Local Activation of Rap1 Contributes to Directional Vascular Endothelial Cell Migration Accompanied by Extension of Microtubules on Which RAPl, a Rap1-associating Molecule, Localizes*

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Endothelial cell migration is promoted by chemottractants and is accompanied with microtubule extension toward the leading edge. Cytoskeletal microtubules polarize to function as rails for delivering a variety of molecules by motor proteins during cell migration. It remains, however, unclear how directional migration with polarized extension of microtubules is regulated. Here we report that Rap1 controls the migration of vascular endothelial cells. We found that Rap1-associating molecule, RAPl, which belongs to the Ras association domain family (Rassf), localized on microtubules and that activated Rap1 induced dissociation of RAPl from microtubules. A Rap1 activation-monitoring probe based on the fluorescence resonance energy transfer enabled us to demonstrate that local Rap1 activation occurs at the leading edge of the cells under the two types of cell migration, chemotaxis and wound healing. Time lapse imaging of microtubules marked by enhanced green fluorescent protein-RAPl showed the directional growth of microtubules toward the leading edge of the migrating cells. Using adenovirus, inactivation of Rap1 by expression of rap1GAPII inhibited wound healing. In addition, disconnection of Rap1 and RAPl by expression of a RAPl mutant also perturbed wound healing. Collectively, the locally activated Rap1 and its association with RAPl controls the directional migration of vascular endothelial cells.

Rap1 belongs to the Ras GTPase family and cycles between a GDP-bound inactive form and a GTP-bound active form (1). Rap1 activation is regulated by guanine nucleotide exchange factor (GEF), whereas Rap1 inactivation is regulated by GTPase-activating protein (GAP). GEFs contain a catalytic domain and regulatory domains that either bind to upstream molecules or are regulated by second messengers such as cAMP and Ca2+. The former GEFs include C3G and PDZ-GEF; the latter includes CalDAG-GEFs and Epac (cAMP-GEF) (reviewed in Ref. 2). Thus, the spatial Rap1 activation depends on the localization of GEF and the spreading of second messengers. Like GEFs, the spatial Rap1 inactivation depends on the localization of GAPs for Rap1 (3). We have currently developed a spatio-temporal activation/inactivation monitoring probe for Rap1 in living cells (4).

Once activated by GEFs, GTP-bound Rap1 associates with effector molecules including Raf-1, B-Raf, RafGDS, and AF-6 (2). Rap1 shares these effector molecules with Ras protein; therefore, Rap1 is suggested to function antagonistically on the Ras-activated intracellular signaling pathway. However, Rap1 may have a unique function in regulating cell adhesion (5, 6). Rap1 was recently reported to be indispensable for cell-extracellular matrix (ECM) contacts by stabilizing the cell-ECM contacts, indicating that Rap1 enhances cell adhesion to ECM (7, 8). Moreover, Bud1, the yeast homologue of Rap1, determines the budding site (9), and Rap1 regulates adherens junction positioning for cell division in Drosophila (10), implying that Rap1 is also involved in cell polarization.

Cells have a polarity determined by cell protrusions and retractions, when moving toward certain chemoattractants or during wound healing. In the protrusions, actin is actively polymerized and depolymerized, whereas in retractions stabilized actin fibers are observed (11). For perpetual movement toward the chemoattractants, asymmetrical polarity of cell contacts to ECM is required. Focal adhesions connecting actin stress fibers are assembled in the protrusions and disassembled in the retractions of migrating cells (reviewed in Ref. 12). Like actin, microtubules are assembled toward the leading edge of the protrusions. By constituting rails for motor proteins carrying the molecules to the protrusive part of the cell, microtubule promotes cell polarity (13). Furthermore, recently, assembly and disassembly of focal adhesions are reportedly regulated by microtubules (14, 15). Thus, microtubule extension toward the leading edge parallels the change in polarity of the motile cells toward the chemoattractants or during wound healing.

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The abbreviations used are: GEF, guanine nucleotide exchange factor; CFP, cyan fluorescent protein; ECM, extracellular matrix; S1P, sphingosine 1-phosphate; SDF-1, stromal-derived factor-1; MTOC, microtubule-organizing center; Rassf, Ras association domain family; S1P, sphingosine 1-phosphate; SDF-1, stromal-derived factor-1; YFP, yellow fluorescent protein; MES, 4-morpholineethanesulfonic acid.

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RAPL/NORE1B (hereafter referred to as RAPL) is identified as a Rap1-binding molecule (16), which contains a Ras/Rap1 binding domain and belongs to the Ras association domain family (Rassf) (17, 18). Whereas Rassf members function as potent suppressors of tumors (19–21), RAPL links Rap1 activation upon T cell receptor cross-linking and stimu- lated-Ras-mediated factor-1 (SDF-1) stimulation to integrin activation. In addition, RAPL mediates the polarization and activation of SDF-1 receptors upon Rap1 activation (16). Recently, Rassf1 has been shown to localize at and stabilize microtubules (22). However, it is unclear whether other molecules belonging to Rassf function in the association with Ras family GTPases.

We investigate the localization of RAPL in the vascular endothelial cells and how Rap1-RAPL participates in determining the directional migration in response to a chemoattractant, sphingosine 1-phosphate (SIP) (23), and during wound healing. RAPL localizes at the microtubule-organizing center (MTOC) and microtubules. Rap1 is activated at the leading edge of migrating cells. In addition, inactivation of Rap1-RAPL signal perturbed the wound closure. These data suggest that local activation of Rap1 and its association with RAPL regulates the directional cell migration of vascular endothelial cells.

**Experimental Procedures**

**Reagents and Antibodies**—SIP was purchased from Biomol (Plymouth, PA). Protein A-Sepharose was from Calbiochem. Anti-green fluorescent protein (GFP) was developed in our laboratory. Anti-Rap1 was from BD Biosciences. Anti-RAPL antibody was a kind gift from T. Kinashi (Kyoto University, Japan).

**Plasmids**—The coding sequences of human Rassf1A, Rassf1C, Rassf2 (KIAA0168), Rassf3, and RAPL (NORE1B) were amplified by PCR-based mutagenesis and subcloned into pCA-EGFP vector similarly to Rassf1. pCAGGS eukaryotic expression vector and expressed enhanced green fluorescent protein (EGFP)-tagged each Rassf molecules (24). cDNAs encoding RAPL deletion mutants as indicated in Fig. 4 were amplified by PCR and ligated into pCA-EGFP vector similarly to Rassf1. dC1, dC2, dC3, and dN encoded amino acids 1–222, 1–168, 1–100, and 101–1–265 of RAPL, respectively. A mutant of RAPL (hereafter referred to as the RA mutant) in which Lyn123, Lyn124, and Lyn125, 154, 155, 164, and 165 were deleted was replaced with Ala, to be incapable of associating with Rap1 (16). cDNA encoding an RA mutant was amplified by PCR-based mutagenesis and subcloned into pCA-EGFP. pCXN2-FLAG-Rap1 expressed FLAG-tagged Rap1. Either constitutive active or dominant negative forms of Rap1 (Rap1V12 or Rap1N17) cDNAs were similar to those described in Ref. 2. pMR21-Rap1V12 expressed FLAG-tagged Rap1V12 and internal ribosomal entry site-driven dsFP93 as described previously (24). pMR21-FLAG-RAPL expressed both FLAG-tagged rsg1APII and internal ribosomal entry site-driven dsFP93. pCA-DsRed-CrkI was derived from pCAGGS eukaryotic expression vector as described previously (24). pRaichu-Rap1 expressed a chimeric protein consisting of yellow fluorescent protein (YFP), Rap1, and Ras-binding domain of Raf and cyan fluorescent protein (CFP) followed by the CAA motif of Ki-Ras. In pRaichu-Rap1N17, a cDNA encoding Rap1N17 was replaced with that encoding Rap1. pGEX-RAPL was constructed by inserting a cDNA encoding full-length RAPL into pGEX (Amersham Biosciences).

**Adenovirus**—Both an adenovirus-expressing EGFP-tagged RAPL and an adenovirus-expressing EGFP-tagged RA mutant of RAPL were produced using the Adeno-X expression system (BD Biosciences). Briefly, cDNA from pCA-EGFP-RAPL was inserted into Adeno-X viral DNA using pShuttle as a transfer vector. Adenovirus-expressing EGFP-RAPL was produced from HEK293 cells transfected with Adeno-X-EGFP-RAPL. An adenovirus-expressing RA mutant of RAPL was produced in a similar manner to RAPL-expressing adenovirus. The EGFP-expressing adenovirus and the rap1GAPII-expressing adenovirus were generous gifts from H. Kurose (Kyushu University, Japan) and S. Hattori (Tokyo University, Japan), respectively.

**Cell Culture and Transfection**—Human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs) were purchased from Cascade Biologies, Inc. (Portland, OR) and cultured in Humedia-EG2 as previously reported (24). HEK293T cells were generous gifts from Dr. B. J. Mayer (University of Connecticut) and maintained as described previously. Jurkat cells and HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 (Invitrogen) and Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cultured cells were transfected using Lipofectamine 2000 reagent (Invitrogen).

**Reverse Transcription-PCR, Full-down Assay, Immunoprecipitation, and Immunoblotting**—RNAs from cultured Jurkat cells and HUVECs were prepared by TRIzol (Invitrogen). cDNAs were synthesized by reverse transcriptase reaction using random primers and RNAs as templates. The cDNA specific for human RAPL was amplified by PCR using a primer set (5′-RAPL, CTTGGGAGGACGTGAGAGGCAGTCTC; 3′-RAPL, AGGGATGGAGAAGGCATCCCACTCT). GTP-bound Rap1 was detected according to the method of Bos and co-workers (25). Briefly, HUVECs stimulated with 1 μM SIP for the time indicated at the top of Fig. 5 were lysed in a lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid, 1 mM Na3VO4). Preclreated lysates were incubated with GST-Rap1-binding domain of RalGDS and glutathione-Sepharose beads. Proteins collected on the beads were subjected to SDS-PAGE followed by immunoblotting with anti-Rap1 antibody. Immunoprecipitation and immunoblotting were performed as described previously (26). Briefly, HAECs transfected with wild-type and RA mutants as indicated in Fig. 5 were lysed using lysis buffer. Lysates were preclreated by centrifugation at 15,000 × g for 10 min, followed by immunoprecipitation using anti-GFP and Protein A-Sepharose. Immunoprecipitates were subjected to SDS-PAGE and immunoblotting with anti-FLAG antibody and peroxidase-conjugated goat anti-mouse IgG as a primary and a secondary antibody, respectively.

**Proteins reacting with anti-FLAG were visualized by an ECL system (Amersham Biosciences).**

**Microtubule Binding Assay**—Microtubule-associating protein-containing microtubule prepared from bovine brain were gifts from N. Yamagishi (Kyoto Pharmaceutical University, Japan). Glutathione S-transferase-fused RAPL (GST-RAPL) expressed in BL21-Star bacteria (Invitrogen) was collected on glutathione-Sepharose (Amersham Biosciences). GST-RAPL was eluted using 10 mM glutathione, preclreated with microtubule binding buffer (100 mM MES-KOH (pH 6.8), 2 mM EDTA, 1 mM MgCl2, 10 mM Taxol, 4 μM glycerol, and 1 mM GTP). For the microtubule binding assay, 5 μg of purified microtubules and GST-RAPL at the concentration indicated in Fig. 3 legend were mixed in 200 μl of microtubule-binding buffer for 30 min at 37 °C. After centrifugation at 40,000 × g for 15 min, equal amounts of the supernatant and the pellet were analyzed by SDS-PAGE and immunoblotting with anti-GST. Bovine serum albumin fraction V was used as a negative control for GST-RAPL.

**Wound Healing Assay and Responses to Chemoattractant from a Micropipette**—HUVECs or HAECs transfected with plasmids indicated in the figures were cultured on 35-mm glass bottom dishes coated with collagen until they reached the monolayer state. The cells were cultured in regular medium alone along the edge of the glass culture. The medium remained unchanged during wound healing. Monolayer-cultured HUVECs infected with an adenovirus expressing rap1GAPII, an adenovirus expressing EGFP-RAPL, or an adenovirus expressing the RA mutant of RAPL were scratched and time lapsed imaged. HAECs expressing EGFP-RAPL or those expressing Raichu-Rap1 cultured on glass-bottom dishes coated with collagen were exposed to 1 μM SIP supplied by a micropipette (Femtojet; Eppendorf-Japan).

**Fluorescence Microscopy and Confocal Imaging**—HUVECs or HAECs transplanted with plasmids expressing fluorescence-tagged proteins as indicated in the figure legends were imaged on an Olympus IX-81 inverted microscope. The microscope with a 75-watt xenon arc lamp was equipped with a cooled charge-coupled device camera, CoolSNAP-HQ (Roper Scientific), and two filter exchangers, controlled by MetaMorph 5.0 software (Molecular Devices). The EGFP image and DsRed image were obtained through an XF2043 dichroic filter (Omega) and either a set of an S484/15 excitation filter and an S515/30 emission filter or a set of an S555/25 excitation filter and an S630/60 emission filter, respectively, as reported previously (27). HUVECs transfected with pCA-EGFP-RAPL cultured on a collagen-coated glass-base dish were exposed to 1 μM S1P for the time indicated at the top of Fig. 2 and permeabilized with 0.1% Triton X-100. Permeabilized cells were incubated with anti-β-tubulin or anti-γ-tubulin antibody. Immunopositive reaction was visualized with Alexa 546 goat-antimouse IgG (Molecular Probes, Inc., Eugene, OR). Confocal images of EGFP and Alexa 546 were obtained by an Olympus BX50WI microscope controlled by Fluoview. To monitor the cell shape and localization of fluorescence-tagged molecules in living cells, a phase-contrast image and a fluorescent image were obtained using a 40× objective and an FITC channel.
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Fig. 1. The association of RAPL with Rap1 and its expression in vascular endothelial cells. A, HEK293T cells were transfected with plasmids as indicated at the top. Cell lysates were subjected to immunoprecipitation (IP) followed by immunoblotting (IB) with antibodies as indicated on the left or directly subjected to SDS-PAGE followed by immunoblotting with the antibodies as indicated on the left. An arrow denotes GTP-bound Rap1 co-immunoprecipitated with EGFP-tagged Rap1. B, RNA prepared from the cells as indicated at the top was subjected to reverse transcription-PCR analysis. The sequence of Rap1-specific primers is described under “Experimental Procedures.” RNA from Jurkat cells was used as a positive control. The results are representative of more than three independent experiments.

RESULTS

A Rap1-binding Protein, RAPL, Is Expressed in Vascular Endothelial Cells—RAPL associates with Rap1 upon T-cell receptor stimulation or chemokine stimulation, resulting in redistribution of integrin in lymphocytes (16). Vascular endothelial cells and hematopoietic cells originate from common hemangioblasts; therefore, we tested whether vascular endothelial cells express RAPL, since Jurkat cells express Rap1 (16). RAPL expression was examined by reverse transcription-PCR analysis using a RAPL-specific primer set. HUVECs expressed RAPL mRNA similarly to Jurkat cells used as a positive control (Fig. 1A). We further examined the association of GTP-Rap1 with RAPL by the co-immunoprecipitation assay using 293T cells (Fig. 1B).

A member of Rassf1A localizes to microtubules in COS cells (22), whereas the localization of RALP has not yet been clearly demonstrated, although it is reported to accumulate at the leading edge of T lymphocytes (16). Thus, we tested the localization of RALP in the vascular endothelial cells by using EGFP-tagged RALP. Rassf members as listed (Fig. 2A) were tagged with EGFP and expressed in HAEcs. All Rassf members contain the Ras- and Rap1-binding domain (RA domain) (Fig. 2A). The expression of EGFP-tagged Rassf1 is confirmed by the immunoblot analysis from the lysates of HEK293T cells transfected with the plasmids as indicated at the top (Fig. 2B). EGFP-tagged Rassf1A and -1C, splicing variants from the same gene, were found as circular fibers in the central region of cells except the nucleus, whereas EGFP-tagged Rassf3 and EGFP-RAPL were found as fibers emanating from the central to the periphery. These results suggested that the circular fibers on which EGFP-tagged Rassf1A and -1C localized may represent microtubules deformed by Rassf1-induced stabilization, as previously demonstrated (22). Rassf3 and RAPL appeared to localize on normal microtubules originating from MTOC to the periphery. Thus, we examined the colocalization of EGFP-RAPL with β-tubulin-constituting microtubules and with γ-tubulin preferably localized on MTOC. As expected, EGFP-RAPL clearly localized on microtubules from the MTOC to the periphery (Fig. 2D). In clear contrast to these fibrous expressions, EGFP-Rassf2 was found exclusively in the nucleus. We compared the EGFP-RAPL with EGFP-Rassf1A expressed in motile vascular endothelial cells using time lapse imaging. Both RALP-expressing cells and Rassf1A-expressing cells showed membrane ruffling (Supplemental Video 1); however, microtubules marked by EGFP-RAPL moved dynamically as regular microtubules, whereas those marked by EGFP-Rassf1 were static (Supplemental Video 2). These data indicated that Rassf members, Rassf3 and RALP, appear to bind to microtubules without affecting the endogenous microtubule structure.

We further confirmed the localization of RALP on microtubules by immunostaining using anti-RAPL antibody. Endogenous RALP localized on microtubules in HAEcs (Fig. 3A). The association of RALP and microtubules were examined by biochemical analysis using purified microtubules and GST-RAPL. The microtubule binding assay revealed that GST-RAPL co-sedimented with microtubules in a concentration-dependent manner. Although GST-RAPL and tubulin closely migrated in SDS-PAGE (Fig. 3B, top panel), GST-RALP was clearly separated by immunoblotting with anti-GST (Fig. 3B, middle panel). In agreement with these observations, when microtubule formation was inhibited by nocodazole, the filamentous expression of RAPL was not observed (Supplemental Fig. 1).

Collectively, these results indicated that endogenous RALP localizes on microtubules in vascular endothelial cells.

RAPL Requires Rap1-associating Domain for Localizing on Microtubules but Not Its Association with Rap1—To define the region responsible for the association of RALP with microtubules, we constructed a series of truncated mutants and a mutant incapable of associating with Rap1 (RA mutant) (Fig. 4A). The expression of EGFP-tagged RALP and its mutants was confirmed by the immunoblot analysis from the lysates of HEK293T cells transfected with the plasmids as indicated at the top of Fig. 4B. We examined the expression of EGFP-tagged RALP and its mutants in HAEcs (Fig. 4C). EGFP-tagged full-length RALP and coiled-coil domain-lacking mutant (dC1) localized on microtubules. Intriguingly, RA mutant also localized on microtubules. However, neither RA domain-lacking mutant (dC2 and dC3) nor a mutant lacking the amino-terminal 100 amino acids (dN) localized on microtubules. The EGFP-tagged RA domain alone was not expressed on the microtubules. These data indicated that the association of RALP with Rap1 is not required for the localization of RALP on microtubules and that the amino-terminal part of RALP and the RA domain are essential for its targeting to microtubules.

Rap1 Locally Activated by S1P Triggers directional Migration Preceding Microtubule Extension—To understand the significance of Rap1 activation and RALP localizing on microtubules, we examined RALP localization in HAEcs expressing either Rap1V12 or rap1GAPII. EGFP-RALP dislocated from microtu-
bules in HAECs expressing Rap1V12, whereas it localizes on microtubules in HAECs expressing rap1GAPII (Fig. 5A). These results suggested that activated Rap1 appears to determine the localization of RAPL. Thus, we investigated the effect of local activation of Rap1 on localization of EGFP-RAPL. Since vascular endothelial cells become motile upon S1P stimulation, S1P is thought to function as a chemoattractant (23). S1P did activate Rap1 as demonstrated by pull-down assay (Fig. 5B). GTP-Rap1 was increased at 1 min after S1P stimulation, and its activation persisted until 15 min after stimulation.

To examine the orientation of microtubule growth upon local S1P stimulation, we applied S1P to HAECs using micropipette. HAECs expressing EGFP-RAPL were monitored for cell movement and microtubule growth by phase-contrast and EGFP observations, respectively. HAECs cultured on the collagen-coated glass dish were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with anti-β-tubulin (top) or anti-γ-tubulin (bottom). Immunoreactive proteins were visualized by Alexa546 goat anti-mouse IgG. Both EGFP and Alexa546 images obtained through a BX50WI confocal microscope controlled by Fluoview were shown as RAPL (green), tubulin (red), and superimposed (merge). Note that EGFP-RAPL localizes on microtubules from the MTOC to the peripheral microtubules. Bar, 20 μm.

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**Fig. 2.** RAPL localizes to the microtubules in vascular endothelial cells. A, Rassf members are schematically illustrated. All Rassfs contain a Ras/Rap1-associating domain (RA). Only Rassf1A contains the diacylglycerol-binding motif (DAG). Rassf3 and RAPL have coiled-coil domain (CC) in the carboxyl terminus. The localization of each gene to human chromosome is indicated on the right. aa, amino acids. B, HEK293T cells were transfected with EGFP-tagged plasmids as indicated at the top. Cell lysates were subjected to SDS-PAGE followed by immunoblotting with anti-GFP. -, untransfected. Molecular weight markers are indicated on the left. C, HAECs were transfected with the plasmids used in B and imaged through an Olympus IX81 fluorescent microscope. Note that EGFP-tagged Rassf1A and Rassf1C localize on the spiral fibers, whereas EGFP-tagged Rassf3 and RAPL localize at fibrous structures emanating from the center of the cell to the periphery. The tubular structure observed in HAECs expressing EGFP-RAPL is enlarged in the right bottom panel. Motile HAECs transfected with either EGFP-Rassf1A or EGFP-Rassf3 were video-imaged for phase contrast and EGFP. A series of phase-contrast images and EGFP images of each cell were converted to two videos, Supplemental Video 1 (phase contrast) and Supplemental Video 2 (EGFP). Elapsed time in video is indicated as h: min. Noticeably, the spiral structure surrounding the nucleus in the cell expressing Rassf1A does not move in the protrusive area at all. In clear contrast, the array from the center to the periphery of the cell expressing EGFP-Rassf3 moves toward the ruffled membrane, although both cells move spontaneously, similar to the untransfected cells. Bar, 20 μm. D, HAECs expressing EGFP-RAPL plated on a collagen-coated glass base dish were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with anti-β-tubulin (top) or anti-γ-tubulin (bottom). Immunoreactive proteins were visualized by Alexa546 goat anti-mouse IgG. Both EGFP and Alexa546 images obtained through a BX50WI confocal microscope controlled by Fluoview are shown as RAPL (green), tubulin (red), and superimposed (merge). Note that EGFP-RAPL localizes on microtubules from the MTOC to the peripheral microtubules. Bar, 20 μm.
microtubule growth for directional migration by examining whether endothelial cells extend membranes in response to S1P in the presence or absence of nocodazole. Before the nocodazole treatment, endothelial cells responded to S1P and extended their membranes, whereas after nocodazole, the cells did not extend their membranes (Supplemental Video 4). These data indicated that microtubules grow toward the chemotactrant, which promotes the directional cell movement.

To monitor the spatio-temporal activation of Rap1 in response to S1P from a micropipette, HAECs expressing Raichu-Rap1 were subjected to time lapse FRET imaging. Raichu-Rap1 consists of YFP, Rap1, the Ras-binding domain of Raf, CFP, and a CAAX box of Ki-Ras. This probe enabled us to show Rap1 activation by the increased ratio of YFP/CFP, based upon FRET from CFP to YFP (Fig. 5D). Raichu-Rap1-expressing HAECs exhibited remarkable membrane ruffles when stimulated with S1P from a micropipette (Fig. 5E, third column, top). At this time point, the increased FRET reflecting Rap1 activation was observed at the ruffled membrane (Fig. 5E, third column, bottom). When S1P was released from the relocated micropipette tip, the same cell responded to S1P and showed membrane ruffles toward the micropipette, similar to the first test. The similar Rap1 activation demonstrated by increased FRET was observed at the ruffled membrane (Fig. 5E, right column). A video image for both phase-contrast view and that for FRET images is shown as Supplemental Video 5. Rap1 activation at the ruffled membrane upon S1P stimulation was confirmed by the observation that Rap1 was not activated at the ruffled membrane before the S1P stimulation (Supplemental Fig. 2A). In addition, FRET observed at the ruffled membrane using Raichu-Rap1 was not detected when Raichu-Rap1N17 was used, although S1P-induced membrane ruffling was observed (Supplemental Fig. 2B and Video 6). By stimulating cells with S1P-free medium, we also excluded the possibility that fluid pressure or the proximity of the pipette tip to the cell might cause FRET (Supplemental Video 7). These data indicated that chemotactrant-induced local activation of Rap1 may become a trigger of directional migration accompanied by extension of EGFP-RAPL-marked microtubules.

Rap1 Activated during Wound Healing Is Accompanied by Microtubule Extension—To assess the consequence of the Rap1 activation and the association of activated Rap1 with RAPL, we examined the activation of RAPL and EGFP-RAPL-marked microtubules during wound healing. Microtubules grow in the protruding region of motile polarizing fibroblasts (28). It has been unclear what determines the polarized growth of microtubules. During wound healing, monolayer vascular endothelial cells migrated to the wound unidirectionally (Fig. 6A, top panels, phase-contrast observations). Crk is an adaptor protein linking signaling from integrins as well as receptor tyrosine kinases to its Src homology 3 domain-binding proteins via Src homology 2 domain. It localizes at focal adhesions by constituting complexes with Src homology 2 domain-binding partners, paxillin and p130Cas (29, 30). To monitor the focal adhesion assembly and growth of microtubules simultaneously, endothelial cells were transfected with the plasmids expressing DsRed-CrkI and EGFP-RAPL. Before scratching, DsRed-CrkI was punctually expressed in the focal adhesions at the cell periphery and the cell body (Fig. 6A, bottom, left panel). When cells started to move toward the wound, the focal complexes and focal adhesions marked by DsRed-CrkI developed profoundly in the leading edge of the cells (24, 31); meanwhile, those in the retracting region were disassembled (Fig. 6A, bottom, right panel). During cell migration upon scratching, microtubules marked by EGFP-RAPL grew in the protrusive region and developed toward the leading edge marked by DsRed-CrkI (Fig. 6A). A series of images for microtubules marked by EGFP-RAPL and those for focal adhesions marked by DsRed-CrkI were converted to a video file (Supplemental Video 8). We further confirmed that endogenous RAPL localized on microtubules in the protruding area of migrat-
Rap1 is activated downstream of Crk via its Src homology 3 domain-binding protein, C3G, which is a GEF for Rap1 (32). Thus, we examined whether Rap1 was activated during cell migration using Raichu-Rap1. HAEcs cultured as a monolayer sheet were transfected with plasmids expressing Raichu-Rap1 and time lapse-imaged under the phase-contrast and FRET view after scratching. Cells were moving into the wound as revealed by phase-contrast observations (Fig. 7, left panels). Cell expressing Raichu-Rap1 as well as untransfected cells moved toward the wound. Rap1 was activated at the membrane ruffles in the leading edge (Fig. 7, right panels), where Crk was localized at focal complexes or growing focal adhesions (Fig. 6). These results suggested that Crk-Rap1 signaling upon focal adhesion assembly may contribute to the directional migration.

**Rap1-RAPL Signaling Is Required for Directional Movement during Wound Healing**—To test whether Rap1 is required for wound healing, we examined the effect of inactivation of Rap1 by overexpression of rap1GAPII on the directional cell migration. Rap family consists of Rap1A, Rap1B, Rap2A, and Rap2B (1). These molecules share common GEFs and GAPs for their activation and inactivation, respectively. To examine the effect of the Rap family on wound healing, inactivation of Rap by rap1GAPII, a common GAP for all of the Rap family members (33), is preferable rather than knocking down these Rap molecules using the small interfering RNA technique. In addition, rap1GAPII is suitable for the inactivation of Rap1, because it is reported that Rap1N17 does not work as a dominant negative form of Rap1 (34). HUVECs cultured as a monolayer were infected with adenovirus expressing either EGFP or rap1GAPII for 24 h. Infection efficiency exceeded 90%, as confirmed by fluorescence microscopy (Fig. 8C). There was no difference in cell confluence between HUVEcs expressing rap1GAPII and those expressing EGFP. The EGFP-expressing cells separated by the wound moved toward the center line of the wound, whereas rap1GAPII-expressing HUVECs did not. The wound was almost closed by mobilized EGFP-expressing HUVECs 24 h after scratching, whereas it was not closed by rap1GAPII-expressing HUVECs (Fig. 8A).

To test the requirement of Rap1-RAPL signaling for directional movement, we used the RA mutant of RAPL to interfere with the association of RAPL with Rap1. HUVECs expressing EGFP-RA mutant were compared with those expressing EGFP-RAPL during wound healing. HUVECs infected with EGFP-RAPL-expressing adenovirus closed the wound 24 h after scratching, whereas those infected with EGFP-RA mutant-expressing adenovirus did not (Fig. 8B). To exclude the possibility that RA mutant-expressing cells moved more slowly than EGFP-expressing cells, we monitored randomly migrating cells expressing either RA mutant or EGFP. There was no significant change of the migratory velocity between two groups (Supplemental Fig. 4).

**Fig. 4.** The sequence besides the essential amino acids in RA domain required for the association of RAPL with Rap1 is necessary for the localization of RAPL to microtubules. A, schematic illustration of RAPL and its mutants. RAPL consists of an uncharacterized amino terminus, followed by a Ras/Rap1-associating domain (RA domain) and coiled-coil domain. The amino acid (aa) number encoding each domain is indicated at the top. RA mutant, the mutant incapable of associating with Ras and Rap1. The stars indicate the seven amino acids required for the association of RAPL with Rap1 that are replaced with Ala. dC1 and dC2 lacks the coiled-coil domain and both the RA domain and the coiled-coil domain, respectively. dC3 consists of the amino-terminal 100 amino acids, dN lacks the amino-terminal 100 amino acids, which have not been characterized. The coiled-coil and the RA consist of the only coiled-coil domain and the RA domain, respectively. The localization of RAPL and its mutants on microtubules is summarized on the right. B, HEK293T cells were transfected with the plasmids encoding amino-terminally EGFP-tagged DNA, as indicated in the top. Cell lysates were subjected to SDS-PAGE, followed by immunoblot probed with anti-GFP antibody. Molecular weight markers are indicated at the left. C, HAEcs transfected with the plasmids used in B were imaged using an Olympus IX-81 fluorescent microscope. Note that RA mutant and dC1 localize on microtubules as well as full-length RAPL. Bar, 40 μm.
FIG. 5. Rap1 regulates the localization of RAPL and precedes the extension of microtubules toward the leading edge of migrating cells upon chemotactic S1P stimulation. A, HAECs were co-transfected with pCA-EGFP-RAPL and either pIRM21-Rap1V12 (top) or pIRM21-rap1GAPII (bottom). Note that EGFP-RAPL is dissociated from microtubules in Rap1V12-expressing cells, whereas EGFP-RAPL is associated with microtubules in rap1GAPII-expressing cells. B, HAECs stimulated with 1 μM S1P during the time period indicated at the top were lysed and analyzed by Bos’s pull-down method using the GST-Rap1 binding domain of RapGDS. C, HAECs expressing EGFP-RAPL were time lapse-imaged before (~19 min) and after (17 min) the point (0) when 1 μM S1P was applied from a micropipette. Note that at time point 0, the cell
FIG. 6. Microtubules marked by EGFP-RAPL grow toward the leading edge during wound healing. A, monolayer-cultured HAECs expressing both EGFP-RAPL and DsRed-CrkI were time lapse-imaged after scratching. Phase-contrast (top), EGFP (middle), and DsRed (bottom) images were obtained through Olympus IX81 fluorescent microscope at the time point after scratching as indicated at the top. The arrows in the bottom panel indicate the nascent focal complexes at the leading edge, whereas the arrowheads indicate the focal adhesions in the retracting region. Note that microtubules marked by EGFP-RAPL grow toward the leading edge marked by DsRed-CrkI. Wound, scratched area. Bar, 50 μm. A series of time lapse images of phase-contrast, EGFP, and DsRed view were converted to a video (Supplemental Video 7). B, monolayer-cultured HAECs were immunostained with anti-RAPL 4 h after scratching. Phase contrast (left) and immunostaining with anti-RAPL (right) followed by visualization with Alexa488-conjugated secondary antibody are shown. 

DISCUSSION

The directional migration is accompanied with microtubule growth toward the leading edge of migrating cells. The microtubule extension depends on the localization of microtubule-capturing or -attracting molecules. Microtubules cooperatively promote cell migration accompanied with cell polarization together with actin cytoskeleton (35, 36). Extracellular stimuli activating Rho family proteins, Rho, Rac, and Cdc42, via plasma membrane receptors and cell-ECM complexes determine the direction of microtubule growth (28). Therefore, the downstream effectors of Rho family proteins are proposed to function as microtubule-capturing molecules at the cell cortex. Such candidate systems include Cdc42-Par6-protein kinase Cζ, dynein and Rac/Cdc42-IQGAP-CLIP170 (35, 38). Here we demonstrate, for the first time, that Rap1-RAPL signaling contributes to determining the direction of cell migration accompanied with microtubule growth upon chemoattractant stimulation and during wound healing.

Given that RAPL was expressed in vascular endothelial cells and associated with GTP-Rap1, it is important to ask where and how RAPL is regulated by active Rap1 in living cells. To answer this question, we first examined the localization of RAPL and found that RAPL localized on microtubules from MTOC to the periphery. Previously, it has been reported that Rassf1A localizes on microtubules in a variety of cells (22) and participates in mitosis by inhibiting the binding of anaphase-promoting complex to Cdc20 (39) or by stabilizing microtubules for tumor suppression (22). Rassf members were originally isolated as tumor suppressors. Thus, Rassfs have been mainly focused on as regulators of tumor suppression. We noticed that microtubules found in HAECs transfected with pEGFP-Rassf1A and -1C were different from those found in HAECs transfected with pCA-EGFP-RAPL. Rassf1 appeared to deform and thicken microtubules, whereas RAPL seemed to localize on endogenous microtubules. Recently, Rassf1A and -1C are re-moving toward the left top corner exhibited microtubules growing in the same direction as the cell movement. An enlarged image is shown in the white box (bottom left). Upon start of the S1P application, the cell turn to the micropipette tip (black arrows), began to move and exhibited a protrusion toward the tip, in which the microtubules extended forward along the direction of movement. An enlarged image is shown in the white box (bottom right). The white arrows indicate the moving direction. A series of phase-contrast images and EGFP images were converted into a video (Supplemental Video 3). Bar, 40 μm. D, schematic illustration of Raichu-Rap1. FRET efficiency depends on the guanine nucleotide binding. GDP-bound Raichu-Rap1 emits 475-nm fluorescence when excited at 433 nm, whereas GTP-bound Raichu-Rap1 emits 527-nm fluorescence due to FRET. Raf, Ras/Rap1 binding domain of Raf. E, HAECs expressing Raichu-Rap1 were imaged during exposure to S1P from the micropipette tip. Phase-contrast images and FRET images were obtained before and after the S1P stimulation. The time points indicated at the top show the first location and stimulation with S1P (black), the relocation of the tip, and the stimulation with S1P (red). Note that increased FRET reflecting Rap1 activation was observed at the edge of protrusion toward the micropipette tip. Red and blue hue indicate the increased and decreased FRET, respectively. The arrowheads indicate the activated Rap1 shown by the increased FRET. A series of phase-contrast images and FRET images were converted to a video (Supplemental Video 4). HAECs expressing Raichu-Rap1N17 was FRET-imaged during exposure to S1P from the micropipette tip similarly to Raichu-Rap1. Note that FRET does not occur at the ruffled membrane induced by S1P (Supplemental Video 5). Untransfected HAECs do not exhibit membrane ruffles in response to S1P-free medium (Supplemental Video 6).
ported to suppress tumors by stabilizing microtubules and maintaining genomic stability (40). The circular fibers found in the EGFP-Rassf1-expressing cells appear to reflect the stabilized microtubules. Furthermore, although Rassf1A-transfected cells exhibited the membrane ruffling, microtubules marked by EGFP-Rassf1A did not grow or shrink at all (Fig. 2 and Supplemental Videos 1 and 2, left). In clear contrast, EGFP-tagged RAPL and endogenous RAPL localizes on microtubules (Figs. 2D and 3 and Supplemental Videos 1 and 3, right). RAPL dislocated from microtubules when cells were transfected with Rap1V12-expressing plasmids (Fig. 5A). In addition, inactivation of Rap1 and disconnection between Rap1 and RAPL perturbed the directional migration (Fig. 8). These results imply that Rap1-RAPL signal may participate in regul-
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Rap1-associating Molecule, Localizes Migration Accompanied by Extension of Microtubules on Which RAPL, a Rap1-associating Molecule, Localizes

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