Characterization of Interactions of Adapter Protein RAPL/Nore1B with RAP GTPases and Their Role in T Cell Migration \*\*\^\#\@

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Using a model of integrin-triggered random migration of T cells, we show that stimulation of LFA-1 integrins leads to the activation of Rap1 and Rap2 small GTPases. We further show that Rap1 and Rap2 have distinct roles in adhesion and random migration of these cells and that an adapter protein from the Ras association domain family (Rassf), RAPL, has a role downstream of Rap2 in addition to its link to Rap1. Further characterization of the RAPL protein and its interactions with small GTPases from the Ras family shows that RAPL forms more stable complexes with Rap2 and classical Ras proteins compared with Rap1. The different interaction pattern of RAPL with Rap1 and Rap2 is not affected by the disruption of the C-terminal SARAH domain that we identified as the \( \text{Rap2} \) is not affected by the disruption of the C-terminal SARAH domain family (Rassf). RAPL, has a role downstream of Rap2 in addition to its link to Rap1. Further characterization of the RAPL protein and its interactions with small GTPases from the Ras family shows that RAPL forms more stable complexes with Rap2 and classical Ras proteins compared with Rap1. The different interaction pattern of RAPL with Rap1 and Rap2 is not affected by the disruption of the C-terminal SARAH domain that we identified as the \( \text{Rap2} \) is not affected by the disruption of the C-terminal SARAH domain family (Rassf). RAPL, has a role downstream of Rap2 in addition to its link to Rap1. Further characterization of the RAPL protein and its interactions with small GTPases from the Ras family shows that RAPL forms more stable complexes with Rap2 and classical Ras proteins compared with Rap1. The different interaction pattern of RAPL with Rap1 and Rap2 is not affected by the disruption of the C-terminal SARAH domain that we identified as the \( \text{Rap2} \) is not affected by the disruption of the C-terminal SARAH domain family (Rassf). RAPL, has a role downstream of Rap2 in addition to its link to Rap1. Further characterization of the RAPL protein and its interactions with small GTPases from the Ras family shows that RAPL forms more stable complexes with Rap2 and classical Ras proteins compared with Rap1. The different interaction pattern of RAPL with Rap1 and Rap2 is not affected by the disruption of the C-terminal SARAH domain that we identified as the

Ras family members (about 15 related small GTPases, including classical Ras and Rap proteins) have been linked to a number of distinct functional classes of putative effectors (1–3). Three of these classes, Raf kinases, Rap guanyl nucleotide exchange factors, and phosphatidylinositol-3-kinase isoforms, have been studied more extensively as effectors of classical Ras proteins. Although there are several examples where members of Ras the family have been clearly linked to a particular downstream effector protein within a specific signaling context, in many instances, effector utilization by Ras proteins needs to be defined more precisely (4, 5). Conversely, known effectors for Rap GTPases can interact with different members of the Ras family. Since some of the Ras GTPases are closely related proteins (in particular within the effector-binding domains) (3) and several effector classes contain functionally related isoforms (1), understanding the importance of these often subtle differences remains a complex issue. Furthermore, despite several strategies that combine Ras/effector characterization in vitro with establishing functional links in cells (4), the task of substantiating the physiological relevance of many possible Ras-effector combinations has been particularly difficult to accomplish.

One class of Ras effectors, designated as the Ras association domain family (Rassf),\(^\#\) comprises structurally related gene products encoded by six distinct genes (rassf1 -- rassf6) and their splice variants (6). Rassf proteins contain a variable amino-terminal segment followed by a Ras association (RA) domain (7) and a putative coiled-coil structure (SARAH domain) (8) extending to the carboxyl terminus. These proteins are likely to function as adapters linking Ras GTPases, binding to the RA domain, to downstream signaling components. Some of the binding proteins, such as MIST kinases, can bind to Rassf proteins by interaction with the SARAH domain (9).

Among the Rassf family members, the function of Rassf1 and Nore1 (Rassf5) has been studied more extensively. Most notably, Rassf1 and Nore1 exhibit biological properties compatible with a tumor suppression function and growth inhibition (6, 10). The mechanisms of action of these Rassf members and a link to the Ras family, however, need further clarification. The evidence for the role of Nore1 in Ras-mediated growth inhibition is limited (11), and it was also found that Nore1 growth inhibition of human tumor cells did not require Ras (12). More recently, Rassf family members have also been implicated in

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regulation of cell adhesion and migration. In this respect, a splice variant of Nore1, designated as Nore1B or RAPL, have been best characterized (13, 14). RAPL is expressed preferentially in lymphocytes and has been implicated in inside-out signaling controlling cell adhesion and motility by modulating the affinity and/or special organization of LFA-1 integrins (15). In T and B cells, these responses are triggered by chemokines or antigen. It has been shown that in T cells, RAPL interacts with LFA-1 integrins and that, in response to a chemokine (CXCL12), RAPL relocates to the leading edge and immunological synapse (15). Analysis of RAPL function in vivo using mice with homozygous deletion of the exon encoding Nore1B/RAPL-specific sequences demonstrated defective migration of lymphocytes to secondary lymphoid organs and defective adhesion of dendritic cells (16). The function of RAPL in lymphocyte signaling has been linked to activation of a small GTPase from Ras family, Rap1 (15, 17), known to be one of the critical components of inside-out signaling to integrins in several cellular systems (18). Since RAPL has been initially isolated as a putative interacting protein using specifically Rap1 as bait, most of the work focused on the link between RAPL and Rap1 (13, 14). It is, however, possible that in other cellular contexts and signaling pathways, RAPL can be regulated by other Ras GTPases in parallel or instead of Rap1.

In this study, we show that RAPL has a role in regulation of random migration of HSBC2 T cells in a similar way to Rap GTPases, particularly the less characterized Rap2. The link between Rap2 and RAPL is further supported by studies of Rap2/RAPL interaction, providing new insights into characteristics required for the observed effects of Rap2 on T cell migration.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—The following antibodies were used in this study: Rap1 rabbit polyclonal IgG (epitop 121; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse anti-Rap2 (BD Pharmingen), rabbit anti-Nore1 (Novus Biologicals), rat anti-RAPL (15), GFP (B2) mouse monoclonal IgG (Santa Cruz Biotechnology), mouse anti-Myc (made in house), mouse anti-RAPL (15), GFP (B2) mouse monoclonal IgG (Santa Cruz Biotechnology), and rabbit anti-Nore1 (Novus Biologicals), rat anti-RAPL (15), GFP (B2) mouse monoclonal IgG (Santa Cruz Biotechnology), and anti-GAPDH mouse monoclonal (Fitzgerald Industries International Inc.), anti-CD11a mouse monoclonal antibody 38 (19). Horseradish peroxidase-labeled secondary antibodies, such as ECL anti-mouse IgG1, ECL anti-rabbit IgG1, and ECL anti-rat IgG1, were bought from GE Healthcare. ICAM-1Fc was produced as a chimeric protein consisting of five extracellular domains of ICAM-1 fused to the Fc fragment of human IgG1 (20). The fluorescent cell label 2',7'-bis-(carboxyethyl)-5(6)carboxyfluorescein was purchased from Calbiochem. The nucleotides, GDP, GTP, and GMP-PNP were purchased from Calbiochem. Isopropyl β-D-thiogalactopyranoside and arabinose used for protein induction were purchased from Sigma.

**Construction of Plasmids for Protein Expression**—The following RAPL plasmids used in this study were described previously: a plasmid encoding the Myc-tagged full-length human RAPL protein (pcDNA3RAPLmyc) (15) and a plasmid encoding mouse sequences corresponding to amino acid residues 52–265 or amino acid residues 52–212 in human RAPL fused to GST (pGEXNore199–413 and pGEXNore199–358) (7, 21). These plasmids were used to generate other constructs (namely RAPL42 (aa 42–265myc), RAPL42ΔC (aa 42–222myc), RAPLΔC (aa 52–212), and RAPL SARA1 (aa 212–265)). All proteins were expressed using the pTriEx™-4Ek/LIC system (Novagen; Merck) containing His6 and S tag preceding a Tev precision protease cleavage site in addition to sequences specified for each construct. Various constructs for members of the Ras small GTPase family and the effector RapGDS were described previously (22). GFP constructs pEGFP-RAPL, pEGFP-Rap1AV12, and pEGFP-Rap2BV12 were generated using the pENTR TOPO® system along with the GATEWAY™ cloning technology (Invitrogen). All point mutations were introduced using the QuikChange® site-directed mutagenesis kit from Stratagene. The following point mutations were made: K123L, R124L, K135L, H138A, Q136L, K154L, K155L, D160L, D132L, and I134L in pTriExRAPL42–265myc; K123L, R124L, K154L, and K155L in pEGFP-RAPL; F39S, A65T, S66A, and I72M in pTriEx-Rap2BV12; and S39F in pTriEx-Rap1AV12. The V12G mutation in Rap1AV12 and pEGFP-Rap2BV12 was reverted back to wild type (WT) to generate pGFP-Rap1A and pEGFP-Rap2B.

**Protein Modeling**—The RAPL RA domain predicted by SMART to range from amino acid 119 to 212 was modeled using the optimized mode of Swissmodel (available on the World Wide Web) due to sequence identity of less than 15%. First, an alignment was created with RA domains of Ral–GDS (Protein Data Bank code 1LFD) and Rap–RBD (Protein Data Bank code 1GU8) (23, 24), taking into account secondary structure elements of the ubiquitin superfold and conserved residues important for interaction with small GTPases. Secondary structure prediction was performed with the Psipred secondary structure prediction algorithm (available on the World Wide Web). For the models of RAPL complexes with Rap1 and Rap2, the template was the known structure of the Rap1GDS RA domain with H-RasD31K (Protein Data Bank code 1LFD) (23) and the known structures of Rap1A (Protein Data Bank code 1GU8) (24) and Rap2A (Protein Data Bank code 2RAP) (25) were combined with the RAPL model. The programs ProtParam, SMART, PyMol, and Swiss PDB viewer (all available on the World Wide Web) were used to manipulate and analyze structures and models. The stereochemical quality of the model was evaluated with Procheck (26).

**Protein Expression and Purification**—Escherichia coli CD41(DE3) GroELCamR was transformed with the specified constructs and grown in LB medium (containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol). The expression of GroEL was induced by 100 μM isopropyl β-D-thiogalactopyranoside and incubation at 20 °C overnight. Cells were lysed using BugBuster® reagent (Novagen) containing a 0.2 mg/ml final concentration of lysozyme (Sigma). The homogenate was spun for 1 h at 11,000 rpm at 4 °C. His6- or GST-tagged expressed proteins were isolated from the supernatant using His TrapTM FF column or GST TrapTM FF column (GE Healthcare) and eluted with a buffer containing 50 mM Tris/HCl, pH 8.0, 500 mM NaCl, 1 mM TCEP, and 500 mM imidazolium or 20 mM reduced glutathione.
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In Vitro Pull-down Assays—S-tagged small GTPases were bound to S-agarose beads (Novagen; Merck) and were loaded with nucleotide in nucleotide loading buffer (100 mM Tris/HCl, pH 7.5, 15 mM EDTA, and 1 mM TCEP) in the presence of 1 mM GTP or GDP. The nucleotide was adjusted to a final concentration of 20 mM MgCl₂. The beads were washed with 50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, and 1 mM TCEP (WB-A). The second interaction partner that was diluted in the same buffer containing an additional 2 mg/ml bovine serum albumin was incubated with loaded beads at room temperature for 30 min. The beads were washed three times in WB-A, boiled in sample buffer, and subject to SDS-PAGE and Western blotting. For the investigation of protein interactions in a cell context, HSB-2 T cells were co-transfected with different DNA constructs (10 μg/construct) and lysed after 24 h in Nonidet P-40 lysis buffer (1% Nonidet, 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 20% glycerol, proteinase inhibitor, and phosphatase inhibitors) for 30 min on ice. The cell lysate was incubated with S-agarose beads (prewashed with Nonidet P-40 buffer) for 1 h at 4 °C with constant agitation and then washed three times with Nonidet P-40 buffer, and the interaction was analyzed by Western blotting. The same protocol was applied to the GST-tagged proteins bound to glutathione-Sepharose beads (GE Healthcare).

Rap Activation Assay—The Rap activation assay was carried out as described by McLeod et al. (27). Briefly, glutathione-Sepharose beads (GE Healthcare) were loaded with RasGDS-RA-GST protein in Nonidet P-40 buffer (incubation on the rotating wheel for 1 h at 4 °C). HSB-2 cells were washed once in HBSS (Invitrogen) containing 20 mM Hepes, pH 8.0, and then placed for 30 min on coverslips coated with ICAM-1Fc in the presence of 5 mM MgCl₂ in HBSS buffer or left in suspension. Subsequently, the HSB-2 cells were lysed with Nonidet P-40 buffer for 30 min on ice, and the cell lysate was added to RasGDS-RA-GST beads followed by incubation for 30 min at room temperature. The glutathione beads were washed three times with Nonidet P-40 buffer, and the samples were analyzed by Western blotting for the amount of Rap1-GTP and Rap2-GTP present compared with total Rap in the supernatant.

Circular Dichroism—The protein folding and content of α-helical and β-sheet structural elements were measured via CD using a circular dichroism spectrophotometer (Aviv 215). The proteins were buffer-exchanged and diluted to a concentration of 0.5 or 0.25 mg/ml in 10 mM Na₂HPO₄/NaH₂PO₄, pH 7.0, and 50 mM NaCl. Every CD spectrum was measured twice across the spectrum from 190 to 240 nm at 25 °C (and for the RAPL212–265 also at 80 °C) by taking points every 0.2 nm with a 100 nm/min scan rate, interrogation time of 1 s, and 1-nm band width. Cells with a path length of 0.01 cm were used. The measured CD spectrum was compared against a data base of CD spectra of known secondary structures using Selcon3.

Protein Cross-linking—The RAPL SRH-domain (aa 212–265) was “buffer-exchanged” using NAP5 columns (GE Healthcare) into an amine group free buffer (25 mM Na₂HPO₄/NaH₂PO₄, 50 mM NaCl, and 1 mM MgCl₂). The protein was then diluted to the indicated concentrations below 5 mg/ml, and the cross-linker BS3-do (Pierce) was resuspended in Me₂SO was added in a 10-fold molar excess. The reaction was left incubating for 30 min at room temperature and stopped by quenching the reaction by adjusting to a final concentration of 20 mM Tris/HCl, pH 7.5. The samples were analyzed by SDS-PAGE and Coomassie staining.

Isothermal Calorimetry (ITC)—The thermodynamic parameters for the interaction of RAPL42myc and RalGDS RA domain with small GTPases were measured using an isothermal titration calorimeter (MSc-ITC; MicroCal, Inc.) at 25 °C in ITC buffer containing 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, and 1 mM TCEP as described previously (22). The small GTPases were loaded overnight at 4 °C with nonhydrolyzable GMP-PNP in a buffer containing 50 mM Tris/HCl, pH 7.5, 0.2 mM (NH₄)₂SO₄, and 1 mM EDTA using alkaline phosphatase. The nucleotide was fixed to a final concentration of 20 mM MgCl₂, and the buffer was exchanged into ITC buffer using NAP10 columns (GE Healthcare) and dialyzed overnight at 4 °C. A 1 μM solution of small GTPase was titrated into the sample cell containing 100 μM effector protein. Titrations were carried out by injecting volumes of 12 μl into the sample cell for the length of 25 injections with an injection delay of 300 s. Further data evaluation was done using the MicroCal Origin program.

Kinetic Parameters Using Biacore—The dissociation constant and the kinetic parameters were measured using the Biacore3000 (Biacore AG; GE Healthcare) in 25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM TCEP. A CM5 chip was coated with 10,000 response units of GST-antibody using the GST immobilization kit and linked to the CM5 chip using the amine coupling kit (Biacore AG; GE Healthcare). 1000 response units of GST RAPLΔC were fixed on the chip. Small GTPases were loaded with nonhydrolyzable GMP-PNP as described for ITC. Different concentrations of small GTPases (from 125 nM up to 40 μM) were flown over the prepared surface at 30 μl/min, with at least two concentrations measured twice; association was measured for 5 min, and dissociation was measured for 10 min. Measurements took place either at 25 or 4 °C. The data were evaluated using the BIAevaluation software.

Cells Culture and Transfection—The HSB-2 T cell line was maintained in RPMI 1640-glutamine (Invitrogen BLR) sup-
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implemented with 10% fetal bovine serum and 2.4 mM glutamine at cell numbers between 1 and 4 \times 10^6 cells/ml (28). Transfections of HSB-2 cells with DNA or siRNA were carried out as previously described (20). Briefly, HSB-2 cells were washed in Opti-MEM combined with GlutaMax (Invitrogen BLR) and resuspended to a final cell concentration of 4 \times 10^7 cells/ml. siRNA against RAPL (single Ambion probe, ID 131879), Rap1 (Dharmacon smart pools for Rap1A (gene ID 5906) and Rap1B (gene ID 5908)), Rap2 (Dharmacon smart pools for Rap2A (gene ID 5911), Rap2B (gene ID 5912), and Rap2C (gene ID 57826) and Ambion single probes (ID 120623, ID 120287 and ID 12069)) or control siRNA (siCONTROL nontargeting smart pool from Dharmacon (scrD) and Silencer® negative control from Ambion (scrA)) was added at 200 nmol or 10 \mu g of DNA to 2 \times 10^7 cells and electroporated at 950 microfarads/260 V electroporator. The cells were then added to prewarmed RPMI 1640, 10% fetal bovine serum, 2.4 mM glutamine and incubated for 24 h for DNA and 72 h for siRNA transfection at 37 °C with 5% CO_2. The efficiencies of transfections were assessed by fluorescence-activated cell sorting analysis. Expression of GFP-RAPL relative to endogenous RAPL was analyzed by Western blotting using anti-RAPL antibody and quantified. The amount of total RAPL in GFP-RAPL-expressing cells was about 2-fold higher than control cells. The expression of GFP and GFP-RAPL was also confirmed by Western blotting using anti-GFP antibody and compared with endogenous \alpha-actinin as loading control (bottom, right panel). Random migration on ICAM-1Fc in the absence or presence of control antibody (monoclonal antibody 38 (mAb38)). Control HSB-2 cells (WT) were compared with the cells expressing either GFP or GFP-RAPL (left panel) or the cells treated with the nontargeting scrambled (ScrA) or RAPL-specific (RAPL siRNA) siRNA (right panel). Experiments were carried out in triplicate in 96-well plate format, and adhesion was determined as a percentage of total cells using fluorescence measurements.

FIGURE 1. RAPL in HSB-2 T cells is involved in random migration without marked effect on adhesion. A, untreated HSB-2 cells (WT) were compared with the cells treated with a 100 nm concentration of either nontargeting scrambled siRNA (ScrA) or siRNA specific for RAPL (RAPL siRNA) (top panels). Knockdown of protein expression of endogenous RAPL was analyzed by Western blotting using anti-RAPL antibody and compared with \alpha-actinin (\alphaAc) (top, left). Random migration on ICAM-1Fc of the control (WT) and siRNA-treated cells is presented as tracking diagrams (top, middle panels; WT, ScrA, and RAPL siRNA) and as box and whiskers plots (top, left panel). Untreated HSB-2 cells (WT) were also compared with HSB-2 cells transfected with either plasmid expressing GFP or GFP-RAPL protein (bottom panels). The proportion of green fluorescent cells, 65% for GFP and 62% for GFP-RAPL, was determined by fluorescence-activated cell sorting analysis. Expression of GFP-RAPL relative to endogenous RAPL was analyzed by Western blotting using anti-RAPL antibody and quantified. The amount of total RAPL in GFP-RAPL-expressing cells was about 2-fold higher than control cells. The expression of GFP and GFP-RAPL was also confirmed by Western blotting using anti-GFP antibody and compared with endogenous \alpha-actinin as loading control (bottom, right panel). Random migration on ICAM-1Fc of WT-, GFP-, or GFP-RAPL-expressing cells was analyzed by video microscopy, and the data are presented as tracking diagrams (bottom, middle panels; WT, GFP, and GFP-RAPL) and as box and whiskers plots (bottom, left panel). The data shown for all migration experiments are representative of at least five independent experiments. The speed is shown as arbitrary units (AU), where 100 arbitrary units represents 1.65 \mu m/min. B, adhesion of HSB-2 cells to ICAM-1Fc was analyzed using immobilized ICAM1-Fc in the absence or presence of control antibody (monoclonal antibody 38 (mAb38)). Control HSB-2 cells (WT) were compared with the cells expressing either GFP or GFP-RAPL (left panel) or the cells treated with the nontargeting scrambled (ScrA) or RAPL-specific (RAPL siRNA) siRNA (right panel). Experiments were carried out in triplicate in 96-well plate format, and adhesion was determined as a percentage of total cells using fluorescence measurements.

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**Figure 2.** Rap2 and Rap1 differently affect random migration and adhesion of HSB-2 T cells. A. The activation state of Rap1 and Rap2 was assessed in a pull-down assay to immobilize Rap1-GTP and Rap2-GTP to glutathione-Sepharose beads loaded with GST-RalGDS RA. The cell extracts were prepared from HSB-2 cells present in suspension in a standard medium (control; lane 1) and from cells following plating on ICAM-1Fc in the presence of 5 μM MgCl₂ for 30 min at 37°C (ICAM1-Fc; lane 2). Aliquots of Rap1-GTP, Rap2-GTP, and Rap proteins in the cell lysate (aliquots containing 50 μg of protein) prior to pull-down (Rap in CL) were analyzed by Western blotting (left panels). Western blots were quantified by densitometry, and the amounts of Rap-GTP were calculated and expressed as a percentage of total Rap (right panels). B. Levels of endogenous Rap1, Rap2, and phospholipase Cγ2 in HSB-2 cells treated with 200 nM siRNA specific for Rap2 (100 nM Rap2A + 100 nM Rap2B probe) were analyzed at the indicated time points by Western blotting using corresponding, specific antibodies (top, left). Rap2 knockdown was about 50%. Expression of GFP, GFP-Rap2 WT, and GFP-Rap2BVT was analyzed by Western blotting using anti-GFP antibodies and compared with amounts of endogenous GAPDH (top, left panel). The proportion of cells expressing GFP fusion proteins Rap2BVT and Rap2BVT was 47 and 56%, respectively. Based on analysis of the expression of GFP-Rap2 proteins and the amount of endogenous Rap using anti-Rap2 antibodies, it was calculated that the total amount of Rap2 increased within the range of 1.8–2.2-fold. Random migration of HSB-2 cells expressing GFP, GFP-Rap2 WT, and GFP-Rap2BVT and of HSB-2 cells treated with Rap2 siRNA (as above) for 72 h is represented as tracking diagrams and their statistical analysis (bottom panels). C. Levels of endogenous Rap1, Rap2, and phospholipase Cγ2 in HSB-2 cells treated with either 200 nM siRNA specific for Rap1 (100 nM Rap1A + 100 nM Rap1B probe) or with 200 nM scrambled siRNA (Scr D) were analyzed at the indicated time points by Western blotting using corresponding, specific antibodies (top, left). Rap1 knockdown was about 50%. Levels of endogenous Rap1 were reduced by 50–60% (top, left). Levels of endogenous Rap2 increased within the range of 1.8–2.2-fold (top, right). Random migration of HSB-2 cells treated with scrambled siRNA (Scr D); with or without Rap2 siRNA (as above) for 72 h is represented as tracking diagrams and their statistical analysis (bottom panels).
mouse 38 anti-LFA-1 blocking antibody was added to each well and left incubating for 15 min on ice. The plate was subjected to brief centrifugation (1000 rpm for 1 min) before an incubation of 40 min at 37 °C to allow adhesion. Nonadherent cells were removed (two wash steps with HBSS, 20 mM Hepes, pH 8.0), and the T cell attachment was quantified using a Cytofluor multiwell plate reader (PerSeptive Biosystems, Hertford, UK). The adhesion was quantified as a percentage of total fluorescence.

RESULTS

RAPL and Rap2 Are Critical for Random Migration of HSB2 T Cells—To participate in an immune response, T cells must migrate across the vasculature and within tissues (30). The migration of HSB-2 T cells in vitro on ICAM-1, following engagement of integrin LFA-1, has been previously characterized as a model for this T cell migration (20). Although signaling pathways triggered by T cell integrins have not been fully elucidated, there is evidence that several small GTPases, including Rap proteins, are involved (31, 32). T cell motility responses to antigen and chemokines have been characterized, and studies have mainly focused on one Rap member, Rap1 (18), and one of its proposed effectors, RAPL/Nore1B (13, 14). To test the possible role of RAPL and Rap GTPases in random migration of HSB-2 T cells on ICAM-1, we performed specific knockdown of these proteins using siRNA. This approach was complemented by introduction of the wild type and constitutively active forms of these proteins into HSB-2 cells (Figs. 1 and 2).

The random migration of control HSB-2 T cells was compared with cells in which the amount of endogenous RAPL protein has been depleted by ~70% using a specific siRNA probe. HSB-2 T cells where RAPL was depleted show a significant reduction of the rate of migration (Fig. 1A, top). The role of RAPL in positive regulation of migration in this cellular system was further supported by the observation that overexpression of GFP-RAPL fusion protein led to an increase in the rate of migration on ICAM-1Fc (Fig. 1A, bottom). It was estimated (see the legend to Fig. 1A) that the expressed protein exceeded the endogenous RAPL level about 2-fold. We further tested whether the effect seen on migration was linked to a change of adhesiveness of LFA-1 integrins by measuring adhesion to ICAM-1Fc. The adhesion was analyzed using the same conditions as for random migration. Use of the LFA-1 function-blocking monoclonal antibody 38 demonstrated that effects observed are due to the LFA-1/ICAM-1Fc interaction. Under these conditions, neither reduction of endogenous RAPL protein by siRNA or overexpression of GFP-RAPL had an effect on adhesion of HSB-2 cells to ICAM-1Fc (Fig. 1B).

HSB-2 cells express both Rap1 and Rap2 proteins (data not shown). Furthermore, using immobilized Ras binding domain from RaLGDS, which specifically binds to activated, GTP-bound Ras and Rap proteins, plating of cells on ICAM-1Fc resulted in an increase of GTP-bound Rap1 and Rap2 compared with total Rap present in the supernatant (Fig. 2A). Since these are closely related proteins, we examined the effects on migration and adhesion of HSB-2 cells of knockdown of each Rap protein by siRNA treatment as well as overexpression of the wild type and constitutively active Rap2 (Rap2B12) or Rap1 (Rap1AV12) proteins (Fig. 2, B–D). In both cases, an increase of endogenous Rap levels by exogenous GFP fusion protein expression was about 2-fold. Endogenous levels of Rap1 and Rap2 were similarly reduced by siRNA (~50%).

Expression of GFP-Rap2B12 enhanced HSB-2 migration, whereas Rap2 knockdown reduced the migration (Fig. 2B). In
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There are several reports indicating that RAPL/Nore1B and a longer splice variant Nore1A that contains the same RA domain (Fig. 3A) could interact with several GTPases from the Ras family (5, 7, 33, 34). These studies, however, have not been focused on interactions with Rap1 and Rap2 proteins. One of the methods commonly used to assess interactions of small GTPases with candidate effectors is to demonstrate formation of protein complexes specific for the active, GTP-bound form of these proteins (35). Using a panel of purified and immobilized Ras GTPases in such a pull-down assay, we could show that RAPL/Nore1B could directly interact with Rap2BV12 to enhance migration in RAPL-depleted HSB-2 cells (supplemental Figs. 1 and 2) and Rap2 could interact with RAPL. Based on these observations, we further explored whether Rap2 could directly interact with RAPL and the possible role of such interactions in T cell migration. Our main approach was to characterize their interaction in vitro and subsequently apply our findings to a cellular setting.

**RAPL Forms More Stable Complexes with Rap2 than with Rap1**

In this experimental setup, the positive involvement of RAPL in migration and its possible link with RAPL. Based on these observations, we further explored whether Rap2 could directly interact with RAPL and the possible role of such interactions in T cell migration. Our main approach was to characterize their interaction in vitro and subsequently apply our findings to a cellular setting.
vated, GTP-loaded but not GDP-loaded forms of H-Ras, K-Ras, M-Ras, TC21, and R-Ras and also with Rap2A and Rap2B. However, using the same conditions, formation of stable complexes with Rap1A or Rap1B could not be detected (Fig. 3B). The RA domain from the well characterized effector protein RalGDS bound Rap1 and Rap2 proteins equally (Fig. 3B), as previously published (22, 36). Using different conditions (such as absence of detergent) that enhance nonspecific (to GDP-loaded Rap) and specific binding (to GTP-loaded Rap), some interaction of Rap1A and Rap1B with RAL could be detected; nevertheless, it appeared to be weak compared with Rap2/ RAL binding (Fig. 3C). Analysis of the complex formation using analytical gel exclusion chromatography also demonstrated that the amount of full-length Rap1A or truncated Rap1A (aa 1–166) proteins present in higher molecular weight fractions, complexed with RAL, was much smaller than that of corresponding constructs of Rap2B (Fig. 3D).

To understand the nature of the observed differences between Rap1 and Rap2 proteins, we attempted to measure their binding constants and kinetic parameters (Fig. 3, E and F). Using ITC or plasmon surface resonance, we found that indicate lower values for Rap1 than for Rap2 and comparable with H-Ras, whereas values for the RAL/Rap1 interations were about 2-fold higher, as illustrated in Fig. 3E. The RA domain from RalGDS, analyzed in the same experiment by ITC, had the same binding constants for Rap1 and Rap2 (of about 0.1 μM for both Rap isoforms), similar to those reported previously for Rap1/RalGDS interaction (7).

The assessment of protein-protein interactions by a pull-down assay would reflect not only the strength of binding but, more importantly, the kinetic parameters of an interaction, in particular indicate lower values for Rap1 than for Rap2 and comparable with H-Ras, whereas values for the RAL/Rap1 interations were about 2-fold higher, as illustrated in Fig. 3E. The RA domain from RalGDS, analyzed in the same experiment by ITC, had the same binding constants for Rap1 and Rap2 (of about 0.1 μM for both Rap isoforms), similar to those reported previously for Rap1/RalGDS interaction (7).

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**FIGURE 4.** The C-terminal region of RAL (SARAH domain) has a role in dimerization without an effect on binding to Ras GTPases. A, RAL variants, Myc-tagged RAL42 (aa 42–265), and Myc-tagged RAL42ΔΔC (aa 42–222) were analyzed by size exclusion chromatography on a Superdex200 PC3.2/30 column. The calculated values for their apparent sizes were indicated in the legend to Fig. 4D. Similarly, when purified GST-RAL with the intact or deleted SARAH domain (GST-RAL ΔC) was immobilized on beads and tested for binding of Myc-RAL variants, only RAL proteins containing the intact SARAH domain were able to interact with each other (Fig. 4D, left).

To test whether the SARAH domain contributed to binding and selectivity for Ras family GTases, Myc-RAL and its variant lacking the SARAH domain were compared in a pull-down assay. As shown in Fig. 4E, the deletion of the SARAH domain that affected RAL dimerization had no effect on binding of Ras and Rap proteins compared with the control; although the binding of H-Ras, M-Ras, and Rap2B was clearly detected, Rap1A was not bound to RAL variants under the conditions used (Fig. 4D). This was further confirmed by ITC (data not shown). Therefore, the SARAH domain was not involved in the binding of RAL to GTase family members, and the determinants for differential binding to Ras, Rap2, and Rap1 are likely to involve only residues within the RAL RA domain.

**Residues Critical for Differential Binding of RAL to Rap2 and Rap1 and Their Functional Importance**—The structural features of interaction surfaces between Ras-binding domains

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**Residues Critical for Differential Binding of RAL to Rap2 and Rap1 and Their Functional Importance**—The structural features of interaction surfaces between Ras-binding domains
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FIGURE 5. Three-dimensional model of the RAPL RA domain and interactions with Rap1 and Rap2. A, schematic presentation of predicted interactions between the RAPL RA domain and Rap2, suggesting formation of a hydrophobic pocket due to extensive interactions between Phe39 in Rap2 and Ile134 in Ala131 in RAPL RA. This feature is not predicted in the RAPL/Rap1 interaction surface, where Phe39 is replaced by Ser39. The dotted lines represent hydrophobic interactions, double-headed arrows represent side chain interactions, and continuous lines show main chain interactions. Switch I and switch II regions are marked. B, a model for interaction between the RAPL RA domain, shown as a yellow ribbon, with Rap2A (top) and Rap1A (bottom). Residues Leu137, Ile134, and Ala131 in RAPL RA and residues Ile36 and Phe39 in Rap2A or Ile36 and Ser39 in Rap1 are highlighted.

(designated as RA or RBD domains that adopt the same ubiquitin-like fold) from several effectors and Ras GTPases have been reported (3, 37). In each case, a unique subset of residues from the RA/RBD domain, specific to a particular effector, interacts with residues of Ras within the regions (switch I and switch II) that change their conformation upon GTP binding. Typically, the residues within switch I are involved in RA/RBD binding with a minor contribution from switch II residues in some cases. Manipulation of these interaction surfaces unique to each effector/Ras family member pair could create protein variants that specifically preserve or abolish particular Ras/effector interactions (38). To apply this approach and identify residues that are critical for differential binding of RAPL to Ras family members, in particular Rap1 and Rap2, we first generated a model of RAPL RA domain and its complexes with Rap1 and Rap2.

The RAPL RA domain was modeled by manual alignment with the most similar, structurally known RA domain of RalGDS (supplemental Fig. 1A). The predicted interaction surface of this RA domain suggests several distinct characteristics in distribution of surface charge and position of hydrophobic surfaces compared with RalGDS and Raf (supplemental Fig. 1B, left). Comparison of known structures of Rap1A and Rap2A, proteins with a sequence identity of 60%, shows considerable difference in their structural features (supplemental Fig. 1B, right). Interaction of RAPL RA domain with Rap1 and Rap2 was modeled using structures of Rap1A and Rap2A, and the structure of the RalGDS RA domain-H-RasD31K complex as a template (Fig. 5). The schematic representation of RAPL-Rap complex depicts residues in RAPL RA that mainly interact with the switch I region of Rap (Fig. 5A). The main difference between Rap1 and Rap2 within the switch I region is residue 39 exposed at the surface, Ser39 in Rap1 and Phe39 in Rap2. Furthermore, based on the models of RAPL/Rap1 and RAPL/Rap2 interactions, only Phe39 in Rap2 would be favorably positioned to make interactions with Ile134 and Ala131 in RAPL, thus creating stronger interactions by generating a hydrophobic pocket (Fig. 5, A and B). Since a higher degree of hydrophobic interactions at protein interfaces has been previously associated with more stable complexes (39), this feature is likely to contribute to the more stable nature of RAPL/Rap2 interactions described in Fig. 3.

Based on the model of the RAPL RA domain (supplemental Fig. 3A and Fig. 5), several residues that are likely to be involved in binding of Ras proteins were replaced as single point mutations (Fig. 6A, top). When analyzed in a pull-down assay using immobilized H-Ras, M-Ras, and Rap2B, some replacements (K154L, K155L, and D160L) abolished binding of RAPL to all tested Ras GTases, whereas several mutations (R123L, K124L, and H138A) reduced the binding to these GTases to a different degree (Fig. 6A, middle and bottom). For example, R123L completely abolishes the binding to Rap2B but retained some interactions with H-Ras and M-Ras. Although these mutations support the concept that interactions with different Ras GTases could involve different critical residues, we were not able to generate RAPL variants that spared Rap2B binding while reducing other interactions. Nevertheless, several RAPL RA domain mutants lacking binding to Rap2B with other interactions affected to a lesser degree were tested in HSB-2 cells. Transfection of HSB-2 T cells with R123L and K124L RAPL variants had an inhibitory effect on random migration (Fig. 6B). K154L and K155L RAPL variants that were reported previously to act as dominant negative proteins (12, 33) were also tested and found to reduce migration (data not shown), supporting further the role of RAPL as an effector of Ras GTases in HSB-2 cells.

Since the RAPL variants were not informative regarding selective interactions with Rap1 and Rap2, further mutational analysis was performed with Rap1 and Rap2 proteins. As already highlighted in the three-dimensional model (Fig. 5), within the switch I region, residue 39 is phenylalanine in Rap2A, Rap2B, and Rap2C, whereas in Rap1A and Rap1B, this residue is a serine (Fig. 7A, top). There are also several differences in the switch II region (Ala65, Ser66, and Ile72 in Rap2, corresponding to Thr65, Ala66, and Met72 in Rap1) (Fig. 7A, top); however, these residues were not predicted to directly interact with Rap proteins. Single point mutations of residues in Rap2B to the residues present in Rap1 proteins showed that Phe39 was critical for the Rap2 binding, whereas the other mutations only reduced the binding to RAP1 (Fig. 7A, middle). Furthermore, the replacement of Ser39 in Rap1A by phenylalanine resulted in binding to RAP1 comparable with that of
None of the tested mutations in the Rap proteins had an effect on binding to RalGDS in the in vitro pull-down assay (Fig. 7A, middle). Analysis of Rap2B incorporating the F39S mutation using ITC confirmed that interactions of RAPL with this Rap2 variant were similar to RAPL/Rap1 interactions (data not shown). This Rap2B variant (S39F) was further used to assess the functional importance of stable interactions between RAPL and Rap2 proteins in the HSB-2 migration assay. Transfection of the Rap2BV12 F39S mutant did not enhance HSB-2 migration, as observed for Rap2BV12 (Fig. 2C), and the rate of migration of cells expressing the Rap2BV12 F39S mutant was reduced in comparison with Rap2B V12 at the same expression level and similar to the migration rate of untreated cells (Fig. 7B). Interestingly, expression of the Rap1AV12 S39F variant did not enhance the inhibitory effect of Rap1A V12 on HSB-2 migration but demonstrated properties more similar to Rap2 (supplemental Fig. 4). This, together with the binding studies, illustrates how relatively small differences in protein structures, in this case between switch regions of Rap1 and Rap2 proteins, can determine the different nature of protein/protein interaction with important consequences for cellular function.

**DISCUSSION**

Although some of the Ras family members and effector molecules have been studied in great detail, others still remain poorly defined (1, 2, 40). Previous studies of Rap2 proteins were limited and often focused on comparison with Rap1 (36, 41–44), whereas Nore1B/RAPL protein was mainly considered as an effector of Rap1 in lymphocytes (13, 14). Although Rap1 and Rap2 proteins are closely related (40), the functional link between Rap2 and RAPL has not been explored. Since our initial experiments suggested the possibility of a Rap2/RAPL link, we analyzed the LFA-1-mediated random migration of T cells on ICAM-1, where both RAPL and Rap1 have been demonstrated to have a role. We confirmed the positive involvement of RAPL in migration. However, under the same conditions, the lack of an effect on adhesion of HSB-2 T cells was more similar to our observations for Rap2 than for Rap1, which was clearly required for cell adhesion (Figs. 1 and 2).

In addition to potentially linking RAPL and Rap2, these experiments also provided new insights into the function of RAPL and Rap GTPases in T cells. The finding that RAPL is not critical for adhesion was somewhat surprising, since previous studies in T cells showed a marked reduction of adhesion to ICAM1 using a RAPL variant considered to have a dominant negative function and also colocalization of RAPL with Rap1 regulating the adhesion of T cells in response to cytokines and antibody stimulation (15). However, studies using mice deficient in RAPL showed that RAPL was not critical for basal levels of adhesion of T cells to ICAM-1 or VCAM-1, although it con-
tributed to adhesion stimulated by CCL21, and the migratory properties leading to redistribution of T-lymphocytes were clearly affected (16). Thus, it is possible that RAPL, depending on the cell type and environment (in particular the presence or absence of antigens or chemokines), could contribute to cell motility without affecting cell adhesion.

The findings that Rap2 can be activated under the same conditions as Rap1 and that it can affect migration (Fig. 2) are consistent with several previous observations in B cells, where supporting evidence was mainly obtained from overexpression of activated Rap2 and the use of Rap GAPII that blocks the activation of both Rap1 and Rap2 (42, 43, 45). We also show that expression of constitutively active Rap2 enhances migration and, more importantly, that partial depletion of endogenous Rap2 by specific siRNA reduces migration of HSB2 T cells. However, the differences between requirements for Rap1 and Rap2 seen in HSB-2 cells (Fig. 2) have not been observed in previous studies of adhesion and migration of B cells (42, 43). In some other cell types, such as hippocampal neurons, some functional differences have been suggested, and in this system, only Rap2 had an effect on axon and dendrite morphology (46). The possibility that these closely related GTPases could have functional differences is also supported by the findings that Rap1 and Rap2 are differentially activated by various guanylnucleotide exchange factors and that different pathways can be involved in their activation (18, 47). The data presented here (Fig. 2) suggest that Rap1 and Rap2, although both involved in T cell motility, can have overlapping but distinct roles. It is possible that Rap1 could affect T cell motility through its role in adhesion, whereas Rap2 may affect motility through involvement in other aspects of regulation of this complex cellular function.

To obtain further supporting evidence that RAPL can function as an effector of Rap2, we analyzed these proteins and their possible interaction both in HSB-2 cells and in vitro. Studies of co-localization revealed a relatively broad distribution of RAPL in HSB-2 cells moving on ICAM-1 that partially overlapped with localization of Rap2 (Fig. 2). The variants of Rap1 and Rap2 proteins used for the pull-down were analyzed by SDS-PAGE followed by Coomassie Blue staining; lanes 1–7 correspond to Rap2BV12I72M, Rap2BV12S66A, Rap2BV12A65T, Rap2BV12F39S, Rap1AV12S39F, and Rap1AV12 (bottom panel). B, untreated HSB-2 cells (WT) were compared with HSB-2 cells transfected with either plasmid expressing Rap2BV12 or Rap2BV12F39S protein (left panels and top right panel). The expression of Rap2BV12 and Rap2BV12F39S was also confirmed by Western blotting using anti-His6 antibody (bottom, right panel). Compared with endogenous Rap2 protein, the expression of exogenous Rap2 increased Rap2 levels by 1.5-fold. Random migration on ICAM-1Fc of WT-, Rap2BV12-, or Rap2BV12F39S-expressing cells was analyzed by video microscopy, and the data are presented as tracking diagrams and their statistical analysis.

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FIGURE 7. Structural determinants of differential binding to Rap1 and Rap2. A, alignment of the amino acid sequences in the effector binding regions (residues 28–82) of human Rap1 and Rap2 proteins. Residues different within the indicated switch I and switch II region are shown in boldface type (top panel). Rap1 and Rap2 proteins with the indicated point mutations were used in a pull-down experiment with Myc-tagged truncated RAPL (RAPL42) or GST-tagged RalGDS RA domain (RalGDS) (middle panel) as described in the legend to Fig. 3B. The variants of Rap1 and Rap2 proteins used for the pull-down were analyzed by SDS-PAGE followed by Coomassie Blue staining; lanes 1–7 correspond to Rap2BV12I72M, Rap2BV12S66A, Rap2BV12A65T, Rap2BV12F39S, Rap1AV12S39F, and Rap1AV12 (bottom panel). B, untreated HSB-2 cells (WT) were compared with HSB-2 cells transfected with either plasmid expressing Rap2BV12 or Rap2BV12F39S protein (left panels and top right panel). The expression of Rap2BV12 and Rap2BV12F39S was also confirmed by Western blotting using anti-His6 antibody (bottom, right panel). Compared with endogenous Rap2 protein, the expression of exogenous Rap2 increased Rap2 levels by 1.5-fold. Random migration on ICAM-1Fc of WT-, Rap2BV12-, or Rap2BV12F39S-expressing cells was analyzed by video microscopy, and the data are presented as tracking diagrams and their statistical analysis.
acterized, we first determined binding properties and critical structural requirements for this interaction.

Previous studies of the Ras binding RA domain common to Nore1A and Nore1B/RAPL protein were limited to interactions with H-Ras and Rap2 (7, 34). Using a combination of methodologies, we found that Rap2 has a $K_d$ value similar to that of H-Ras, whereas Rap1 binding was consistently lower (Fig. 3). In agreement with this, a previous comparison of the binding of the Nore1 RA domain with H-Ras and Rap1 showed an even larger, 10-fold difference (7, 34). A lower dissociation rate is likely to be an important factor contributing to greater stability of RAPL-Rap2 complexes compared with RAPL-Rap1 (Fig. 3). In fact, it was not possible to detect binding of RAPL to Rap1 in a pull-down assay (Fig. 3). We demonstrated further that the conversion of residue 39 of Rap2 to the corresponding residue in Rap1 was sufficient to destabilize the complex with RAPL and that a Rap2 variant incorporating an S39F replacement in the context of Rap2V12 was no longer capable of enhancing migration of HSB-2 cells (Fig. 7). Thus, this single residue difference between Rap2 and Rap1 in the region involved in effector binding (switch I) is functionally important and could be critical for effector selection or properties of complexes formed with the common effectors. Applying a similar strategy to RAPL that was also previously used to support RAPL regulation by Ras family GTPases (48), we show that RAPL requires a functional RA domain to participate in HSB-2 cell migration (Fig. 6).

Since Ras GTPases interact with multiple effectors, it is possible that other interactions of Rap2 could be affected by F39S replacement in a way similar to the interaction with RAPL. However, several previous studies using an isolated RA domain from well-characterized effectors and several Ras family members showed the same binding pattern of Rap1 and Rap2 to Rap1, B-Raf, RafGDS, Rgl, Rif, and phospholipase Cε (5, 22, 36). Based on these observations, it is unlikely that the F39S mutation would affect Rap2 interaction with these proteins, as shown here for RafGDS (Fig. 7). The available structural data for Ras proteins and RA/RBD domains (3) and the model of RAPL interaction with Rap1 and Rap2 (Fig. 5) also support the possibility that the selectivity of the RAPL RA domain could be unique for the RA domain of this effector. Several Rap2-binding proteins without the RA domain, which apparently do not interact with Rap1, have been identified (RPI8 and its homologue RPI8, TNIK, and MAP4K4) (49–51). However, the function of these proteins as Rap2 effectors has not been extensively explored, and the involvement of these proteins in regulation of T cell migration has not been reported.

Although several lines of experimental evidence suggest that RAPL and other Rassf proteins can function as adaptors, the properties and mechanism of assembly of these proposed complexes that would include Ras, RAPL, and downstream proteins, such as MIST kinases, have not been studied. A study of Nore1A and possible consequences of Ras binding focused on the unique region containing the C1 domain (34). However, RAPL does not contain this region. The other common and potentially important region for regulation of many Rassf members is the SARAH domain that has also been identified in Drosophila proteins and predicted to be region of homo- or heterodimerization and possibly a feature that could be involved in formation of larger oligomers (8). We here provide the first direct evidence that the SARAH domain is an α-helical segment involved in dimerization (or oligomerization) of RAPL in vitro and in cells (Fig. 4). However, it remains to be established whether this provides a mechanism for clustering of different small GTPases that could bind RAPL. It is also possible that binding of Ras GTPases could allosterically regulate the SARAH domain to affect interactions with the MIST kinase that also contains a SARAH domain capable of mediating dimerization (52).

In conclusion, we here provide new insights into properties of RAPL and Rap proteins and characterize unique features of RAPL-Rap1 and RAPL-Rap2 complexes. Further analysis in cells, using this information, supports the signaling link between Rap2 and RAPL in the context of T cell migration. These observations suggest a wider role for Rassf adapter proteins and Rap GTPases in cell motility and show that subtle differences between highly similar Rap proteins could be reflected in distinct interactions with common effectors and their cellular function.

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