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Rap1 GTPase: Functions, Regulation, and Malignancy

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Rap1 is a member of the Ras family of small GTPases that is activated by diverse extracellular stimuli in many cell types. It is activated by distinct types of Rap1 guanine nucleotide exchange factors coupled with various receptors or second messengers, while activated Rap1 is down-regulated by Rap1 GTPase-activating proteins, through which Rap1 activation is controlled spatio-temporally. Functionally, Rap1 either interferes with Ras-mediated ERK activation or activates ERK independently of Ras in a cell-context dependent manner. Accumulating evidence also indicates that Rap1 is a major activator of integrins, playing important roles in the regulation of a variety of integrin-dependent cellular functions. Most recently, significant evidence has emerged that dysregulation of Rap1 activation is responsible for the development of malignancy. Recent extensive research has begun to unveil the roles of this controversial small G protein in physiology and diseases.

Key words: ERK, integrin, myeloid leukemia, Rap1, T cell anergy.

Abbreviations: GEF; guanine nucleotide exchange factor, GAP; GTPase-activating protein, GRD; GAP-related domain, PKA; protein kinase A, A-cyclase; adenyl cyclase, DAG; diacylglycerol, CD-; Ca $^{2+}$, DAG-activated, PLC; phospholipase C, S-SCAM; synaptic scaffold molecule, LZ; leucine-zipper motif, HPV; human papillomavirus, ERK; extracellular signal-regulated kinases, LFA-1; leukocyte function-associated antigen-1, CDM; *C.elegans* ced-5, vertebrate DOCK180 and *Drosophila* Myoblast City, CML; chronic myelogenous leukemia, MDS; myelodysplastic syndrome, APC; antigen-presenting cells, HSC; hematopoietic stem cells, ECM; extracellular matrix.

Rap1 GTPase was discovered by Kitayama and colleagues in 1989 as a gene (*K-rev*) product that restored a malignant phenotype of *K-Ras*-transformed fibroblasts (*1*). Rap1 is a member of the Ras family of small GTPases bearing the highest homology to Ras (*2*). Although its biological functions remained controversial for a decade, recent extensive studies have begun to elucidate the roles of Rap1 in physiology and diseases.

Regulation of Rap1 signaling

Rap1 binds to either GTP or GDP, and the transition between the two states represents a molecular switch (*3*). The GTP- and GDP-bound Rap1 differ in the conformation of two regions, switch 1 and 2, allowing downstream effector molecules to discriminate between two states, GTP-form "on" and GDP-form "off" signals. The GDP-GTP cycle is regulated by guanine nucleotide exchange factors (GEF) and GTPase-activating proteins (GAP). GEFs facilitate release of the bound nucleotides, allowing Rap1 to rebind more abundant GTP in the cells, whereas GAPs enhance the intrinsic GTPase activity of Rap1 to hydrolyze the bound GTP to GDP.

Rap1 GEFs

Several types of GEFs sharing a catalytic GEF domain are coupled with distinct receptors or secondary messengers (Fig. 1). C3G, the first Rap1 GEF to be isolated, binds to SH3 domains of Crk adaptor proteins, through which it is recruited to the membrane signaling domains and phosphorylated for activation (*4*). Epac family proteins have auto-inhibitory domains capable of binding c-AMP, and the binding of c-AMP results in the activation of their GEF activity via conformational change (*5*, *6*). Recently, a c-AMP analogue that selectively inhibited the c-AMP-mediated activation of Epac without affecting PKA activity was reported (*7*), and, by using it, it was shown that Epac mediated some of the c-AMP-induced cellular functions previously thought to be mediated by PKA (*8*). Members of CalDAG (CD)-GEFs have Ca^{2+} and DAG-binding sites (*9–11*). CD-GEF III is translocated to the cell membrane by DAG, and CD-GEF I is regulated by Ca^{2+}, suggesting that they are major Rap1 activators operating downstream of PLC. PDZ-GEFs bear a Rap1-binding domain and PDZ domain, and interact with Rap1GTP and other proteins such as PDZ-containing synaptic scaffold molecules (S-SCAM) respectively (*12*). Most recently, a CDM family member protein, DOCK-4, has been shown to activate Rap1, although it does not share the conserved GEF catalytic domain and its mechanism of Rap1 activation remains to be seen (*13*).

Rap1 GAPs

In contrast to the marked molecular diversity of GEFs, only two families of Rap1-specific GAPs sharing a catalytic GAP-related domain (GRD) have been identified: rapGAPs and SPA-1 family proteins (*2*). Proteins bearing the GRD are conserved from *C. elegans* and *D. melangomaster* to mammals. rapGAP-I was the first GAP to be isolated (*14*), and rapGAP-II bearing an additional N-terminal region binds to Gα and is translocated to the membrane *via* G protein-coupled receptors (*15*). The SPA-1 family consists of structurally related proteins including

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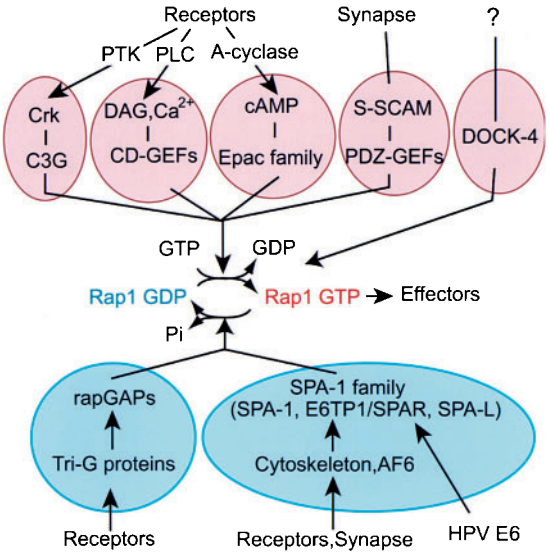

Fig. 1. **Regulatory molecules for Rap1 activation.** Rap1 is activated by several distinct types of specific guanine nucleotide exchange factors (GEFs) that are coupled with various receptors *via* adaptor molecules, scaffold proteins, or second messengers, while it is inactivated by two groups of specific GTPase-activating proteins (GAPs) that associate with receptors or cytoskeletal structures. Intracellular activation status of Rap1 is controlled spatio-temporally by the balance between GEFs and GAPs. SPA-1 family proteins are targeted for degradation by human papillomavirus E6 protein (HPV-E6). Tri-G; trimeric G proteins.

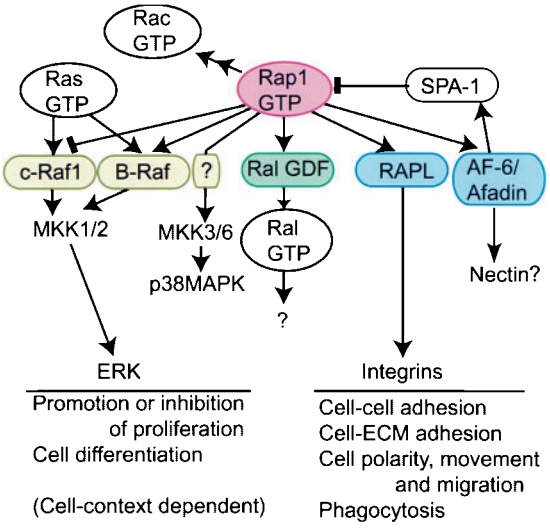

Fig. 2. **Effector molecules of Rap1.** Rap1GTP specifically interacts with various effector molecules to exert the biological functions. Rap1 has an effector domain essentially identical to Ras, and some of the effector molecules are shared with Ras. A part of Rap1 functions is Ras-dependent, and also Rap1 may cross-talk with other small GTPases including Ral and Rac. AF-6/afadin binds to both Rap1GTP and Rap1GAPs (SPA-1) and facilitates Rap1 inactivation. AF-6/afadin is also associated with nectin, but it remains to be seen whether Rap1 affects the nectin-mediated cell adhesion.

SPA-1 (*16*, *17*), E6TP1/SPAR (*18*, *19*), and SPA-Ls (*20*), all of which have additional domains such as PDZ, LZ, and actin-binding domains. A prototypic SPA-1 was isolated as a protein induced by the mitogenic stimulation

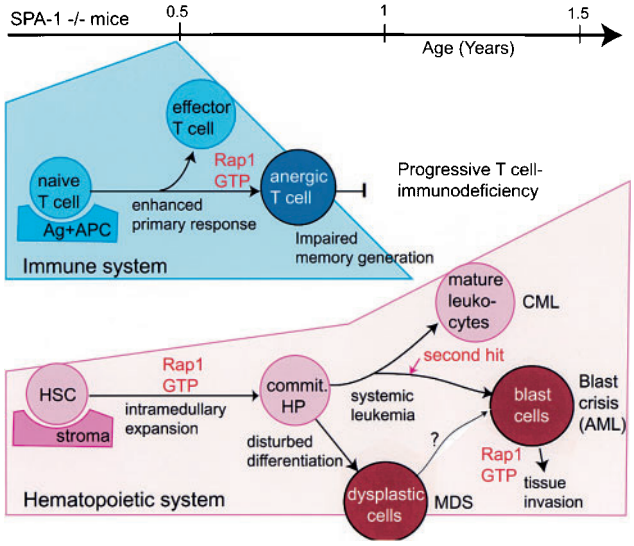

Fig. 3. **Dysregulated of Rap1 activation in the immune and hematopoietic systems causes characteristic disease complex.** SPA-1 is a non-redundant Rap1 GAP in the peripheral T cells and hematopoietic progenitors in bone marrow, and SPA-1-deficient mice reveal age-dependent progression of T cell-immunodeficiency followed by myeloproliferative disorders. Excess Rap1GTP is accumulated in the antigen-stimulated T cells and the early hematopoietic progenitors in bone marrow leading to the T cell unresponsiveness (anergy) and abnormal myeloprolirefation, respectively. Majority of SPA-1-deficient mice with CML develop lethal blast crisis likely due to the second genetic hits. A portion of SPA-1-deficient mice develops MDS with severe anemia, but it remains to be seen whether they also ultimately progress into AML as in human MDS. Leukemic blast cells, but not mature leukocytes in CML, continue to exhibit excess Rap1GTP, which is suggested to play a critical role in the invasion of blast cells into vital organs. Commit.HP; committed hematopoietic progenitors.

in lymphohematopoietic cells (*16*), and E6TP1 as a protein targeted for degradation by human papillomavirus (HPV) E6 oncoprotein (*18*). More recently, SPAR, which may be a human counterpart of rat E6TP1, was reported to regulate the formation of the dendritic spur in neurons (*19*). Also, several isoforms of SPA-1-like proteins (SPA-Ls) were identified in the neuronal synaptic vesicles (*20*). Thus, each GAP appears to show a unique expression profile and subcellular localization in different cells of various tissues (*17*). Rap1GAPs play a crucial role in the spatiotemporal control of Rap1 activation in cells (*21*).

Biological functions of Rap1

Recent studies have highlighted two major biological functions of Rap1, regulation of ERK activation and integrin-mediated cellular functions (Fig. 2). Several effector molecules that bind Rap1GTP have been identified, many of which are also capable of binding RasGTP, including c-Raf1, B-Raf, RalGEFs (RalGDS, Rlf, Rgl), AF-6/Afadin, and PI3K p110 (reviewed in Ref. *2*).

Rap1 signaling in cell proliferation

Following the discovery of Rap1, efforts were initially directed to investigating its effects on Ras-ERK signaling, and it was found that a dominant active Rap1 mutant (RapV12) attenuated the Ras-mediated ERK activation, probably via competitive interference with c-

Raf1 activation by Ras (*22*, *23*). On the other hand, Rap1 was reported to stimulate the cell growth in another fibroblast line (*24*) and to be capable of activating B-Raf independently of Ras in certain cell types (*25*, *26*). The differential effects of Rap1 on c-Raf1 and B-Raf activation were suggested to be due to much stronger binding of Rap1GTP to the cysteine-rich domain of c-Raf1 than to that of B-Raf (*27*). In the immune system, anergic T cell clones that failed to proliferate by specific antigen stimulation exhibited unusually high Rap1GTP levels (*28*), and forced expression of RapV12 in normal T cell clones induced an anergic state with compromised ERK activation in response to the antigens (*28*, *29*). On the other hand, it was reported that CD2 promoter-driven RapV12 transgenic mice showed no evidence of T cell anergy *in vivo* (*30*). While a RapV12 mutant was used widely as a constitutively active form (*31*), recent analysis has revealed that RapV12 is less yet significantly susceptible to Rap1 GAP (*32*), thus indicating the need for careful interpretation of experimental results using the mutant, particularly in the cells with abundant GAPs. Using *SPA-1* gene-targeted mice, we found that deregulated activation of endogenous Rap1 in hematopoietic progenitors resulted in enhanced proliferation leading to ERK activation (*33*), suggesting that Rap1GTP induced ERK activation independently of Ras possibly *via* B-Raf. In contrast, persistent Rap1 activation following antigen activation in T cells of SPA-1 –/– mice ultimately caused anergy due to uncoupling of Ras-mediated ERK activation (*34*). It appeared that the amount of Rap1GTP attained in SPA-1 –/– T cells was much higher than in RapV12-transgenic mice, and thus the level of Rap1GTP was suggested to play a role in setting the threshold for activation of T cells by antigens through the modulation of Ras-ERK signaling. The results reinforced the notion that the effects of Rap1 signaling on cellular proliferation were highly cell-context-dependent. In addition, Rap1 was shown to be responsible for the activation of MKK3/6-p38MAPK pathway by cell stretching stimuli (*35*) as well as in NMDA receptor-dependent removal of synaptic AMPA receptors for long-term depression in neurons (*36*).

Rap1 signaling in cell adhesion

The first indication that Rap1 regulated cell adhesion came from the observation that overexpression of membrane-targeted C3G in the adherent cells induced markedly enhanced cell spreading, while that of SPA-1 resulted in cell rounding followed by detachment from the matrix (*37*). Consistent with this, it was reported that *C3G* gene-targeted mice were embryonic-lethal due to aberrant epithelial cell adhesion in developing tissues (*38*). In *Drosophila*, it was reported that Rap1 mutation disrupted normal cell shape and morphogenesis in the eye, ovary and wing in the embryos (*39*), and that Rap1 regulated the position of adherens junction markers in the epithelium lining the wing (*40*). Since then, much evidence has accumulated that Rap1 mediates the activation of integrins (*2*, *41*, *42*). Rap1 was shown to play critical roles in various integrin-dependent cellular functions such as immunological synapse formation (*29*), macrophage phagocytosis (*43*, *44*), and chemokine-induced migration of leukocytes (*45*, *46*). It was found that Rap1 signaling mediated the inside-out activation of integrins *via* various stimuli, inducing an increase in integrin affinity, avidity, or both (*30*, *41*). Furthermore, integrin-mediated cell adhesion induced by Mn^{2+} or by an activatory antibody that was capable of activating integrin still required Rap1 activation (*47*), suggesting that Rap1 signaling was required for the entire process of integrin-mediated cell adhesion. Most recently, it was found that a new Rap1 effector, RAPL, mediated the spatial regulation of LFA-1 by Rap1 and regulated the lymphocyte movement and migration by a chemokine (*48*), and we reported that AF-6/Afadin bound to both Rap1GTP and Rap1GAPs including SPA-1 and regulated integrin-mediated cell adhesion by modulating the GAP activity (*49*). Afadin is shown to bind to nectin involved in adherens junctions (*50*), and it remains to be seen whether Rap1 affects cell adhesion systems other than integrins.

Rap1 in malignancy

In spite of the initial attention paid to the possible roles of Rap1 in malignancy, little evidence was obtained for such roles until very recently. It was reported that the degradation of E6TP1 by oncogenic HPV-E6 *via* E6AP ubiquitin ligase was highly correlated with the cellular transformation *in vitro* (*51–53*). It was found also that CD-GEF I was activated recurrently by proviral integration in leukemia-prone BXH-2 recombinant strain of mice, and such leukemia cells exhibited constitutive Rap1 activation (*54*). Most recently, two independent works have provided more direct evidence for Rap1 involvement in malignancy.

Good Rap and bad Rap

During the search for genes homozygously mutated in tumor cells, recurrent mutation of a CDM (*C. elegans* ced-5, vertebrate DOCK180, and *Drosophila* Myoblast City) family gene called *DOCK-4* was found in some human and mouse tumor cells (*13*). DOCK4 was shown to be a specific Rap1 activator, the mutation of which in cancer cells caused the loss of function. Introduction of a wild-type *DOCK-4* into such cancer cells suppressed their growth and invasion *in vivo*, which was associated with the restoration of adherens junctions among the cancer cells (*13*). This effect of *DOCK-4* was replicated by RapE63 expression, and thus it was indicated that loss of Rap1-mediated intercellular adherens junctions due to *DOCK-4* disruption played a critical role in the growth and invasion of some human cancers.

On the other hand, we found that SPA-1-deficient mice developed a spectrum of myeloid disorders of late onset that resembled human chronic myelogenous leukemia (CML) in the chronic phase, CML in blast crisis and myelodysplastic syndrome (MDS) (*33*). In the bone marrow of preleukemic SPA-1 –/– mice, there was a marked expansion of multipotential hematopoietic progenitors with significant Rap1GTP accumulation and ERK activation. Retroviral transduction of RapE63 in the normal hematopoietic progenitors induced enhanced hematopoiesis both *in vitro* on the stroma cells and *in vivo* (*33*), and we speculate that constitutive Rap1 signaling enhanced the interaction of progenitors with stroma cells in the hematopoietic microenvironment (niche), ultimately leading to the deregulated expansion and/or survival of the progen-

itors. The majority of mice further developed lethal blast crisis probably *via* second genetic hit, and the blast cells continued to show constitutive Rap1 activation (*33*). Restoring *SPA-1* gene in the SPA-1 –/– blast cell line abrogated the Rap1GTP accumulation and concomitantly suppressed the leukemogenesis *in vivo*, strongly suggesting that excess Rap1 activation was responsible for not only the initial leukemogenesis but also the maintenance of malignant phenotype of the blast cells *in vivo*. Interestingly, leukemia development in SPA-1 deficient mice was preceded by the progression of T cell immunodeficiency, suggesting that the independent effects of Rap1 dysregulation on immune and hematopoietic systems collaboratively resulted in the pleiotropic myeloproliferative disease complex (Fig. 3). Deficiency of *NF1* (neurofibromatosis-1) gene encoding RasGAP is known to cause leukemia, CML in mice and juvenile CML in human, *via* deregulated Ras activation in response to GM-CSF (*55*, *56*), and that of *TSC-2* (tuberous sclerosis-2) gene encoding GAP specific for Rheb (*57*) may be related to the tumors in this inherited human disease (*58*). Myeloid disorders in SPA-1 deficiency are another example of the tumor-suppressor functions of GAPs for small G proteins (*59*). These results have indicated that either defective or excess Rap1 activation can contribute to malignancy *via* distinct biological effects in different cell types.

Future prospects

Recent studies have begun to unveil the biological functions of long-enigmatic Rap1 GTPase in cell proliferation and adhesion, and other functions such as protein transport via cross-talk with other small G proteins has been also reported (*60*). We recently developed the conditional gene-engineered mice for SPA-1 and RapE63, analysis of which might help to reveal new functional aspects of Rap1. Roles of Rap1 signaling in physiology and diseases can be highly diverse depending on the cell types of different tissues with various specified functions, and dissecting the molecular mechanisms should provide novel clues for the control of human diseases including malignancy.

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