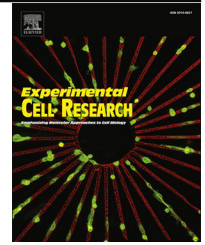


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Research Article

Rap G protein signal in normal and disordered lymphohematopoiesis

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ABSTRACT

Rap proteins (Rap1, Rap2a, b, c) are small molecular weight GTPases of the Ras family. Rap G proteins mediate diverse cellular events such as cell adhesion, proliferation, and gene activation through various signaling pathways. Activation of Rap signal is regulated tightly by several specific regulatory proteins including guanine nucleotide exchange factors and GTPase-activating proteins. Beyond cell biological studies, increasing attempts have been made in the past decade to define the roles of Rap signal in specific functions of normal tissue systems as well as in cancer. In the immune and hematopoietic systems, Rap signal plays crucial roles in the development and function of essentially all lineages of lymphocytes and hematopoietic cells, and importantly, deregulated Rap signal may lead to unique pathological conditions depending on the affected cell types, including various types of leukemia and autoimmunity. The phenotypical studies have unveiled novel, even unexpected functional aspects of Rap signal in cells from a variety of tissues, providing potentially important clues for controlling human diseases, including malignancy.

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Introduction

Small G proteins control a wide variety of essential cellular processes. Rho family members control cell shape and movement via regulation of the cytoskeleton, members of the Rab and Arf families play important roles in protein transport associated with vesicles involved in endocytosis and exocytosis, and Ran is crucially involved in the regulation of nuclear transport. Ras family members mediate the cell signaling that controls cell proliferation, differentiation, and gene activation, and the biological effects may be highly diverse depending on the cell types and states. Importantly, deregulated Ras activation is potentially oncogenic and may cause cellular senescence or apoptosis in normal cells by inducing intrinsic tumor suppressor mechanisms to prevent cell transformation [1]. Among the Ras family, Rap G proteins (Rap1a, b and Rap2a, b, c) represent a distinct subfamily.

Although Rap subfamily members bear a high structural similarity to Ras, they have distinct signaling mechanisms in time and space and show unique biological effects [2,3]. Most notably, Rap plays an important role in regulating the activity of integrins and other cell adhesion molecules, thereby controlling cell–cell and cell–extracellular matrix communications [4,5]. Beyond basic cell biological analysis, an increasing number of studies in the past decade have focused on the specific roles of Rap signal in various physiological functions as well as pathological conditions. It has been reported that Rap signal plays important functional roles in many tissue systems, including immune [6], hematopoietic [7,8], endocrine [9], and nervous tissues [10,11]. Rap signal also affects the tissue invasiveness and metastasis of various human cancers [12–14]. In this brief review, I will summarize the role of Rap signal in normal development and function as well as in disorders of the lymphohematopoietic system.

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Regulation of Rap regulators

Like other small G proteins, the switch function of Rap is regulated by guanine nucleotide (GXP) exchange factors (GEFs) and GTPase-activating proteins (GAPs), which together control time, place, and duration of Rap activation [3,15]. GEFs bind to GXP-bound Rap and competitively dissociate the Rap from the GXP. Due to a much higher cellular concentration of GTP than GDP, the process markedly accelerates the reloading of Rap with GTP, thus converting it to an active form. There are many distinct Rap GEFs, which are linked to a wide variety of upstream signaling receptors via various mechanisms: C3G (RapGEF1) recruited to tyrosine kinase receptors via adapters such as Crk; Epac1, 2 (RapGEF3, 4) and CalDAG GEF1 (RasGRP2) activated by second messengers such as cAMP and Ca²⁺/diacylglyceride (DAG), respectively; PDZ-GEF1, 2 (RapGEF2, 6) bound to cell junctional proteins via the PDZ domain; and Repac (RapGEF5) bound and regulated by GTP-bound Ras or Rap. Discovery of Epac was particularly noteworthy, in that a significant portion of cAMP-mediated effects previously attributed to cAMP-dependent protein kinase were mediated actually by an Epac/Rap pathway [16]. All these molecules bear a catalytic CDC25-homology domain and show GXP exchange activity selective for Rap family members, although some may display broader activity. Through these distinct types of Rap GEFs, Rap is activated in response to highly diverse extracellular stimuli.

RapGAPs interact with GTP-loaded Rap with high affinity. By providing an asparagine residue, called the Asn thumb, in the vicinity of GTP bound at the switch II region of Rap, they strikingly enhance hydrolysis of the bound GTP [17]. The mode of action is distinct from that of RasGAPs, and RapGAPs specifically convert Rap-GTP to an inactive Rap-GDP. There are two families of RapGAPs; Rap1GAP family (Rap1GAP1a, 1b, 2) and Sipa1 family (Spa-1, Spar1, 2, 3). These RapGAP proteins are localized in various cell compartments of different cell types and regulate local Rap activation in different places [3]. For example, Rap1GAP1a binds to G α 's at the plasma membrane, whereas Rap1GAP2 is associated with signal scaffolding protein 14-3-3. Among Sipa1 family members, Sipa1 (Spa-1), a founding member, is predominantly expressed in lymphohematopoietic cells, while Spar1 and Spar 2 are expressed in neurons [4,10]. In these tissue cells, Sipa1 members are associated with a diverse array of proteins via unique motifs such as PDZ and actin-binding motifs and are localized in various places such as plasma and endosomal membranes, cortical cytoskeleton, dendritic spines, and synaptic vesicles. In addition to these authentic RapGAPs, several GAPs bearing a Ras-GAP domain with an arginine residue crucial for Ras-GAP activity called Arg finger may display significant or even better GAP activity for Rap than Ras, including Gap1 family members (GAP1^{IP4BP}, RASAL, CAPRI), SynGAPs (RASA1, 5), and plexins (semaphorin receptors) [18]. Such an unexpected RapGAP activity by some RasGAPs is attributed to the unique interaction of extra-GAP domains with the Rap switch II region, leading to a reorientation of Rap catalytic residues [19]. Such dual-specific GAPs may also contribute to the regulation of Rap signal [20].

The presence of many regulatory proteins with distinct functional features and localization indicates that Rap signaling is under tight regulation depending on the stimuli. Such Rap regulatory proteins should provide a valuable clue in investigating the specific role of Rap signal in unique functions of different tissue cells.

Rap signal in T-cell development and leukemia: Rap meets Notch signaling

Rap signal is essential in normal T-cell development in the thymus [21]. Conditional expression of *Sipa1* or a dominant negative mutant of Rap1 (*Rap1A17*) under *lck* promoter causes a severe defect in the thymic T-cell development at the early CD4/CD8 double-negative stage. At this stage, early T-cell progenitors (ETPs) begin to rearrange T-cell receptor (TCR) β genes, and resulting TCR β chains are assembled with a surrogate TCR α chain ($\rho T\alpha$) to form pre-TCR. The pre-TCR autonomously delivers signal in a ligand-independent manner to rescue pre-T cells from apoptosis and promote robust proliferation, ensuring diversification of the TCR repertoire in a process called β -selection [22]. Rap is activated downstream of pre-TCR and induces PI3K/Akt activation. Inasmuch as the defective pre-T-cell development by Rap signal attenuation was restored on a *p53*^{-/-} genetic background [21], Rap signal is considered to play a crucial role in the rescue of pre-T cells from cell death, in which p53 activation may be involved due in part to the TCR gene recombination events. In addition to pre-TCR, Notch signal also plays an important role in promoting the robust proliferation of pre-T cells by repressing Pten and activating the PI3K/Akt pathway [23]. In the thymic tissue, a Notch ligand (Delta-like 4, Dll4) is expressed on cortical thymic epithelial cells and is indispensable for the expansion of ETPs [24]. Intriguingly, expression of an active form of C3G (farnesylated C3G [C3G-F]) was found to markedly enhance the Notch-dependent proliferation of ETPs in the presence of stroma cells expressing Notch ligand [21], suggesting the presence of a functional crosstalk between Rap and Notch signals.

More convincing evidence for the regulation of Notch activation by Rap signal was derived from a bone marrow transplantation (BMT) study. Hematopoietic stem cells (HSCs) transduced with C3G-F and transplanted into lethally irradiated mice resulted in markedly accelerated thymic repopulation and hyperplasia, eventually leading to lethal T-cell acute lymphoblastic leukemia (T-ALL) spreading into all vital organs [25]. As in many human T-ALLs, the proliferation of these T-ALL cells was abrogated by a γ -secretase inhibitor and thus was crucially dependent on sustained Notch signaling. Since there was a striking increase in *Notch* transcripts in the thymocytes prior to overt T-ALL development, it was suggested that the deregulated Notch signaling, which induces autologous *Notch* activation, was responsible for the initiation of leukemia. Unlike HSCs expressing truncated, active Notch intracellular domain (*NICD*), which caused T-ALL in the BM as well, T-ALL development from the HSCs expressing C3G-F was absolutely dependent on the normal thymic microenvironment. Although many cases of T-ALL developed from HSCs expressing C3G-F showed characteristic gain-of-function mutations of *Notch1*, we also observed frank T-ALL cells with no detectable Notch mutation. Therefore, the *Notch* mutations per se may not be a necessary condition for T-ALL genesis from C3G-F-expressing HSCs, in agreement with a recent report indicating that the mutant *Notch1*'s often observed in human T-ALLs are insufficient for T-ALL development in a BMT system [26].

Intriguingly, we also found that the attenuation of Rap activation in the T-ALL cells resulted in compromised proliferation in vitro as well as reduced leukemogenesis in vivo, irrespective of *Notch1* mutation (submitted for publication). The effect was

observed not only in the T-ALL derived from HSCs expressing C3G-F but also in spontaneously arising T-ALL via proviral translocation into *Notch1*. Attenuated Rap activation in T-ALL resulted in diminished NICD expression and consequently reduced induction of the target *Hes1*, suggesting that Rap signal functions upstream of Notch activation. Notch is activated via stepwise proteolytic processing, the cleavage at a supra-membrane site (S2) by Adam10 followed by intramembrane cleavage (S3) by γ -secretase complex [27]. We recently found that Rap signal was needed for the generation of the mature, active form of Adam10 required for S2 processing of Notch at the plasma membrane prior to the generation of NICD (submitted for publication). These results unveil a unique role of Rap signal in the activation of Notch signal in normal thymic T-cell development as well as T-ALL genesis (Fig. 1).

Rap signal in normal hematopoiesis and its disorder

RapGEF2 (*PDZ-GEF1*) deficiency results in a severe defect of embryonic definitive as well as fetal liver hematopoiesis [28]. Although adult BM hematopoiesis is unaffected, probably due to

the compensation by other types of GEFs, the results strongly suggest that Rap signal plays an essential role in the development and/or maintenance of HSCs (Fig. 1). On the other hand, we previously reported that deregulated Rap activation in *Sipa1* deficiency resulted in the development of a spectrum of myeloproliferative disorders (MPDs), including diseases resembling human chronic myeloid leukemia (CML) and less frequently myelodysplastic disease [29]. The vast majority of human CML is caused by *Bcr-Abl* fusion gene, and we previously reported that the murine *Sipa1*^{-/-} hematopoietic progenitor cells (HPCs) transduced with human *Bcr-Abl* showed enhanced survival of CML-initiating cells with highly accelerated blastic transformation and chromosomal aberrations in vivo [30]. It was noted that overt MPD development in *Sipa1*^{-/-} mice occurred only after long latent periods at ages of more than 1 year. Paradoxically, however, young *Sipa1*^{-/-} mice with no evidence of MPD revealed rather reduced BM hematopoietic activity with significantly diminished numbers and cell cycle progression of HPCs [31]. Furthermore, young *Sipa1*^{-/-} mice showed a markedly increased susceptibility to γ -ray irradiation, leading to lethal BM failure with little regeneration of hematopoiesis following otherwise sublethal whole-body γ -ray irradiation [31]. The effect was attributable to markedly

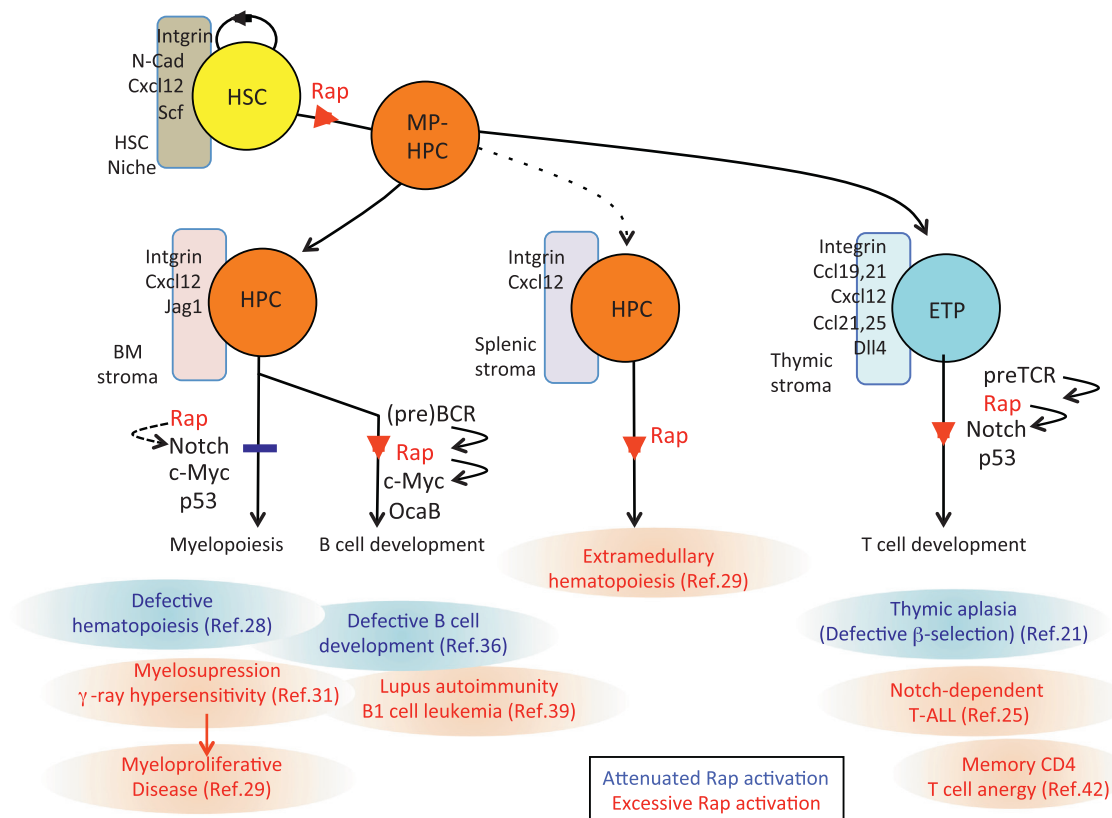


Fig. 1 – Rap signal plays crucial roles in normal lymphohematopoiesis of essentially all lineages, and its dysfunction leads to characteristic disorders depending on the lineages. Maintenance of HSCs as well as the development of myeloid and B- and T-lineage cells depends on the intimate interaction with unique stroma cells in distinct lymphohematopoietic microenvironments such as cell adhesion molecules (integrins, N-cadherin), Notch, chemokines, and growth factors, and Rap signal may be activated in the hematopoietic progenitor cells during the interaction. Attenuated Rap activation at different stages results in the defective development of corresponding hematopoietic cell components (blue), whereas deregulated excessive Rap activation causes unique disorders, including myeloproliferative disease, lupus-like autoimmunity and B1 cell leukemia, T-ALL, and memory T-cell anergy (red). HSC, hematopoietic stem cell; (MP-) HPC, multipotential hematopoietic progenitor; ETP, early T-cell progenitor; T-ALL, T-cell acute lymphoblastic leukemia.

enhanced expression and activation of p53 in *Sipa1*^{-/-} HPCs *in vivo*, and the enhanced radiosensitivity was abrogated on a p53^{-/-} genetic background. The HPCs in the preleukemic stage of *Sipa1*^{-/-} mice concomitantly showed strikingly increased c-Myc in association with γ H2X expression, suggesting that the enhanced p53 activation was due to the c-Myc overexpression and resulting DNA damage. Because *Sipa1*^{-/-} p53^{-/-} mice were found to develop highly aggressive blastic leukemia much earlier than *Sipa1*^{-/-} mice, it was suggested that p53 effectively functioned as a barrier against leukemia in *Sipa1*^{-/-} mice for significant periods of life, if not forever. Thus, it is likely that the MPD of late onset in *Sipa1* deficiency is secondary to the persistent genetic stress on HPCs, rather than due to the direct effect on their proliferation.

Recently we found that C3G-F-transduced *Sipa1*^{-/-} HSCs with maximal activation of endogenous Rap signal were hardly engulfed in the BM of lethally irradiated normal recipients (our unpublished observation), albeit they showed enhanced repopulation in the thymus. Thus, in strong contrast to the positive effects on ETPs in the thymus, deregulated Rap signal is suggested to act rather negatively in the maintenance and proliferation of normal HPCs in the BM microenvironment. Most recently, it was reported that the defective Notch activation in HSCs or the blockade of functional Notch ligand activity in BM stroma cells resulted in the robust expansion of myeloid cells and MPD [32,33]. The results reinforce that Notch signal plays a crucial role in the fate of multipotent HPCs toward myeloid, B-, and T-lineage cells [34]. Considering that Rap signal strongly enhances ligand-induced Notch activation, it is an intriguing possibility that opposing effects of deregulated Rap activation on HPCs and ETPs in BM and thymic microenvironments, respectively, may in part reflect the distinct effects of Notch signal on the different hematopoietic cell lineages (Fig. 1).

Rap signal in self-tolerance and autoimmunity

Development of a diverse array of autoantibodies mostly against cell nuclear components is a hallmark of systemic autoimmune diseases such as systemic lupus erythematosus. Although B cells reactive to DNA are rare in the periphery, unusually high proportions (as much as 50%) of immature B cells in the BM of healthy human beings display DNA-reactive BCR [35]. B cells consist of three major cell subsets, mainstream follicular (FO), marginal zone (MZ), and B1 cells; the latter two populations show characteristic B-cell receptor (BCR) repertoire with “polyspecificity” and may often be involved in autoantibody production [36]. While attenuated activation of all Rap family members in B-lineage cells caused severe defects of mainstream FO B-cell development in BM [37], it was shown that a single deficiency of *Rap1b* resulted in a specific defect of MZ B cells in spleen [38], whose development and maintenance uniquely depend on Notch signaling [39]. On the other hand, we found that deregulated Rap activation in *Sipa1* deficiency resulted in an age-dependent increase of B1 cells, eventually leading to the development of anti-dsDNA antibody and glomerular nephritis reminiscent of human systemic lupus erythematosus [40]. One of the checkpoint mechanisms for potentially autoreactive immature BM B cells is “BCR editing”, in which the BCR signaling via autoantigens induces repetitive *Ig*L rearrangements until alternative, less autoreactive BCR is emerged [35]. The BCR editing process may ensure

the self-tolerance without significant loss of newly emerged B cells. The immature BM B cells in *Sipa1*^{-/-} mice showed remarkably increased transcription co-factor OcaB required for BCR receptor editing, leading to the increase of B1 cells with highly skewed V κ usage and Ig κ /Ig λ isotype inclusion [40]. It is thus suggested that deregulated Rap activation downstream BCR in the immature autoreactive B cells results in the aberrant editing with potential autoreactivity retained, and such B cells may be directed to a differentiation pathway toward B1 cells. B1 and MZ B cells are normally localized extrafollicularly in the peritoneal cavity and splenic MZ, respectively, and are excluded from germinal center reaction, avoiding robust clonal expansion, Ig class-switching, and affinity maturation under T-cell help; this is another checkpoint against autoimmunity [41]. Prior to the development of overt lupus disease, however, *Sipa1*^{-/-} mice also show a marked increase of spontaneous germinal centers in association with follicular T cells (unpublished observation), similar to other lupus-prone mice, suggesting the breakdown of this checkpoint. Thus, Rap signal may also regulate the autoreactivity of BCR repertoire in BM immature B cells as well as the unique tissue localization of potentially autoreactive mature B cells.

One of the major mechanisms for peripheral self-tolerance in T cells is an induction of TCR unresponsiveness for specific self-antigens, called “anergy”. It was reported previously that excessive and persistent Rap activation in CD4⁺ T cells resulted in defective TCR-mediated ERK activation and unresponsiveness to specific antigens [42]. Also, we reported that an increasing accumulation of Rap1GTP in memory phenotype CD4⁺ T cells of aged *Sipa1*^{-/-} mice showed impaired antigen-specific memory response *in vivo* [43]. It was further indicated that the inhibition of TCR signal by CTLA4, an inhibitory T-cell co-stimulatory receptor, was mediated by Rap signal, which interfered with the stable immunological synapse formation with antigen-presenting cells [44]. Thus, in addition to a possible direct inhibitory effect on Ras-mediated ERK activation, deregulated Rap signaling during TCR stimulation may cause disassembling of a signalosome structure in the immunological synapse, indirectly interfering with TCR-mediated activation. More recently, it was reported that transgenic (Tg) mice expressing *Rap1V12*, a putative dominant active form, showed significant resistance to collagen-induced arthritis and experimental autoimmune encephalomyelitis (EAE), a murine model of human multiple sclerosis [45,46]. In the EAE model, it was shown that the size of autoreactive memory CD4⁺ T-cell pool specific for a self-antigen, myelin oligodendrocyte glycoprotein, was significantly reduced in *Rap1V12*-Tg mice. In contrast to the effects on B cells, it is suggested that sustained Rap signaling in autoreactive T cells above a certain threshold level may indeed be involved in the establishment of T-cell anergy for autoantigens and thus in peripheral self-tolerance. This may be another example for the distinct functional consequences of dysregulated Rap activation depending on the cell types.

Concluding remarks

Since their discovery is more than 2 decades ago, there has been a great advance in the understanding of the biology and function of Rap family G proteins. Early studies focused on the function in analogy with classical Ras proteins because of the high structural homology. By subsequent analysis, however, substantial evidence

has been accumulated that indicates the unique signaling function of Rap in various aspects of cell functions, including cell–cell and cell–matrix interactions via regulation of cell adherence molecules. Identification of a number of proteins regulating the Rap signaling has greatly contributed to this understanding. More recently, much effort has been made to delineate the roles of Rap signal in specific physiological functions of various tissues as well as in disease conditions, including malignancy. As expected from its crucial involvement in the cellular communications, the effects of Rap signal can be quite diverse depending on the unique tissue context. Apart from the roles in immune and hematopoietic systems as reviewed here, much information has been accumulated on the involvement of Rap G proteins in the function of neuronal, endocrine, and vascular systems as well as many types of cancers. The phenotypical studies in many other tissue systems may unveil novel, even unexpected roles of Rap signal and provide unique targets for controlling various human diseases including malignancy.

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