Rap1 GTPase-activating Protein SPA-1 Negatively Regulates Cell Adhesion

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Rap1 GTPase is activated by a variety of stimulations in many types of cells, but its exact functions remain unknown. In this study we have shown that SPA-1 interferes with Rap1 activation by membrane-targeted C3G, C3G-F, in 293T cells through the GTPase activating protein (GAP) activity. SPA-1 transiently expressed in HeLa cells was mostly localized at the cortical cytoskeleton and induced rounding up of the cells, whereas C3G-F conversely induced extensive cell spreading. Conditional SPA-1 overexpression in HeLa cells by tetracycline-regulative system suppressed Rap1 activation upon plating on dishes coated with fibronectin and resulted in the reduced adhesion. When SPA-1 was conditionally induced after the established cell adhesion, the cells gradually rounded up and detached from the dish. Both effects were counteracted by exogenous fibronectin in a dose-dependent manner. Retroviral overexpression of SPA-1 in promyelocytic 32D cells also inhibited both activation of Rap1 and induction of cell adhesion by granulocyte colony stimulating factor without affecting differentiation. These results have indicated that Rap1 GTP is required for the cell adhesion induced by both extracellular matrix and soluble factors, which is negatively regulated by SPA-1.

Rap1 is the closest member of Ras family small GTPases (1). Mammalian Rap1 was isolated by cross-hybridization with a Drosophila ras-related gene (2) and was reported to revert "malignant" morphology of oncogenic Ras-transformed 3T3 cells to "normal" phenotype (3). Activation of Rap1 can be regulated by specific guanine nucleotide exchange factors (GEF) catalyzing the conversion from GDP- to GTP-bound forms and GTPase activating proteins (GAPs) accelerating the hydrolysis of bound GTP to GDP (4). C3G, originally isolated as a binding protein to v-crk oncogene product (5), has been shown to exhibit Rap1 GAP activity (6). Most recently, other Rap1 GEFs have been isolated including CalDAG GEFI and Epac, which are regulated by Ca2+ and diacylglycerol and cAMP, respectively (7–9). On the other hand, at least two distinct proteins are shown at present to exhibit specific Rap1 GAP activity in vitro, rapGAP (10) and SPA-1 (11). Expression profiles of rapGAP and Spec-I genes are quite distinct, in that the former is selectively expressed in brain, pancreas, and kidney and the latter predominantly in lymphohematopoietic tissues (11). Recently, an arginine finger motif conserved in the shared catalytic domains of rapGAP and SPA-1 has been proposed to be essential for their GAP activity (12).

Based on the findings that Rap1 shares the effector domain with Ras and binds to several Ras effector molecules (13), it has been proposed that Rap1 GTP antagonizes the Ras signaling pathways (13, 14). This proposed function of Rap1 in normal cells, however, remains controversial (15). Rap1 is activated by the agonistic stimulation of various receptors coupled with tyrosine kinases or G proteins, including thrombin receptor in platelets (16), insulin receptor (17), antigen receptors in lymphocytes (18, 19), GM-CSF receptor and other serpentine receptors in neutrophils (20), and nerve cell growth factor receptor in PC12 cells (21). In some of these, it has been shown that Rap1 is activated by C3G recruited by the CRK adapter protein (18, 21). Rap1 has been shown also to be activated by cAMP (22, 23) and phospholipase C-γ pathway (19), as well as during the cell adhesion in NIH 3T3 cells (24). Together with the presence of multiple Rap1-regulatory proteins with unique signaling motifs and distinct tissue distribution patterns, these results suggest that Rap1 is activated in multiple signal transduction pathways depending on the cell types. The exact functions of Rap1 in the cells, however, still remain largely unknown.

In the present study, we first indicate that SPA-1 and C3G regulate Rap1 activation antagonistically to each other in the cells, in which the former interferes with the activation of endogenous Rap1 by the latter. Conditional overexpression of SPA-1 in HeLa cells resulted in the inhibition of Rap1 activation and reduced adhesion upon contact to the substrate and the detachment of the cells when induced after the establishment of cell adhesion, both of which could be overcome dose-dependently by exogenous fibronectin. Also, retroviral overexpression of SPA-1 in the nonadherent promyelocytic 32D cells inhibited the activation of Rap1 and the induction of cell adhesion by G-CSF stimulation. Thus, the present results have indicated that Rap1 activation is critically involved in the cell adhesion induced by both ECM and soluble factors and that SPA-1 functions as a negative regulator for the activation of Rap1, thereby setting a threshold for cell adhesion.

EXPERIMENTAL PROCEDURES

Antibodies and Other Materials—Rabbit anti-SPA-1 antibody has been described previously (11). Antibodies for VLA-4, LFA-1, and CD44 were provided by Dr. T. Kina, Institute for the Bioregeneration, Kyoto University, Kyoto, Japan. Other antibodies were purchased commercially: anti-Rap1 (Transduction Laboratories), anti-C3G (Santa Cruz Biotechnology), anti-FLAG (Sigma), and anti-CD29 and anti-CD11b (PharMingen). Recombinant human G-CSF was provided by Chugai Pharmaceutical Inc., Tokyo, Japan. Fibronectin was purchased from Life Technologies, Inc.

Cell Cultures—HeLa/Tet-Off (CLONTECH Laboratories, Inc.) and
293T cells provided by Dr. M. Matsuda, Research Institute, International Medical Center of Japan, Tokyo, Japan, were maintained in the Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS). 32Del.3 cells were obtained from the ATCC (CRL 11346) and maintained in RPMI 1640 supplemented with 10% FCS and IL-3 (100 units/ml). An ecotropic cell line, GP+E86, was provided by Dr. T. Sudo, Toray Inc., Kamakura, Japan.

For the cell detachment assay, HeLa/Tet-off cells were seeded at 10^5 cells/ml in 60-mm tissue culture dishes and cultured in the complete RPMI 1640 and G-CSF (2 ng/ml) for the indicated number of days. The cultured cells were rinsed 5 times with phosphate-buffered saline, including Ca^{2+} and Mg^{2+}, and the adherent or nonadherent cells were counted.

**RESULTS**

**SPA-1 Interferes with the Activation of Rap1 by C3G-F in 293T Cells**—We have investigated first whether SPA-1 antagonizes the Rap1 GEF activity of C3G in the cells. Transfection of C3G cDNA failed to activate Rap1 most likely because of its inefficient accessibility to Rap1 (6), and therefore C3G cDNA tagged with a membrane-anchoring CAAX motif at the C terminus (C3G-F) was employed, which induced efficient Rap1 activation in 293T cells (Fig. 1, A and B). As shown in Fig. 1A, cotransfection of varying amounts of Spa-1 (0.06–1 μg) with C3G-F cDNA (1 μg) suppressed the generation of Rap1 GTP in a dose-dependent manner. Conversely, in the presence of a constant level of Spa-1 (1 μg), the efficiency of GEF activity of C3G-F for the endogenous Rap1 was reduced significantly (Fig. 1B). A ΔGRD mutant of Spa-1, which was defective for Rap1 GAP activity in vitro (data not shown), failed to affect the Rap1 GEF activity of C3G-F (Fig. 1A). The results have indicated that SPA-1 interferes with the activation of Rap1 in the cells through the GAP activity.

Overexpression of SPA-1 and C3G-F Differentially Affects the Cell Shape and Size and, SPA-1 Is Probably Associated with Cortical Cytoskeleton—We then intended to examine the cellular effects of SPA-1 overexpression in HeLa cells, which mar-
Overexpression of SPA-1 and C3G-F induces changes in cell shape and size, and SPA-1 is probably associated with cytoskeleton.  

A HeLa cells were transfected with 2 μg of Spa-1 (a, b), C3G (c), or C3G-F (d) with Lipofectin. Two days after the transfection, cytochalasin D (1 μg/ml) was added to a portion of cultures transfected with Spa-1 cDNA (b) and incubated for 30 min. Then the cells were fixed, double stained with anti-SPA-1 (a, b) or anti-C3G (c, d) followed by the fluorescein isothiocyanate-conjugated anti-rabbit IgG and rhodamine-conjugated phalloidin, and analyzed with confocal laser microscopy. Note the difference in magnifications (a–c versus d). B HeLa cells transfected with Spa-1 cDNA, either pretreated with cytochalasin D or not, were lysed with hypotonic buffer and precipitated with centrifugation at 500 × g (PPT). The supernatants were centrifuged again at 100,000 × g to obtain soluble (S100) and precipitated (P100) fractions. SDS sample duffer was added to each fraction and analyzed by immunoblotting with antibodies for SPA-1, Rap1, C3G, and IRP-1.  

Fig. 2. Overexpression of SPA-1 and C3G-F induces changes in cell shape and size, and SPA-1 is probably associated with cytoskeleton. A HeLa cells were transfected with 2 μg of Spa-1 (a, b), C3G (c), or C3G-F (d) with Lipofectin. Two days after the transfection, cytochalasin D (1 μg/ml) was added to a portion of cultures transfected with Spa-1 cDNA (b) and incubated for 30 min. Then the cells were fixed, double stained with anti-SPA-1 (a, b) or anti-C3G (c, d) followed by the fluorescein isothiocyanate-conjugated anti-rabbit IgG and rhodamine-conjugated phalloidin, and analyzed with confocal laser microscopy. Note the difference in magnifications (a–c versus d). B HeLa cells transfected with Spa-1 cDNA, either pretreated with cytochalasin D or not, were lysed with hypotonic buffer and precipitated with centrifugation at 500 × g (PPT). The supernatants were centrifuged again at 100,000 × g to obtain soluble (S100) and precipitated (P100) fractions. SDS sample duffer was added to each fraction and analyzed by immunoblotting with antibodies for SPA-1, Rap1, C3G, and IRP-1.  

originally expressed SPA-1. HeLa cells were transfected with Spa-1, C3G, or C3G-F cDNA, stained with corresponding antibodies, and analyzed by confocal microscopy. As shown in Fig. 2A, a, SPA-1 was detected mostly at the cortical area of the cells. It was noted that Spa-1-transfected HeLa cells tended to become round and smaller as compared with uninfected neighboring cells. To see the possible relationship of SPA-1 with cytoskeleton, the effect of cytochalasin D (1 μg/ml) was examined. At the condition in which cytoskeletal actin organization was disrupted largely, a significant portion of SPA-1 staining was disrupted also into patchy aggregates, mostly colocalizing with F-actin (Fig. 2A, b, merged as yellow staining). C3G was distributed diffusely throughout the cytosol as expected, hardly affecting the cell shape and size (Fig. 2A, c). In contrast, HeLa cells transfected with C3G-F cDNA exhibited an enlarged and spread shape with extensive cytoplasmic protrusions (Fig. 2A, d, note the difference in magnification). A portion of C3G-F was distributed in the mottled pattern at the basal cell surface attached to the dish as revealed by the confocal picture focused on the basal surface (Fig. 2A, d). To confirm the intracellular localization of SPA-1, cell fractionation analysis was done. As shown in Fig. 2B, the majority of SPA-1 was associated with the fraction of hypotonic cell lysate precipitated with the light centrifugation (PPT fraction), which contained cytoskeleton. The majority of Rap1 was also detected in this fraction with a minor portion in the membranous fraction (P100). Conforming to the immunostaining analysis, the significant proportion of SPA-1 in the PPT fraction was released into the S100 and P100 fractions following the cytochalasin D treatment. Although not shown, a similar shift of actin localization was confirmed by immunoblotting using anti-actin antibody. C3G-F was also detected in the PPT and partly in P100, whereas the vast majority of C3G was found in the S100. Iron regulatory protein-1 (IRP-1), used as a control, was exclusively detected in the S100 as expected.

Conditional Overexpression of SPA-1 in HeLa Cells after Establishment of Adhesion Causes Detachment of Cells, whereas That before Adhesion Results in Reduced Adhesion to Fibronectin The effect of SPA-1 overexpression was further investigated by stable transfecants. To avoid possible clonal variance, HeLa cells transfected with Spa-1 cDNA whose expression is regulated by tetracycline (SPA/HeLa) have been established. SPA/HeLa cells cultured in the presence of 1 ng/ml Dox expressed an undetectable level of SPA-1 and exhibited indistinguishable features from parental cells. When SPA HeLa cells were shifted to the medium containing decreasing concentrations of Dox, SPA-1 expression was induced increasingly within a day (Fig. 3A). As shown in Fig. 3B, when the wild-type HeLa cells in suspension were plated onto the tissue culture dishes and allowed to adhere, Rap1GTP was induced above the basal level. In contrast, SPA/HeLa cells that had been induced for SPA-1 in Dox-free medium exhibited negligible generation of Rap1GTP upon plating on the dishes (Fig. 3B). When non-induced SPA-1/HeLa cells that had adhered to dishes were shifted to the medium without Dox, they rounded up progressively and finally detached from the dish within 3 days, whereas all the cells remained adherent in the presence of Dox (Fig. 3, C and D). Round detached cells were all viable (data not shown). Identical results were obtained by using independently isolated SPA/HeLa clones. The results have suggested that Rap1 GTP is required to maintain cell adhesion.  

We then examined the effect of SPA-1 overexpression on the initiation of adherence. SPA/HeLa cells that had been induced in Dox-free medium were made into suspension by trypsinization. Upon plating on the fibronectin-coated dishes, significant generation of Rap1 GTP was detected in the SPA/HeLa cells that had been induced for SPA-1, but the level was much lower than in parental cells that exhibited close to maximal Rap1 GTP even on the plain tissue culture dishes (Fig. 4A). In the short-term adhesion assay at 30 min, the induced SPA/HeLa cells started to adhere to the fibronectin-coated dishes in a dose-dependent fashion, but again it was much less efficient as compared with non-induced SPA/HeLa cells (Fig. 4B). Similarly, detachment of the adhered SPA/HeLa cells following the induction of SPA-1 was prevented by fibronectin in a dose-dependent fashion (Fig. 4C). These results have indicated that SPA-1 interferes with both initiation and maintenance of cell adhesion, which can be counteracted by exogenous fibronectin.

Overexpression of SPA-1 Inhibits the G-CSF-induced Adhesion of Promyelocytic 32D Cells Promyelocytic 32D cells that express significant SPA-1 are nonadherent but are induced to adhere following the G-CSF stimulation. To see the effect of SPA-1 overexpression, 32D cells were infected with either Spa-1 or GRD-Spa-1 recombinant retrovirus. As shown in Fig. 5A, 32D cells infected with the Spa-1 retrovirus (32D/SPA-1) expressed by far more SPA-1 than wild type cells. Upon shift to the medium containing G-CSF, Rap1GTP was generated in the

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The text continues with further details on the experiments and results, including the analysis of cell shape changes, cytoskeletal organization, and the effects of SPA-1 overexpression on cell adhesion and Rap1GTP generation. The discussion points to SPA-1 as a negative regulator of cell adhesion, influencing both the initiation and maintenance of cell adhesion, and its interaction with fibronectin and other cellular components to modulate cell behavior. The text provides a comprehensive overview of the cellular and molecular mechanisms involved in these processes, supported by experimental data and analysis.
wild type cells within a day and increased steadily thereafter (Fig. 5B). As shown in Fig. 5, C and D, the cells concomitantly adhered to the dishes following the shift to G-CSF. The accumulation of Rap1 GTP apparently paralleled with the increased proportion of adherent cells. In contrast, negligible activation of Rap1 was induced in the 32D/SPA-1 following G-CSF stimulation (Fig. 5B), whereas those infected with the ΔGRD-Spa-1 retrovirus (32D/ΔGRD) exhibited comparable Rap-1 activation to wild type cells (data not shown). As also shown in Fig. 5, C and D, 32D/SPA-1 cells remained totally
nonadherent in the presence of G-CSF, whereas those infected with 32D cells infected with pLXSN retrovirus (32D/cont) and 32D/ΔGRD-Spa-1 cells became adherent similarly to wild type cells. Because G-CSF also induces the differentiation of 32D cells into granulocytes, we finally examined the effect of SPA-1 overexpression on the process. As shown in Fig. 6A, 32D/SPA-1 cells differentiated into granulocytes at a degree comparable with that of wild type cells in the presence of G-CSF. The proportions of granulocytes in the adherent and nonadherent fractions of 32D cells were also comparable, suggesting that cell adhesion and morphological differentiation are independent events. Following G-CSF stimulation, the expression of several adhesion molecules such as VLA4, CD18, LFA-1, and CD44 was augmented indistinguishably in wild type and 32D/SPA-1 cells (Fig. 6C), indicating that SPA-1 overexpression barely affected their expression levels per se.

**DISCUSSION**

In the present study, we have indicated that SPA-1 interferes with the activation of Rap1 by C3G bearing a CAAX motif (C3G-F) in 293T cells. In the presence of excess SPA-1, efficiency of Rap1 activation by the transfected C3G-F was markedly reduced, strongly suggesting that SPA-1 can control the threshold against the activation of Rap1 in the cells. HeLa cells transiently transfected with Spa-1 cDNA tended to show smaller and round cell shape, whereas those transfected with C3G-F cDNA conversely exhibited more enlarged and flattened shape with extensive cytoplasmic protrusions. Recently, overexpression of v-Crk that can bind and recruit C3G has been reported to induce cell spreading and focal adhesion formation in PC12 cells (27). The effect was confirmed in HeLa cells that could conditionally overexpress SPA-1 by the tetracycline-regulative system (SPA/HeLa). SPA/HeLa cells were indistinguishable from parental cells, whereas, upon induction of SPA-1 by removing Dox from the culture, the tightly adherent cells gradually became round and detached from the dish. The results have strongly suggested that sustained activation of Rap1 is needed for cells to maintain the adherent state. When non-induced SPA/HeLa cells in suspension were plated on dishes and allowed to adhere, Rap1GTP was significantly increased. In contrast, no detectable activation of Rap1 was observed in SPA/HeLa cells that had been induced with SPA-1. When plated onto dishes coated with fibronectin, such induced SPA/HeLa cells showed significant generation of Rap1GTP, yet much less than in parental cells. Concomitantly, they showed reduced adhesion to exogenous fibronectin. Thus, it has been indicated that SPA-1 negatively regulates both initiation and maintenance of cell adhesion.

The effect of SPA-1 was further investigated in the nonadherent 32D cells, in which cell adhesion is induced by specific soluble factor. Following the stimulation with G-CSF, Rap1 GTP was generated within a day and increased thereafter. In an attempt to prevent the accumulation of Rap1 GTP following the G-CSF stimulation, we transduced Spa-1 cDNA into 32D cells using a recombinant retrovirus (32D/SPA-1). As expected, the generation of Rap1GTP following G-CSF stimulation was nearly completely suppressed in the 32D/SPA-1. Concomitantly, 32D/SPA-1 cells remained totally nonadherent in the presence of G-CSF. The effect was not observed in 32D cells transduced with ΔGRD-Spa-1 retrovirus, indicating that Rap1 GAP activity was required for the effect. We have reported previously that the endogenous SPA-1 is markedly down-regulated in human HL60 promyeloid cells preceding the induction of cell adhesion and extensive spreading by 12-O-tetradecanoylphorbol-13-acetate (11). On the other hand, morphological differentiation into polymorphonuclear granulocytes by G-CSF occurred in 32D/SPA-1 comparably to the wild type cells, indicating that the G-CSF
receptor per se functioned normally in 32D/SPA-1 cells. Expression levels of both integrins β1 and β2 following G-CSF stimulation were unaffected either by SPA-1 overexpression.

These results have indicated collectively that Rap1 is activated either by the adherence to substratum in intrinsically adherent HeLa cells or by the specific soluble factor G-CSF in nonadherent hematopoietic 32D cells, and in both types of cells Rap1GTP is required for the cell adherence and possibly for cell spreading. It appears that sustained presence of Rap1 GTP is required to maintain the cell adhesion. SPA-1 not only inhibits the activation of Rap1 upon contact with substrates but also reduces the accumulated Rap1 GTP pool in the adherent state, thereby interfering with both initiation and maintenance of cell adhesion. Cell adhesion to the ECM via integrins results in the activation of tyrosine kinases such as Src and FAK, which phosphorylate focal adhesion molecules including FAK itself, paxillin, and Cas (28). Because it has been shown that Crk adapter protein is recruited to the focal adhesion complex via SH2 domain (28), it seems possible that Rap1 can be activated there. In addition to C3G that can bind to Crk, distinct Rap1GEF's directly activated by the common second messengers have been recently identified (7–9), and it remains to be seen which Rap1GEF is primarily responsible for Rap1 activation by ECM in HeLa or by G-CSF in 32D cells. Rho GTPases involved in the cytoskeletal reorganization are also activated by both extracellular soluble factors and insoluble ECM's in fibroblasts (28). In human T cells, however, inactivation of Rho GTPase by Clostridium botulinum C3 exoenzyme was reported to show no effect on the cell adhesion to ECM per se, whereas it inhibited the costimulatory activity for T cell receptor-mediated activation (30).

How Rap1GTP is involved in the cell adhesion and spreading remains to be investigated. Recently, molecules that specifically bind to Rap1GTP have been identified, including a group of Rap1GEF's such as RapGDS (31) and Rgr (32, 33) that can activate Ral GTPase. Furthermore, one of the Ral GTP-binding proteins (RalBP1) has been shown to be a GAP for Rho family GTPases (34). These results may imply that ECM- or receptor-coupled Rap1 signaling pathway converges to other small GTPases including Rho family GTPases at the cytoskeletal compartment. In this aspect, it is particularly noted that a substantial portion of SPA-1 is located at the cytoskeletal compartment along with Rap1. It has been reported also that Rap1 is translocated rapidly to the cytoskeleton upon activation in platelets (35, 36). SPA-1 has a PDZ domain followed by leucine zipper motif (37), which potentially mediates the interaction with cytoskeletal elements (38). Our unpublished results, on the other hand, have indicated that overexpression of rapGAP, which lacks above domains and is located mostly in the cytosolic soluble fraction, hardly affects the cell adhesion in the same HeLa/Tet-off system.2 These results altogether suggest that the cytoskeletal association of SPA-1 is crucially important for the regulation of cell adhesion by setting a threshold for Rap1 activation via various external signals.

We have indicated previously that the Spa-1 gene is expressed most abundantly in normal lymphohematopoietic cells (11), in particular mature peripheral lymphocytes.3 One of the most characteristic features of them is to recirculate in the blood and lymphatics. Under the particular situations such as antigenic stimulation and production of chemotactic factors in inflammation, however, they are trapped rapidly at the secondarilymphoid tissues or inflammatory sites, where they are activated and/or proliferate. In such mobile cells, polarized regulation of cell adhesion within a cell is shown to play critical roles for cellular interactions and cell movement such as che-

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2 M. Hattori and N. Minato, unpublished observation.
3 N. Minato, unpublished observation.
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