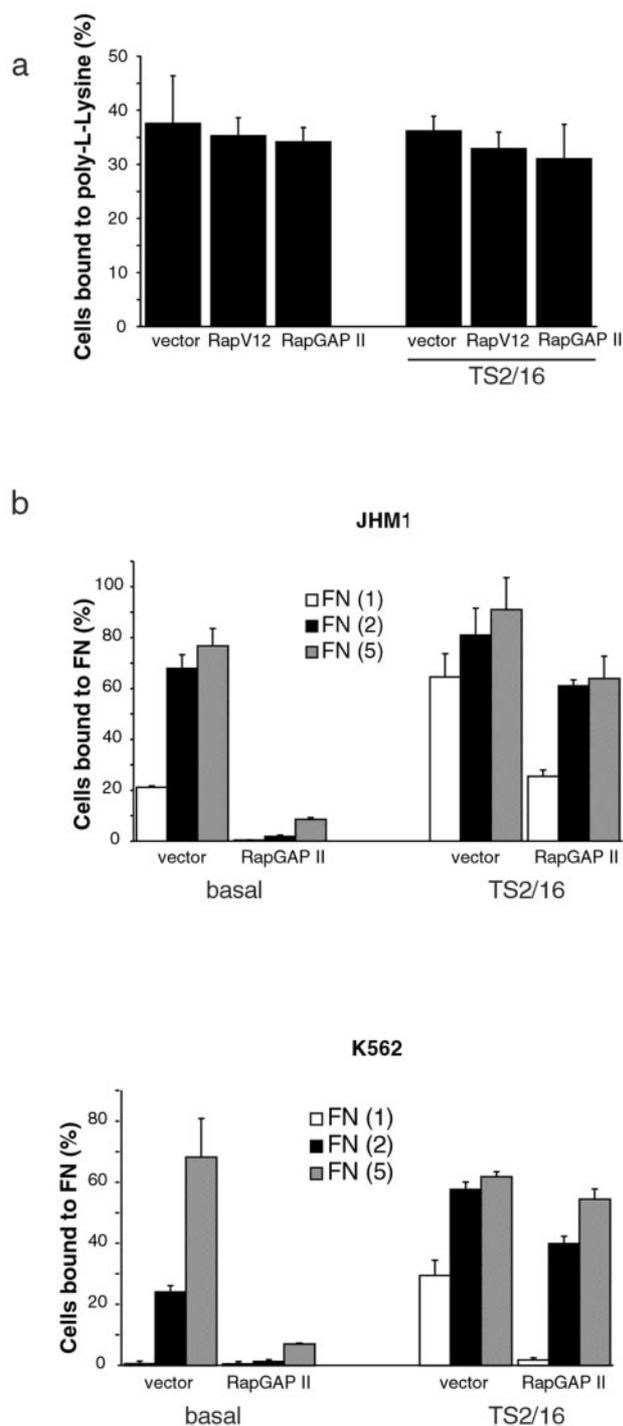


FIG. 4. Inhibition of Rap1 activity abolishes specifically integrin-ligand-mediated cell adhesion. *a*, transient overexpression of RapV12 or RapGAP in K562 cells in the presence or absence of TS2/16 did not affect cell binding to immobilized poly-L-lysine (representative experiment performed in quadruplicate). *b*, inhibition of cell adhesion induced by increased ligand density by transient expression of RapGAP. High ligand densities in combination with TS2/16 treatment overcame cell adhesion inhibition by RapGAP overexpression. The *upper panel* shows adhesion of Jurkat JHM1 cells with and without transient expression of RapGAP to fibronectin (FN, with indicated concentrations in $\mu\text{g}/\text{ml}$ used for coating) in the presence and absence of TS2/16. In the *lower panel* a similar situation is shown for K562 cells (representative experiments performed in triplicate). Jurkat cells and K562 cells were transfected with 5 μg of pG3-TK luciferase reporter plasmid and empty pMT2-SM-HA vector (*vector*), HA-RapV12 (*RapV12*) (10 μg), or HA-RapGAP II (*RapGAP II*) (20 μg). After 42 h cells were allowed to bind to immobilized poly-L-lysine (*a*) or fibronectin (plates coated with indicated dilutions of ligand) (*b*) in the presence and absence of anti-integrin antibody TS2/16.

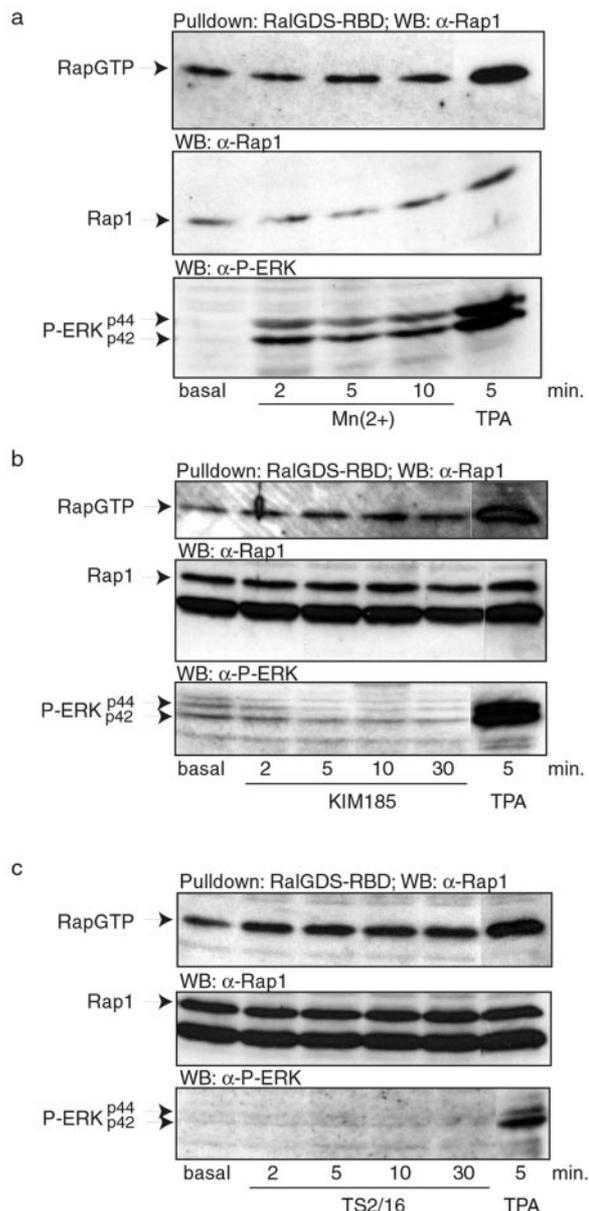


FIG. 5. Rap1 is not activated by Mn^{2+} and activating anti-integrin antibodies. Jurkat cells were unstimulated (*basal*) or treated for the indicated periods of time with either 4 mM Mn^{2+} (*a*), the LFA-1-activating antibody KIM185 (*b*), or the VLA-4-activating antibody TS2/16 (*c*). Cells were lysed, and RapGTP was determined using the RBD pull-down assay. *Upper panels*, RapGTP levels determined by the pull-down assay. *Middle panels*, total Rap1 in the lysates to demonstrate equal input per sample. *Lower panels*, lysates were analyzed for the presence of phosphorylated ERK. Treatment for 5 min with TPA (100 ng/ml) was used as a positive control for Rap1 activation.

diated by integrin-ligand binding, but that integrins and processes required for adhesion are still functional.

Mn^{2+} -/KIM185 Treatment Does Not Activate Rap1—The requirement for Rap1 in Mn^{2+} and antibody-induced integrin-mediated cell adhesion suggested the possibility that both stimuli mediated adhesion via activation of Rap1. We therefore tested whether Mn^{2+} , KIM185, or TS2/16 was able to induce Rap1 activation (Fig. 5). Cells were stimulated for 30 min, corresponding to the duration of the adhesion assay, and Rap1GTP was analyzed using RalGDS-RBD as the activation-specific probe. No activation of Rap1 was observed with Mn^{2+} (Fig. 5*a*). In contrast, strong activation of Rap1 was observed after treatment with TPA. Surprisingly, Mn^{2+} treatment did induce a modest and sustained activation of the MEK/ERK

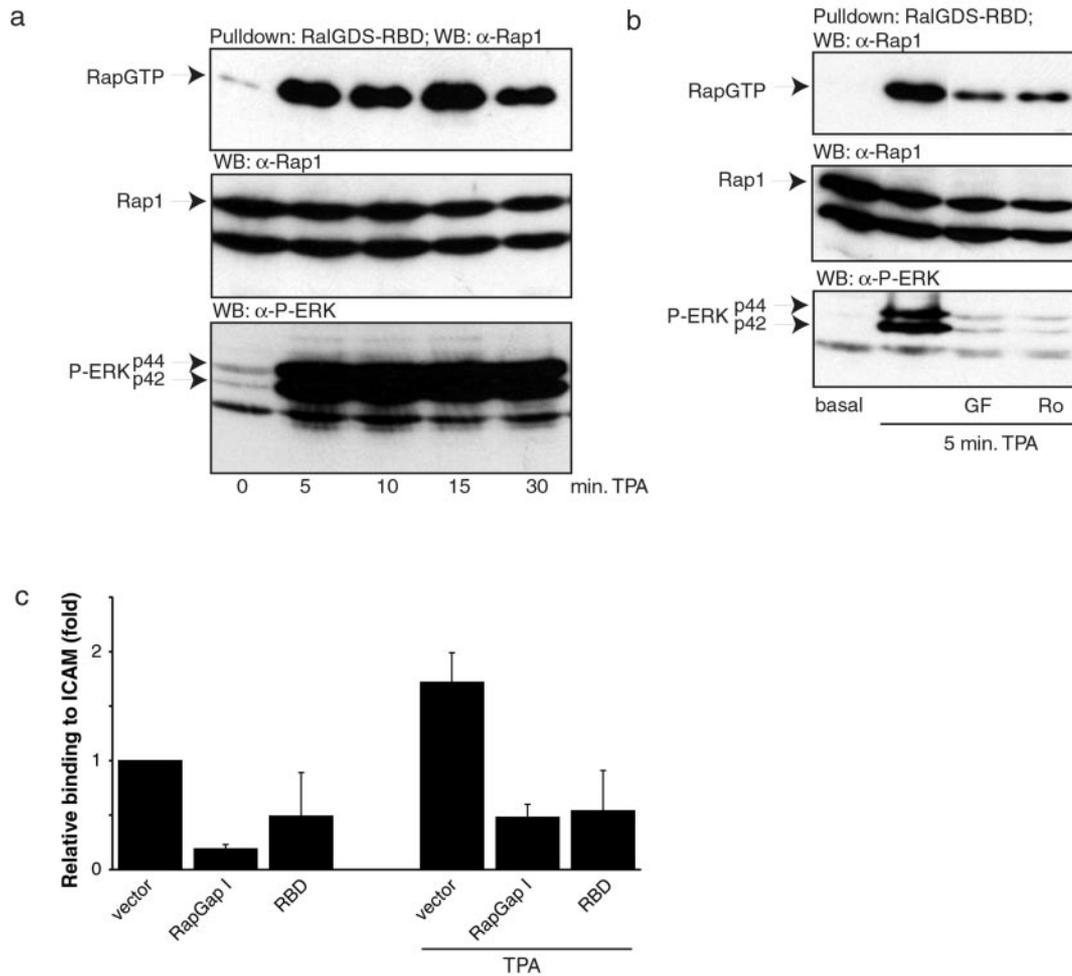


FIG. 6. Effect of inhibiting basal Rap1 signaling on TPA-induced inside-out activation of LFA-1-mediated adhesion. *a*, time course demonstrating RapGTP levels upon TPA treatment (100 ng/ml) for the indicated periods of time. The *upper panel* shows RapGTP levels. The *middle panel* shows total Rap1 in the lysates to demonstrate equal input per sample. The *bottom panel* demonstrates ERK phosphorylation (*P*-) during the time course. WB, Western blot. *b*, TPA-induced Rap1 and mitogen-activated protein kinase activity in the presence of pharmacological protein kinase C inhibitors. After preincubation for 30 min with either GF 109203X or Roche 31-8220 (5 μM) cells were treated with TPA (100 ng/ml) for 5 min. The *upper panel* demonstrates Rap1GTP levels, the *middle panel* shows the presence of equal amounts Rap1 in each sample, and the *bottom panel* shows ERK phosphorylation. *c*, TPA-induced LFA-mediated adhesion to immobilized ICAM-1 is dependent on basal Rap1 activity. Jurkat cells were transfected with 5 μg of pG3-TK luciferase reporter plasmid and either empty pMT2-SM-HA vector (*vector*), HA-RapGAP I (20 μg), or HA-RalGDS-RBD (20 μg), after which binding to immobilized ICAM-1 in the presence and absence of TPA (100 ng/ml) was analyzed (average of 2–3 independent experiments).

signaling pathway, as indicated by phosphorylation of ERK1 (p44) and ERK2 (p42) (Fig. 5*a*). Treatment of cells with KIM185 (Fig. 5*b*) or TS2/16 (Fig. 5*c*) neither increased the Rap1GTP level nor induced ERK activation. Thus, although both divalent cations and activating anti-integrin antibodies stimulate adhesion in a Rap1-dependent manner, this is not a result of acute activation of Rap1.

Inhibition of TPA-induced Adhesion by Rap1-interfering Proteins—The phorbol ester TPA is generally used to induce integrin-mediated cell adhesion by a still elusive pathway. Therefore, we investigated the possible requirement for Rap1 in TPA-induced integrin-mediated cell adhesion as well. Treatment of Jurkat cells with TPA resulted in a rapid and prolonged activation of Rap1 as well as induction of ERK phosphorylation (Fig. 6*a*). Pretreatment of Jurkat cells with pharmacological inhibitors of protein kinase C (Roche 31-8220 and GF 109203X) inhibited TPA-induced activation of both Rap1 and ERK (Fig. 6*b*). Although previous studies provide evidence that excludes the requirement for Rap1 activation in TPA-induced adhesion (14), overexpression of RapGAP I or RalGDS-RBD blocked TPA-induced adhesion to ICAM-1 (Fig. 6*c*). Pharmacological inhibition of protein kinase C selectively

blocked TPA- but not Mn²⁺- or KIM185-induced adhesion to ICAM-1 (Fig. 7*a*). These findings show that TPA-induced inside-out activation of adhesion requires Rap1 activity, either basal or induced.

Rap1-induced Adhesion Requires Intracellular Calcium and Calmodulin—To study the mechanism by which Rap1 regulates integrins, we tried to identify common elements between RapV12- and Mn²⁺- or activating antibody-induced cell adhesion. Several reports provide strong evidence that agonist-induced adhesion in a number of hematopoietic cell types requires influx of calcium from extracellular sources (19, 27, 28). Indeed, the calcium chelator BAPTA-AM strongly inhibited basal and Mn²⁺-, TPA-, and Kim185-induced LFA-mediated adhesion (Fig. 7*a*). We therefore examined next whether RapV12-induced adhesion also displayed a similar requirement. Chelation of intracellular calcium with BAPTA-AM strongly inhibited both basal and RapV12- induced adhesion (Fig. 7*b*). Preincubation of RapV12-transfected Jurkat cells with the imidazole compound SK&F 96365, which inhibits calcium channels in leukocytes and, thus, lowers intracellular calcium, efficiently blocked basal and RapV12-induced adhesion in a dose-dependent manner (Fig. 7*c*). The calmodulin

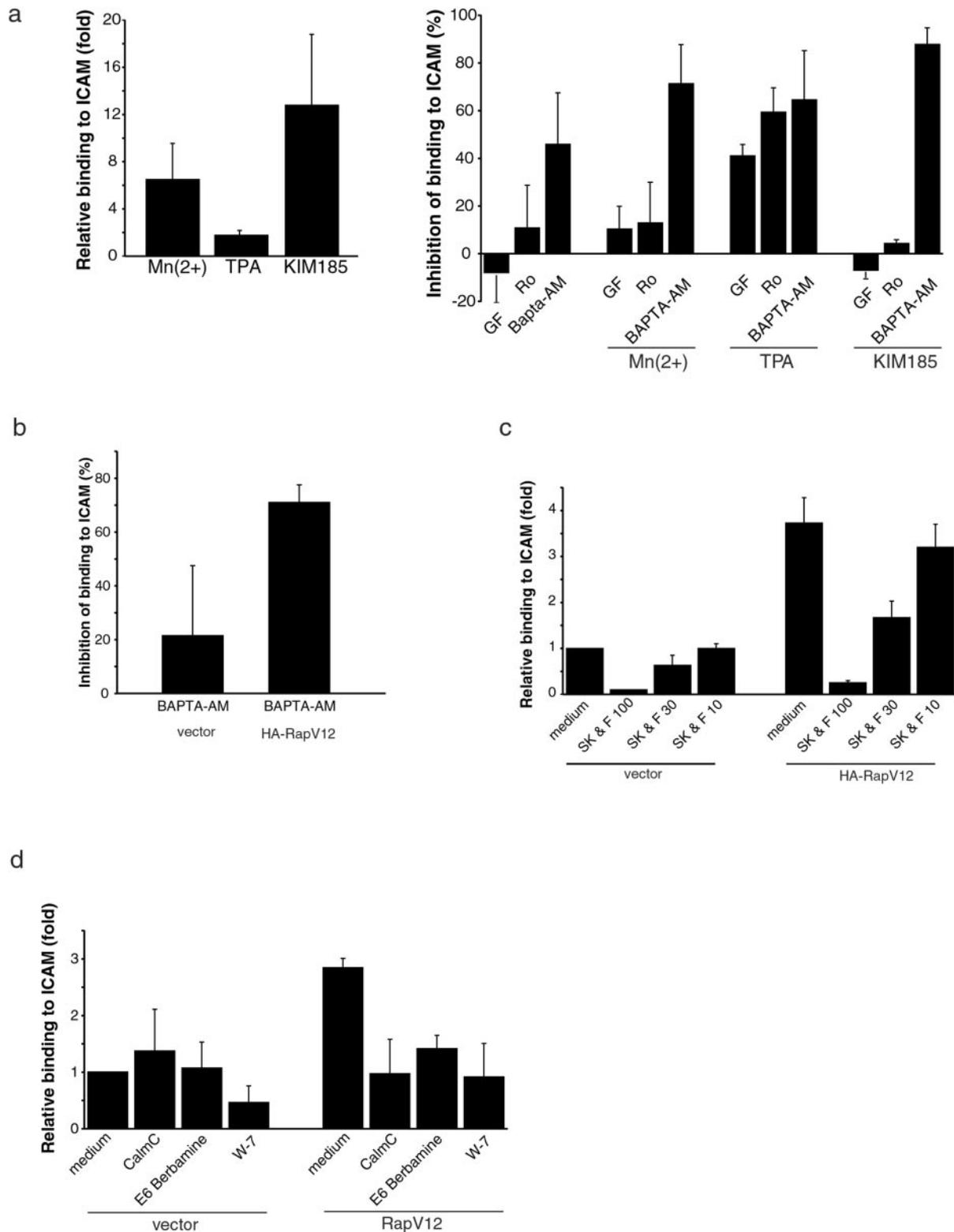


FIG. 7. Involvement of intracellular calcium in RapV12-induced LFA-1-mediated cell adhesion. *a*, effect of protein kinase C inhibitors and the calcium chelator BAPTA-AM on LFA-1-mediated adhesion to immobilized ICAM-1. Jurkat cells were transfected with 5 μ g of pG3-TK luciferase reporter plasmid and empty pMT2-SM-HA vector. The next day, cells were serum-starved overnight, and 42 h after transfection, cells were pretreated for 30 min with intracellular Ca²⁺-chelator BAPTA-AM (20 μ M) or one of the protein kinase C inhibitors, Roche 31-8220 (*Ro*) (5 μ M) and GF 109203X (*GF*) (5 μ M) for 30 min at 37 °C, after which binding to immobilized ICAM-1 in the absence and presence of Mn²⁺, TPA, or KIM185 was determined. *Left panel*, fold inductions of cell adhesion to immobilized ICAM-1 upon treatment of Jurkat cells with Mn²⁺, TPA, or KIM185. *Right panel*, percentages inhibition of cell binding to ICAM-1 upon treatment with the inhibitors is shown (average of 3–6 independent experiments performed in quadruplicate). RapV12-induced LFA-1-mediated adhesion to ICAM-1 could be blocked by chelation of intracellular calcium with BAPTA-AM (20 μ M). *b*, inhibition of calcium channels with the imidazole compound SK&F 96365 (*SK&F*) (100, 30, and 10 μ M) (*c*), and inhibition of calmodulin with calmidazolium chloride (*CalmC*; 10 μ M), E6 berbamine (10 μ M), and W-7 (100 μ M) (*d*). Jurkat cells were transfected with 5 μ g pG3-TK luciferase reporter plasmid and either empty pMT2-SM-HA vector (*vector*) or HA-RapV12 (10 μ g). After 42 h they were left untreated or preincubated for 30 min with the inhibitors, and subsequently cells were incubated for 30 min at 37 °C to allow binding to immobilized ICAM-1. Data represent the average of 3 independent experiments performed in quadruplicate.

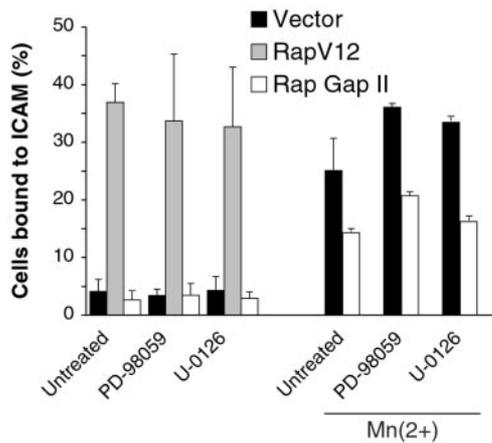


FIG. 8. MEK-ERK pathway is not involved in Rap1-mediated cell adhesion to ICAM-1. Jurkat cells were transfected with 5 μ g pG3-TK luciferase reporter plasmid and empty pMT2-SM-HA vector (*vector*), HA-RapV12 (10 μ g), or RapGAP I (20 μ g). After 42 h cells were either left untreated or preincubated with the MEK inhibitors PD 98059 (10 μ M) or U0126 (10 μ M) for 30 min at 37 $^{\circ}$ C, after which the cells were allowed to bind to immobilized ICAM-1 for 30 min in the presence and absence of Mn^{2+} . Data represent the mean and S.E. of two independent experiments performed in quadruplicate.

inhibitors W-7, E6 berbamine, and calmidazolium chloride each blocked RapV12-induced adhesion (Fig. 7d). From these results we conclude that RapV12-induced adhesion, like adhesion induced by other stimuli, requires calcium signaling.

The MEK-ERK Pathway Is Not Involved in Rap1-mediated Cell Adhesion—We were particularly interested in a possible role of the MEK-ERK pathway in Rap1-mediated cell adhesion, since positive and negative regulation of this pathway by Rap1 is a predominant effect of Rap1 reported in T lymphocytes and other cell types (29–31). To measure whether the MEK-ERK pathway mediates Rap1-induced adhesion, we used the MEK inhibitors PD 98059 and U0126. Although both MEK inhibitors completely inhibited TPA-induced ERK activation (data not shown), they did not affect RapV12- or Mn^{2+} -induced adhesion (Fig. 8). Alternatively, Rap1 may mediate cell adhesion by inhibiting ERK activation, suggesting that RapGAP might inhibit cell adhesion by preventing the inactivation of ERK. However, in the presence of the two MEK inhibitors, RapGAP II still inhibited cell adhesion. From these results we conclude that MEK and, therefore most likely ERK, is not involved in Rap1-mediated cell adhesion. This is confirmed by recent findings that active Rap1 did not interfere with the Ras signaling pathway (17). By utilizing the phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 and the phospholipase C inhibitor U73122, we did not find a role for phosphatidylinositol 3-kinase or phospholipase C in either RapV12- or Mn^{2+} -induced adhesion (data not shown).

DISCUSSION

Previously, we and others have shown that the small GTPase Rap1 is required for cytokine-induced inside-out activation of various integrins, including LFA-1, VLA-4, and $\alpha_M\beta_2$ (14, 15, 32, 33). In this manuscript we now demonstrate that also direct activation of LFA-1-, VLA-4-, and VLA-5-mediated adhesion by Mn^{2+} - or integrin-activating antibodies (monoclonal KIM185 for LFA-1 and TS2/16 for VLA-4 and VLA-5) is sensitive to inhibition of Rap1 signaling. This is shown by overexpression of either Rap-specific GAPs, which interfere with Rap1 signaling by reducing levels of endogenous GTP-bound Rap1, or overexpression of RalGDS-RBD, which inhibits Rap1 signaling by binding to GTP-bound Rap1 and sequestering it from potential effector proteins in Jurkat and K562 (Figs. 1 and 3). As an

assay to monitor enhanced integrin function, we measured binding to immobilized integrin ligands, *i.e.* ICAM-1 for LFA-1, VCAM-1 for VLA-4 and fibronectin for VLA-4 and VLA-5. This unexpected finding raised the question of whether the inhibition is due to an integrin-specific effect or whether Rap1 affects a general requirement for integrin-mediated cell adhesion, like cytoskeletal rearrangements and cell spreading. However, inhibition of Rap1 signaling did not affect integrin-independent adhesion to poly-L-Lysine (Fig. 4a). In addition, inhibition of TS2/16-induced adhesion by blocking Rap1 signaling could be rescued by increasing the fibronectin concentration (Fig. 4b). Moreover, inhibitors of actin cytoskeleton dynamics, cytochalasin D (14), and jasplakinolide,² did not influence RapV12-induced adhesion.

Basal Rap1 signaling may be sufficient to play a role in integrin-mediated adhesion, since we have not observed an increase in Rap1GTP upon treatment with either Mn^{2+} or activating antibodies (Fig. 5). This may imply that Rap1 only provides a favorable setting for enhanced integrin function. In agreement with a facilitating function for Rap1 are the recent findings that overexpressing Rap1bV12 in megakaryocytes itself had no effect, but augmented fibrinogen binding to $\alpha_{IIb}\beta_3$ induced by a PAR4 thrombin receptor agonist (34). In addition, we have not observed increased adhesion of human megakaryoblasts overexpressing active Rap1 to immobilized fibrinogen, whereas we have found inhibition of TPA-induced adhesion by RapGAP.² However, RapV12 as well as Rap1-specific GEFs that activate endogenous Rap1 are able to induce increased integrin-mediated cell adhesion (Fig. 2), showing that activation of Rap1 is sufficient to enhance the function of LFA-1.

We have also investigated the involvement of putative Rap1 effectors in the regulation of integrins. Although regulation of the MEK-ERK pathway is reported to be a predominant effect of Rap1 in T lymphocytes and other cell types (29–31), MEK inhibitors neither blocked RapV12- nor Mn^{2+} -induced adhesion (Fig. 8). Interestingly, this issue was recently analyzed in an *in vivo* model system (17). In agreement with our results, transgenic mice constitutively expressing Rap1aV12 in their T cell lineage did not demonstrate a modulating role for Rap1 in the MEK-ERK pathway. Furthermore, inhibitors of the proposed Rap1 targets phospholipase C and phosphatidylinositol 3-kinase did not interfere with RapV12- or Mn^{2+} -induced adhesion. However, pretreatment of cells with the intracellular calcium chelator BAPTA-AM inhibited both basal adhesion and RapV12-, Mn^{2+} -, or integrin-activating antibody-induced adhesion to a similar extent (Fig. 7). Utilization of the SK&F 96365 inhibitor, which blocks extracellular calcium channels required for leukocyte adhesion, also blocked RapV12-induced adhesion. This result is compatible with recent evidence that extracellular stimuli inducing T lymphocyte adhesion via LFA-1, such as T cell receptor stimulation and phorbol esters, do so by inducing an influx of extracellular calcium (28). Although in several studies Rap1 has been implicated in the regulation of cellular calcium levels (35–37), clear evidence is still lacking. A recent study demonstrated that Rap2b via interaction with phospholipase C ϵ is able to regulate intracellular calcium signaling in HEK293 cells (38). A large number of calcium-responsive signaling proteins have been shown to regulate integrin-dependent adhesion in various cell types, like calpain (28, 39, 40), calmodulin (41), and calcium/calmodulin-dependent kinase II (42–44). In the case of RapV12-induced adhesion, calmodulin is required (Fig. 7d). However, we could not demonstrate a role for calpain, calcium/calmodulin-dependent kinase II, or cal-

² Kim M. T. de Bruyn, S. Rangarajan, K. A. Reedquist, C. G. Figdor, and J. L. Bos, unpublished results.

cincurin in RapV12-induced integrin function (Fig. 7). The function of this calmodulin requirement is therefore still unclear.

Our results indicate that all stimuli that enhance the function of integrins tested, including inside-out signaling and direct activation, require Rap1. Previously, it was reported that the phorbol ester TPA may induce integrin activation independent of Rap1 activation (14). However, we have found that overexpression of RapGAPs or RalGDS-RBD efficiently blocked TPA-induced adhesion, indicating that Rap1-GTP is also required for TPA-induced integrin activation. How Rap1 enhances integrin function is still elusive. Cell surface expression of integrins is not affected by Rap1 signaling interference (14, 15). This indicates that Rap1 is involved in the increase in integrin function either by inducing a conformational change or by inducing clustering of integrins, two nonexclusive modes of integrin activation (8, 9, 45). Importantly, the effect of Rap1 on integrin function occurs with both β_1 and β_2 integrins. The most plausible explanation for the role of Rap1 is regulation of auxiliary factors for integrin function. These factors may be signaling or structural proteins that associate with integrin cytoplasmic tails (46). Matsuda and co-workers utilize fluorescent resonance energy transfer analysis to visualize Rap1 activation *in vivo* and note that growth factor-induced Rap1 activation initiates in the perinuclear region (47). Perhaps Rap1 influences integrin processing, the repertoire of integrin-associated proteins that is delivered to surface, or modulates membrane microdomain organization (lipid rafts) (48, 49) influencing integrin function (50).

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