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Review

The role of Ras and other low molecular weight guanine nucleotide (GTP)-binding proteins during hematopoietic cell differentiation

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Abstract. Recent progress in the understanding of signal transduction and gene regulation in hematopoietic cells has shown that many intracellular signalling pathways are modulated by low molecular weight guanine nucleotide (GTP)-binding proteins (LMWGs). LMWGs act as molecular switches for regulating a wide range of signal-transduction pathways in virtually all cells. In hematopoietic cells, LMWGs have been shown to participate in essential functions such as growth control, differentiation, cytoskeletal organization, cytokine and chemoattractant-induced signalling events, reduced nicotinamide adenine dinucleotide phosphate oxidase activity, intracellular vesicle transport and secretion. In human leukemias, myelodysplastic syndromes and myeloproliferative disorders, Ras activation occurs by point mutations, overexpression or by alteration of NF-1 Ras-GTPase activating protein (GAP). These are post-initiation events in leukemia but may modulate growth-factor-dependent and independent leukemic growth. Two animal models of mutated N-ras expression resulting in myelodysplastic and myeloproliferative features are discussed. The role of Ras in organ development is discussed in the context of transgenic knockout mice. More LMWG functions will certainly be identified as we gain a better understanding of regulatory pathways modulating myeloid signal transduction. This review will summarize our current understanding of this rapidly advancing area of research.

Key words. Cell differentiation; gene expression; Ras; Rap; Rho; Rab; ARF; Ran; Rad.

Introduction

LMWGs are involved in the regulation of cellular functions in virtually all living organisms and play key roles in signal transduction in myeloid cells as well as in the regulation of gene expression (reviewed in [1–9]). These specialized proteins are separated into two distinct classes consisting of (i) the heterotrimeric GTP-binding proteins (known as G proteins) and (ii) the monomeric LMWGs. In general, the heterotrimeric G proteins (which will not be discussed here) function in transducing signals from cell surface receptors to intracellular effectors that generate a variety of second messengers [1]. Activation of these proteins frequently leads to Ras activation via both α and β/γ subunits of G-protein-mediated signalling pathways [10, 11]. In addition, second messengers that function downstream from G proteins like calcium [12] and cyclic AMP (cAMP) [13, 14] activate both Ras and Rap, and both Ras- and Rap-GTPs have been identified as binding partners for G subunits [10, 11]. There is a functional link between G proteins and Rho family members through the regula-

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Table 1. Mammalian low molecular weight GTP-binding proteins of the Ras superfamily and their major functions.

Subfamily	Members	Function
Ras	part of mitogen-activated protein kinase pathway; immunoglobin capping; cell growth and differentiation; regulation of cell survival and apoptosis; cell cycle regulation and integrin signaling	K-Ras A, K-Ras B, N-Ras, M-Ras, R-Ras, TC21, H-Ras, D-Ras
Rap1a Rap1b	antagonist of Ras; involvement in leukocyte NADPH oxidase involvement in protein kinase C activation and calcium flux in platelets; direct regulation by second messengers; regulation by second-messenger-regulated GEFs; morphogenic differentiation in *Drosophila* independent of Ras	Rap2a, Rap2b Rheb
Ral	role in vesicle transport	Ral B
Rho	cytoskeletal assembly; cell morphology; cell–cell adhesion; cytolytic function; vesicle trafficking + secretion	Rho A, B, C
Rac	membrane ruffling; activation of the phagocyte NADPH; oxidase; cell-cell adhesion; cell polarization and chemotaxis; cell morphology; cytolytic function; cytoskeletal assembly	Rac 2, TC10
Rab	role in intracellular transport; endocytosis; degranulation + secretion	Rab1A,B;Rab2, Rab3A,B,C; Rab4-11; smg p25B,C
ARF	required for vesicle transport from endoplasmic reticulum to Golgi membranes; activation of phospholipases C and D; degranulation and secretion	ARF1-6
Rad	induced early after mitogenic activation of resting cells with role in control of proliferation	
M-Gem	induced regulatory protein that plays role in receptor-mediated signal transduction; cell growth and differentiaion	H-Gem
Ran	nuclear import and export	

tors of G protein signalling (RGS) proteins [15]. This review will focus on the second class of GTP-binding proteins, the LMWGs, and their role in myeloid gene expression, signal transduction and cell differentiation. LMWGs are involved in a wide range of cellular functions in myeloid cells, including growth control and differentiation.

General features of LMWGs

The LMWGs are members of a growing family of GTP-binding proteins known as the Ras superfamily of GTP-binding proteins [2–4], which consists of more than 50 proteins that are structurally related to Ras. LMWGs exhibit common features, including low molecular weights ranging from 20–29 kDa and the presence of consensus GTP-binding motifs. The Ras superfamily of LMWGs can be divided into six subfamilies based primarily on common structural features [5, 6]. Although we do not completely understand the function of each member of a subfamily, there appears to be extensive redundancy within each subfamily (see table 1).

All GTP-binding proteins, including LMWGs, utilize a common functional mechanism that is based on their ability to cycle between inactive GDP- and active GTP-bound forms (reviewed in [2, 4, 16, 17]). As illustrated in figure 1, the inactive LMWG releases GDP when it receives an upstream activation signal. This process is facilitated by GDP dissociation stimulators (GDSs) also called guanine nucleotide exchange factors (GEFs), which catalyze the release of GDP from the GTP-binding protein [4, 18, 19]. GTP then binds to the transiently empty guanine nucleotide-binding site, thereby activating the LMWGs that carry out downstream cellular

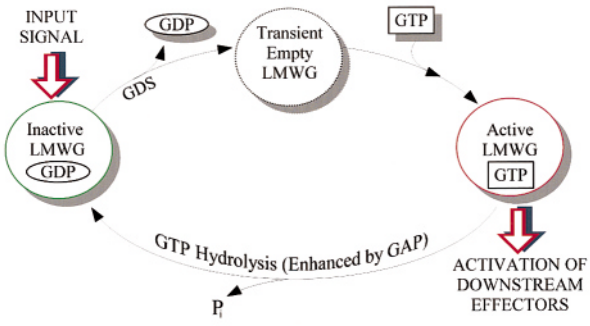

Figure 1. The inactive low molecular weight GTP-binding protein (LMWG) releases GTP when it receives an upstream activation signal. This process is facilitated by GDP-dissociation stimulators (GDPs), also called guanine nucleotide exchange factors (GEFs), which catalyze the release of GDP from the GTP-binding protein. GTP then binds to the transiently empty guanine nucleotide-binding site, thereby activating the LMWGs that carry downstream cellular functions. Simultaneously, GTP is hydrolyzed by an intrinsic GTPase activity, resulting in termination of the activated state. In cells, this normally low GTPase activity is enhanced significantly by GTPase-activating proteins (GAPs).

functions. Simultaneously, GTP is hydrolyzed by an intrinsic GTPase activity, resulting in termination of the activated state. In cells, this normally low GTPase activity is enhanced significantly by GTPase-activating proteins (GAPs, NF1-GAP/neurofibromin) that may be the target protein or a component of the target protein complex [4, 18, 19]. Thus, the ability of GTP-binding proteins to switch between two distinct states, an inactive GDP-bound state and an active GTP-bound state, enables them to act as timed molecular switches that can precisely control various cellular processes. Although there are other known cellular switches, such as ion channels and transcription factors, the GTP-binding proteins are distinctive because they contain an intrinsic timer and can be modulated by external regulatory factors.

The Ras subfamily of LMWGs

The three human ras genes (H-ras, N-ras and K-ras) encode four 188–189-amino-acid (p21) proteins that function as GDP/GTP regulated switches (H-Ras, N-Ras, K-Ras4A and K-Ras4B) [18, 20–23]. The two forms of K-Ras diverge solely in their COOH-terminal 25 amino acids as a consequence of alternative exon use. Ras proteins are located at the inner surface of the plasma membrane, where they serve as binary molecular switches to transduce extracellular ligand-mediated stimuli into the cytoplasm, to control signal transduction pathways that influence cell growth, differentiation and apoptosis [19, 24]. There is differential lipid modification of the three mammalian isoforms [25]. The low intrinsic GTP hydrolytic activity of Ras is modified by GDSs (Ras-GRF/mCDC25, SOS1/2), which promote formation of the active GTP-bound state [13]. The calcium-regulated Ras-GDP releasing protein (GRP) [12] is of special interest because it links different signal transduction pathways. Single amino acid substitutions at 12, 13 or 61 create transforming mutant proteins that are insensitive to GAP stimulation [26, 27] and therefore locked in the active GTP-bound state. Many transformed cell lines contain mutant forms of Ras, including the promyelocytic cell line HL-60 (N-Ras; codon 61). There is also evidence that reversing this transforming paradigm by Krev-1 (Rap1a) activation leads to monocytic differentiation [28, 29].

On the other hand, Ras proteins are activated transiently in response to a wide spectrum of extracellular signals such as growth factors, cytokines and hormones that stimulate cell surface receptors. The latter include receptor tyrosine kinases (RTKs), non-RTK-associated receptors and G-protein-coupled seven-transmembrane receptors (SRs) [20]. The best-characterized Ras-mediated signal transduction pathway involves the activation of peptide mitogen-stimulated RTKs such as the epidermal growth factor (EGF) receptor (EGFR) [30, 31] (fig. 2). Substantial biological, biochemical and genetic evidence has implicated the Raf-1 serine/threonine kinase as a critical effector of Ras function [31]. A key observation is that biologically active Ras forms a high-affinity complex with Raf-1 [32–36], whereas the inactive form of Ras does not. The Ras-Raf association initiates a translocation of the normally cytoplasmic

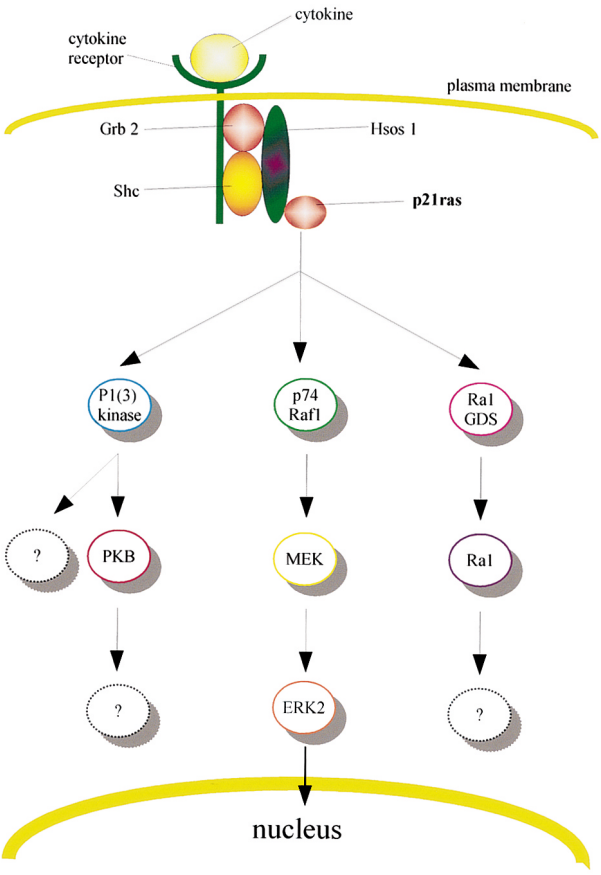

Figure 2. Ras proteins are activated transiently in response to a wide spectrum of extracellular signals such as growth factors, cytokines and hormones that stimulate cell surface receptors. The best-characterized Ras-mediated signal transduction pathway involves the activation of peptide mitogen-stimulated receptor tyrosine kinases (RTKs) such as cytokines and epidermal growth factors (EGF). Substantial evidence has implicated Raf –1 serine/throenine kinase as a critical effector of Ras function. Upon activation, Raf phosphorylates and activates two mitogen-activated protein kinase (MAPK) kinases (MAP-KKs, also called MEK1 and MEK2). Activated MEKs, which function as dual-specificty kinases, phosphorylate tandem threonine and tyrosine residues in two MAPKs also referred to as extracellular signal-regulated kinases (ERKs). Once activated, MAPKs translocate to the nucleus where they activate a variety of substrates. Ras signals through a number of effectors in addition to Raf, including Ral-GDS and phospho-3-inositol kinase (PIK).

Raf protein to the plasma membrane, where subsequent events lead to the activation of its kinase function. These events are complex and incompletely understood [37]. Raf-1 is only one member of a family of isoforms including A- and B-Raf, which can be regulated differentially by Ras [37]. Rap1 can inhibit Raf-1 and stimulate B-Raf activity [38]. Upon activation, Raf phosphorylates and activates [39] two MAPK kinases (MAP-KKs, also called MEK1 and MEK2). Activated MEKs which function as dual-specificity kinases phosphorylate tandem threonine and tyrosine residues (TEY motif) in two MAPKs (also referred to as extracellular signal-regulated kinases, ERKs), designated p42MAPK/ERK2 p44MAPK/ERK1, which activates them [40]. Once activated, MAPKs translocate to the nucleus where they phosphorylate and activate a variety of substrates, including the Elk1 nuclear transcription factor [41]. Elk1 forms a complex with serum response factor (SRF) at the serum response DNA element (SRE) present in many promoters, such as the c-fos promoter [42]. Ras signals through a number of effectors in addition to Raf isoforms, including the Ral-GDS [43], AF6 [44], Rin1 [45], Kinase Suppressor of Ras [46] and PI3K [47].

Recently, the Ras subfamily has been extended by new members like M-Ras, R-Ras [48], TC21 [49] and two phosphoproteins, pp21-1 and pp21-2 [50] involved in hexamethylene-bis-acetamide (HMBA)-induced differentiation of DS-19 erythroleukemia cells which are being further characterized. Murine erythroleukemia (MEL) cells deficient in cAMP-dependent protein kinase (A kinase) activity are impaired in chemically induced differentiation [51]. We identified two low molecular weight proteins (referred to as pp21-1 and pp21-2) by two-dimensional polyacrylamide gel electrophoresis, which are phosphorylated when parental MEL cells but not A-kinase-deficient MEL cells are treated with the membrane-permeable cAMP analog 8-bromo-cAMP [50]. We showed that pp21-1 and pp21-2 are direct A-kinase substrates, bind GTP and belong to the LMWG-superfamily of proteins.

Ras activation in malignant hematopoiesis

Ras mutations are found in a wide variety of human leukemias (table 2). In the absence of Ras mutation, alternative mechanisms of activation are operative in some leukemias. For instance, in chronic myelogenous leukemia (CML) where Ras mutations are rare [52, 53], the underlying molecular defect is the formation of the aberrant bcr-abl protein as a result of the Philadelphia t(9;22) translocation [54]. Recently, it was shown that constitutive Ras activation and reduced Ras-GAP activity are present in CML cells [55]. The mechanism by which Bcr-abl activates Ras-mediated pathways seems to be via interaction with the Grb2 adapter protein [55, 56]. In addition, Bcr can interact with Rho family members to regulate and coordinate signalling [57]. The importance of these pathways in CML is suggested by experiments demonstrating that suppression of Ras inhibits the transforming ability of Bcr-Abl in bone marrow cells [55]. In chronic myelomonocytic leukemia (CMML) patients, a translocation joining the platelet-derived growth factor receptor-β (PDGFR-β) to the transcription factor TEL has been described [58, 59]. PDGFR is known to elevate Ras-GTP levels in the presence of exogenous growth factor [52, 53]. It has been hypothesized that the aberrant fusion receptor (PDGFR-β-TEL) may still be capable of localizing to the plasma membrane [59], and once at its site of function the helix-loop-helix motif of Tel may aid in the dimerization of adjacent receptors [59, 60]. This might allow activation of Ras-mediated pathways in those CMML patients who lack Ras mutations.

In leukemias with a t(6;11) translocation, a fusion protein containing part of the AF-6 gene has been described [61]. AF-6 has been found to bind the Ras

Table 2. Ras mutations in hematopoietic malignancies*.

Myelodys plastic syndrome (all subtypes) Type of Hematological Disorder	% with RAS Mutations	RAS-gene mutated	References
	9-33	N-RAS	[101, 104]
chronic myelomonocytic leukemia	32–65	N- and K-Ras	[52, 53]
refractory anemia with excess blasts in transformation	26	N- and K-Ras	[53]
acute myelogenous leukemia	25–44	N- and K- Ras	[53, 67]
chronic myelogenous leukemia	Uncommon		[53, 67]
juvenile chronic myelogenous leukemia	21–30	N- and K-Ras	[148, 149]
acute lymphoblastic leukemia	6–18	N- and K-Ras	[75, 76]
chronic lymphocytic leukemia	Uncommon		[75]
multiple myeloma	9–39	N- and K-Ras	[150–152]
Hodgkin's lymphoma	Uncommon		[153]
non-Hodgkin's lymphomas	Uncommon		[70, 154]

* N-ras mutations are much more frequent in the above entities than K-ras mutations.

effector domain when Ras is in the GTP-bound, activated conformation, and this interaction can be inhibited by the cRaf-1 protein [62]. The significance of this finding in terms of Ras activation is currently unknown, but it is conceivable that alterations of AF-6 by translocation may interfere with its functional interaction with Ras. In addition to translocation, other types of genetic alterations may also produce constitutive Ras activation. Children with type 1 neurofibromatosis who have reduced function of neurofibromin, the product of the NF1 gene, are prone to malignant myeloproliferative syndromes. These include monosomy 7 syndrome as well as juvenile chronic myelogenous leukemia (JCML). NF1 contains a GAP-related domain that is thought to downregulate Ras by enhancing the intrinsic Ras GTPase [63]. JCML patients with a loss of NF1 have been found to have elevated levels of Ras-GTP [64]. In addition, mice heterozygous for an NF1 deletion develop a myeloproferative disorder that resembles human JCML [65]. In patients with JCML who do not have loss of NF1, Ras mutations are often present [66]. These data suggest that the central molecular feature in development of these childhood myeloproliferative disorders is Ras activation, which may occur because of mutation or because of loss of the NF1 tumor suppressor. Finally, in acute myeloid leukemia (AML), polymorphisms and possible somatic mutations in the N-ras promoter have been identified by one group [67].

Ras activation in acute leukemias: incidence and timing of point mutations

Among hematological neoplasms, activation of ras genes by heterozygous somatic point mutations of either codon 12, 13 or 61 is most frequent in multiple myeloma and in AMLs. In a large series of adult patients with AML, the incidence of N- or K-ras point mutations is in the range of 20–45% [68–72]. A similar pattern of mutations is found in childhood AML, but their incidence is lower than in adults (3–24% [73–76]). Compared with adult AML, ras mutations are overall less frequent (6–20%) in acute lymphoblastic leukemia (ALL) [77–80]). In both types of acute leukemia, these alterations mostly involve the N-ras gene, less frequently K-ras, and only rarely H-ras. It is well-established that one or even several different ras mutations can be present in independent subclones of the leukemic blasts in AML, implying these mutations not as primary genetic events during leukemogenesis but as postinitiation lesions [81–83]. This is supported by the observation that AML patients with a known ras mutation at initial diagnosis who achieve remission following chemotherapy may relapse with a leukemic clone not harboring the initial activating ras mutation [83].

To address the question of the timing of ras mutation during leukemogenesis on a clonal level, Bashey et al. utilized clonogenic assays combined with a mutation analysis detecting two different N-ras point mutations [84], combined with fluorescent in situ hybridization (FISH) for detection of trisomy of chromosome 8. The latter was employed in an elegant study of a patient with AML and the presence of both genetic alterations in the leukemic blasts was established [85]. Whereas all analyzed colonies were composed of cells, which were trisomic for chromosome 8, only a fraction was positive for one of the two N-ras mutations detected in bulk leukemic cells. However, none of the colonies harbored the second mutation, also indicating that trisomy 8 was acquired before the N-ras mutation during the course of disease [85].

Whereas the incidence, patterns and sequence of acquisition of these activating mutations are thus well-established for AML, the results of several large observational studies regarding correlation with leukemic phenotype and prognosis of AML are conflicting. In earlier studies, a possible association of Ras activation with a myelomonocytic or monocytic leukemic phenotype in AML had been reported, but was not found in several larger patient series. Similarly, the clinical impact of the presence of ras mutations on prognosis in AML has not been settled, with two studies reporting a better response of AML with N-Ras activation to chemotherapy [86, 87] and other studies reporting no influence [88] or a worse prognosis [89]. These conflicting results may be due to different patient populations, treatment approaches and possible variability in the methods of mutation detection, particularly with regard to sensitivity of the assays used. Only two large studies have addressed the role of ras mutations in the prognosis of childhood ALL. Of 100 children analyzed in our study, 6 had an activating mutation, which was associated with a significantly increased relapse rate [78]. In a second study involving 125 cases, 14 mutations were found, but no impact upon the event-free survival rate was noted [80]. Considering different leukemia treatment in these two studies, and the overall low incidence of ras mutations in this disease, meta-analyses are necessary to settle the question of the prognostic value of ras mutation [90].

Functional role of activated Ras in growth and differentiation of myeloid leukemic cells

In view of the 'subclonal' nature of ras mutations often involving a subpopulation of the leukemic cells in AML, several groups have performed analyses of clonal growth of myeloid leukemia cells under semisolid media conditions. Leukemic progenitors are thus detected as

AML colony-forming units (AML-CFU). The aim of these studies was to dissect the functional role of activated Ras upon the growth and differentiation pattern of colonies grown from single cells. Bashey et al. [84] applied an elegant culture system of selection for both AML-CFU as well as more immature leukemic progenitor cells (resulting in macroscopically visible colonies, AML-MCFU) to address the question of the maturation stage of the leukemic cells bearing the mutated allele. Of 10 patients studied, mutation analysis revealed activated as well as wild-type ras alleles in different colonies, with the proportion of colonies bearing no mutation varying between 5 and 57%. Among 4 patients having two different mutations, these were detected in different subclones, and using the AML-MCFU, two mutations (N12Cys, N12Asp) were found exclusively in the AML-MCFU but not the AML-CFU. When utilizing the AML-CFU assay on cells from 2 patients with subclonal presence of N-ras mutations, and under culturing conditions favoring either growth-factor-independent colony growth (no cytokines added to the semisolid media) or growth-factor-dependent growth (IL-3, GM-CSF and G-CSF added), we also observed the growth of colonies bearing either wild-type or mutated ras alleles [71, 91]. Interestingly, in one of these patients a limited number of colonies grew in the absence of additional growth factors. All of these colonies were negative for the N-ras mutation. Thus, activated ras in the leukemic cells from this patient was not necessary for the ability of leukemic cells to form growth-factor-independent colonies. In the presence of growth factors, colonies were readily generated from both patients, and ras mutations versus wild-type N-ras alleles were detected in about equal numbers of colonies. When colonies were scored for their composition (content of blast cells vs. mature cells), the majority of colonies bearing ras mutations were composed of mature elements [91]. Conversely, the majority of colonies lacking the N-ras mutation were composed of blasts. This suggests that in these patients, the subclone bearing activated ras may have a higher propensity for in vitro differentiation than the (initial) leukemic clone lacking the mutation.

Comparative studies of monoblastic U-937 cells (growth-factor-independent proliferation in culture) and early myeloblastic/erythroblastic TF-1 cells (growth-factor-dependent) transfected with an activated H-ras construct also revealed that presence of mutated Ras protein may indeed result in differentiation or enhanced proliferation depending on the cellular background. Whereas in U-937 cells a marked inhibition of growth together with monocytic maturation was noted, expression of the same construct in TF-1 cells resulted in factor-independent growth, as well as enhanced proliferation in the presence of GM-CSF or erythropoietin, but the maturation stage of these cells remained unchanged [92]. On the basis of Ras having been implicated in at least one signal transduction pathway upstream of hematopoietic growth factor expression, it was hypothesized that induction of cytokine expression, presumably resulting in autocrine growth stimulation and growth-factor-independent proliferation, could be a downstream effect of activated Ras. This hypothesis has been tested in several different hematopoietic and mesenchymal cell lines and for different cytokines [interleukin (IL)-1β, IL-3, IL-6, G-CSF, GM-CSF]. In an elegant study using human fibroblasts and mesothelioma cells transfected with a mutant ras (H-12val and N-12asp, respectively), Demetri et al. demonstrated an increased expression of messenger RNAs (mRNAs) coding for G-CSF, GM-CSF and IL-1β [93]. Nuclear 'run-on' and mRNA half-life studies were employed to clarify the mode of upregulation, which was shown to be transcriptional for IL-1β, and posttranscriptional for the other three cytokines as well as for IL-6.

In an attempt to correlate the presence of N-ras mutations with cytokine expression in primary AML, we determined the patterns of N-ras mutations in peripheral blood or bone marrow samples from 50 patients with AML and known patterns of cytokine expression (IL-6, G-CSF, GM-CSF, M-CSF, IL-1β, TNF-α). Whereas the overall incidence of activated N-ras in this patient group was 44%, the respective mutations were prevalent in the large majority of cells of only 6 patients (12%), whereas in the other samples, only a subclone(s) of leukemic cells bore the respective mutation. Of the 6 patients with activated ras in a large proportion of leukemic cells, expression of G-CSF, TNF-α and IL-6 was observed in 2, 2 and 6 cases, respectively, with GM-CSF, M-CSF and IL-1β all being expressed by 1/6 cases. Sample size was limited in this study due to the necessary exclusion of patients with only subclonal presence of N-ras mutation (constituting heterogeneous cell populations not allowing direct inference of growth-factor synthesis). The biological significance of these associations is at present unclear.

However, Moroni et al. have established a model of Ras-mediated growth-factor independence of myeloid cells. By using the IL-3-dependent PB-3c murine mast cells transduced with a v-H-ras or activated human H-ras, they observed a reversible abrogation of growth-factor dependence of these cells [94]. The effect of the Ras activation was transcriptional or posttranscriptional depending upon integration of the viral ras within the IL-3 gene locus [95]. IL-3-independent growth was also mediated by activated H-ras in diploid murine myeloid progenitor FDC-P2 cells [96]. In IL-3-dependent 32Dc13 cells, inactivation of (wild-type) H-ras by a dominant inhibitory mutant blocks proliferation of these cells [97]. In addition, autocrine IL-1

production in leukemic cells has very recently been functionally linked to mutated ras in two leukemic cell lines, giving further support to the notion of ras activation in AML resulting in uncoupled proliferation of these cells [98].

Several studies have been performed to determine the effect of activated N-ras upon IL-6 expression and the growth characteristics of myeloma cells. Expression of the mutant ras in ANBL6 cells resulted in IL-6-independent growth, since neutralizing antibodies against IL-6 did not negatively affect their enhanced proliferation compared with the native ANBL6 cells [99]. Interestingly, when comparing different activated N- and K-ras transfectants, K-ras expression in these cells resulted in growth inhibition, which may be due to enhanced apoptosis [100].

Ras activation in MDSs

Myelodysplastic syndromes (MDSs) are a heterogeneous group of clonal hematopoietic disorders resulting from transformation of an early hematopoietic progenitor cell. The clinical hallmarks are cytopenia of usually two or all three peripheral blood cell lineages in the presence of a hypercellular bone marrow. In addition, a variable 'preleukemic' blast expansion is present in less than 40% of MDS cases. Cytogenetic abnormalities, which are quite similar to those observed in AML of the elderly, are present in about 50% of MDS.

The spectrum of ras mutations in MDS is also similar to that of AML, but large patient series have shown that the incidence is lower than in the former, being in the range of 10–30% [101, 102]. This somewhat lower incidence in a preleukemic disorder is quite compatible with the fact that only portions of MDS cases, particularly those with highest blast excess and chromosomal abnormalities, have a high risk of progression to AML within 0.5–2 years. Most lineage studies have shown the ras mutations to be restricted to the nonlymphoid cell fraction, which is in line with numerous clonality studies in MDS demonstrating that lymphoid cells are not part of the transformed clone in MDS [103]. As in AML, mutations may be restricted to subclones even within the myeloid cell fraction [75, 104] and may thus not be the initiating event during transformation.

PCR offers the opportunity to retrospectively study kinetics of mutational activation in peripheral blood and bone marrow smears from archival samples of AML and MDS [105, 106]. Longitudinal analysis revealed that the appearance of the N-ras mutation in MDS can be quite variable and can persist for up to 5 years without the emergence of AML. However, several large studies have established that the presence of ras mutations is an independent predictor of poor prognosis in MDS [106]: presence of an N-ras mutation is associated with a decreased survival and an increased risk of progression to AML.

Animal models of activated ras inducing a myelodysplastic and myeloproliferative phenotype

Two murine models addressing the effects of a mutated ras on hematopoietic cells have been established. Results of both studies are compatible with the mutant ras, inducing a dysplastic phenotype of the erythropoietic lineage and myeloid lineage, respectively. Darley et al. expressed a mutated N-ras gene in murine CD34-enriched cells transduced with amphotropic retrovirus and studied the proliferation, differentiation and apoptosis of these cells [107]. They observed a proliferative effect mediated by the mutant ras as shown by an increased doubling time and a decrease of cells within the S and G2/M phase of the cell cycle. Similarly, they noted a decrease in differentiation and an increase in apoptosis. Dysplastic features of these erythroid progenitors were reminiscent of MDS.

MacKenzie et al. also developed a model of mutated N-ras in the myeloid lineage. Retrovirally transduced murine bone marrow cells expressing activated Ras were transplanted into lethally irradiated mice [108]. They observed variable hematopoietic disturbances in the regenerated bone marrows of the majority of animals, which were reminiscent of myeloproliferative syndromes of MDS, and of AML, respectively. An increased rate of apoptosis was also noted in this model. The lack of development of acute leukemia in the majority of animals, however, indicates secondary events as being necessary for transformation to acute leukemia. To our knowledge, no studies using leukemogenic agents on these mice were published.

Regulatory proteins

As described above, the activation state of LMWGs is determined by the nucleotide (GDP or GTP) bound to the protein, and this in turn is determined by both the rate of GDP release and the rate of GTP hydrolysis. Therefore, much research on the regulation of the LMWGs has focused on the identification and characterization of factors that interact with LMWGs, since their activity must be enhanced by external regulatory proteins to ensure timely inactivation of these molecular switches [109]. The first LMWG regulatory molecule to be identified was p120 Ras GAP [110]. This protein interacts with the effector domain of GTP-Ras and is able to stimulate GTP hydrolysis by Ras [109]. Subsequent to the identification of Ras GAP, GAPs have been identified for a number of other LMWGs, includ-

ing Rap GAPs, Rho/Rac GAPs, Ral GAPs and Rab GAPs [109].

Recently, a number of proteins have been identified that activate LMWGs by facilitating the exchange of GDP for GTP (i.e. GDSs) (reviewed in [111]) GDSs bind to the GDP-bound form of the LMWG and facilitate or enhance the release of GDP [111], which is normally very slow in the cell, and a stable complex of GDSs associated with the LMWG remains [112]. GTP then binds rapidly to the empty nucleotide-binding site, and the associated GDS is released [111]. A number of mammalian Ras GDSs have been identified [111], including Ras-GRF, a homolog of yeast CDC25 [113]; mSOS-1 and mSOS-2, homologs of *Drosophila* Sos [114].

The role of mSOS in activation of the Ras signalling pathway has recently been elucidated through the concerted effort of a number of groups (reviewed in [115]) who found that recruitment of SOS1 to the plasma membrane facilitated Ras activation and subsequent activation of downstream signalling pathways. In support of this model Quilliam et al. [116] and Aronheim et al. [117] recently showed that membrane targeting of SOS was sufficient for Ras activation, and proposed that Ras function was controlled by the dynamic interaction of its regulatory proteins with the plasma membrane where Ras is located. In any case, these regulatory proteins would also participate in the Ras-mediated signal transduction found in myeloid cells (see below for a discussion of the role of Ras pathways in myeloid cells).

Vav is ubiquitously expressed in hematopoietic cells and appears to play a role in B- and T-cell-receptor activation [118, 119]. In T cells, Vav is stimulated by T cell antigen-coupled protein tyrosine kinase phosphorylation [119] as well as by lipid second messengers such as diacylglycerol [120]. This may allow T cells to integrate signals from distinct protein tyrosine kinase-dependent and -independent receptors to activate Ras. It should be noted however, that recent studies by Bustelo et al. [121] suggest that Vav does not actually function as an upstream regulator of Ras but rather acts by a distinct mitogenic pathway that is interconnected with the Ras mitogenic pathway. It was found that Vav2 is an activator of Cdc42, Rac1, and RhoA [122].

GDSs have been identified for many of the other subclasses of LMWGs, including Ral, Rap, Rho, Rab and Ran [19]. Of particular interest to myeloid signal transduction is smgGDS (smg refers to small GTP-binding protein), whose broad range of specificity includes Rap, K-Ras, Rho and Rac proteins [123, 124]. Smg GDS binds stoichiometrically to these isoprenylated LMWGs but apparently does not distinguish between the various types of isoprenoid groups (e.g. smg GDS binds to farnesylated K-Ras as well as geranyl-geranylated Rho, Rac and Rap) [124] and may use other structural clues on LMWGs to distinguish between appropriate and inappropriate substrates [23, 124]. It is thought that smg GDS plays a role in regulating the intracellular localization of LMWGs by covering or masking their isoprenylated carboxyl termini, thereby allowing them to be released into the cytosol [4, 125].

GDP-dissociation inhibitors (GDIs) function by inhibiting GDP dissociation from their target LMWGs [19]. To date, GDIs have been identified for the Rab [126] and Rho/Rac LMWG subfamilies [127, 128]. However, it is likely that other classes of GDIs will also be identified. These proteins are cytosolic proteins that bind stoichiometrically to the GDP-bound forms of their respective targets [128]. In addition, GDIs have a wide range of binding specificity and appear to interact with more than one member within a particular LMWG subfamily [4, 15]. For example, Rho GDI interacts with Rho, Rac and CDC42Hs [129–131]. Recent studies suggest that GDIs can also facilitate the release of LMWGs from membrane locations in the cell in a process similar to that described for smg GDS above [129]. A number of posttranslationally modified LMWGs have been identified in cytosolic locations complexed with GDIs, including Rho and Rac [4, 129, 130], and it is thought that this ability of GDI to solubilize LMWGs may play a role in regulation by facilitating a membrane association-dissociation cycle for these proteins [131, 132]. The ability to modulate intracellular localization could then be used to regulate the amount of LMWG available for functional activity in the membrane as well as facilitating these proteins among various intracellular membranes [130–133].

Rap: the suppressor of K-ras transformation

Rap1 (Krev1) is a small GTPase first identified as a transformation suppressor of K-Ras based on its homology to a *Drosophila* Ras-related protein [134]. Four forms of Rap have been identified: Rap1a, Rap1b, Rap2a and Rap2b, which are highly homologous. Rap1 is very similar to Ras (53% homology), particularly in the effector region. This could account for the antagonistic effects of Rap on Ras-mediated signalling in overexpression studies. However, recent data indicate that activation of endogenous Rap1 does not necessarily impair Ras-mediated signalling [135]. Novel assays based on the measurement of bound GTP and GDP to determine Rap activation [136, 137] might help to elucidate its function. Rap can act independently of Ras, for example through activation by cell adhesion and a variety of second messengers and growth factors. Rap-GEFs can be regulated by cAMP [13, 14] and calcium [12]. Rap-GAPs can bind to heterotrimeric G proteins

[10, 11]. Nitric oxide-dependent differentiation of HL-60 cells is mediated by Rap1a [28], and a defect in posttranslational modification [29] might play a role in the differentiation block of nitroprusside-resistant cells. Variant HL-60 cells resistant to differentiation induced by nitroprusside and cyclic GMP (cGMP) analogs have normal guanylate cylase and cGMP-dependent protein kinase (G-kinase) activity [138]. We found decreased phosphorylation of a low molecular weight protein (PP23) in the variant cells [28]. By comigration on two-dimensional polyacrylamide gels, phosphopeptide mapping, immunoprecipitation and immunoblotting, we were able to show that pp23 was one of three posttranslationally modified forms of Rap 1a expressed in HL-60 cells. Using an in vitro transcription/translation system, we studied each of the posttranslational processing steps of Rap1a and showed that pp23 represented fully processed Rap1a. By immunoprecipitation, immunoblotting and ^{35}S-methionine/cysteine incorporation, the variant cells were deficient in pp23, and thus in fully processed Rap 1a, but expressed normal amounts of completely unprocessed Rap 1a and geranyl-geranylated Rap 1a. The lack of Rap 1a processing beyond geranyl-geranylation in the variant cells was not secondary to a change in Rap 1a amino acid sequence. The variant cells had normal carboxyl methyltransferase activity, suggesting they are deficient in proteolytic cleavage of Rap 1a. Further experiments are needed to characterize the protease responsible for this posttranslational modification [29].

Tuberous sclerosis (TSC) is a human genetic syndrome characterized by the development of benign tumors in a variety of tissues, as well as rare malignancies. Two different genetic loci have been implicated in TSC. One of these loci, the tuberous sclerosis-2 gene (TSC2) encodes an open reading frame with a putative protein product of 1784 amino acids [139]. The putative TSC2 product tuberin contains a region of limited homology with the catalytic domain of Rap1GAP. Tuberin stimulates the intrinsic GTPase activity of Rap1a but not Rap2 and Rho [139]. These results suggest the loss of tuberin leads to constitutive activation of Rap1 in tumors in patients with tuberous sclerosis, indicating that Rap 1 is not only an antagonist of Ras but has an independent function in signal transduction. Recently, binding of tuberin to Rab5 has been observed, which would allow an alternative mechanism [140].

The Rho subfamily of LMWGs

A recent study has demonstrated a role for Rho in T cell development. Transgenic mice that lacked Rho function by targeting of a transgene encoding *Clostridium botulinum* C3-transferase, which specifically inhibits Rho proteins to the thymus, displayed maturational proliferative and cell survival defects [141]. Thymi were strikingly smaller, and the generation of thymocytes and mature peripheral T cells was severely impaired. However, analysis of the maturation stages of thymocytes lacking Rho function showed the existence of a subpopulation of thymocytes representing all stages of thymocytic development, although they were severely reduced in numbers. Thus, differentiation of progenitor cells to mature T cells could occur in the absence of rho function. It has been proposed that the decrease in cellularity in thymi lacking rho function could be attributable to the failure of thymocytes to traverse through the G1 phase of the cell cycle [141]. Thus Rho signalling appears to be selectively required in early development for proliferative expansion and survival of thymocytes. However, there seem to be different functions of the GTPase Rho in prothymocytes and late pre-T cells [142]. Loss of Rho function in the thymus is accompanied by the development of thymic lymphoma [143].

The ARF subfamily of LMWGs

The ADP-ribosylation factor (ARF) proteins are another group of LMWGs that have been implicated along with the Rab proteins in the regulation of intracellular vesicle transport [144]. ARF was first discovered as a membrane-bound cofactor for cholera-toxin-dependent ADP ribosylation of Gs-α (ARF), and is a 21-kDa protein with homology to Ras. ARF also exhibits structural features similar to heterotrimeric Gs-protein α-subunits, and it has been proposed that ARF represents an intermediate type of LMWG with characteristics of Ras-related proteins and heterotrimeric G proteins. ARF is located in the Golgi complex in mammalian cells and is required for vesicle transport from endoplasmic reticulum to Golgi membranes, a process which requires multiple GTP-binding proteins, including ARF, Rab and heterotrimeric G proteins. In addition, ARF is a component of non-clathrin-coated vesicles, where it is thought to be involved in vesicle formation and coat assembly. Thus ARF LMWGs play important roles in the regulation of the transport of proteins between early compartments of the secretory pathway. In myeloid cells ARF has recently been shown to participate in the regulation of phospholipase D activity. As described above, phospholipase D acts as an important signal-transducing enzyme in myeloid cells and plays an important role in the activation of the human phagocyte nictotinamide adenine dinucleotide phosphate (NADPH) oxidase and the secretory process.

Perspective

The generation of transgenic mouse models and transgenic knockout mouse models will allow us to find out how extended the redundancy is within each Ras family and which small G proteins are essential for vital cellular functions. Knockout mice for H-ras [145], N-Ras [146] and K-Ras [145] have been developed. In contrast to the findings that H-Ras-deficient mice and N-Ras-deficient mice are born and grow normally, the K-Ras-deficient embryos die progressively between embryonic day 12.5 and term, indicating that the different forms of Ras are not totally redundant. The generation of Rap1-deficient mice will especially assist in the study of the multifunctional role of this protein. The combination of these mouse models with improved methods to determine the activation of various LMWGs [141, 144] will permit new insights into the role of GTP-binding proteins in signal transduction in hematopoietic cells. There is increasing evidence that growth inhibition by farnesyltransferase inhibitors is mediated by accumulation of alternate prenylated (geranyl-geranylated) RhoB, which induces elevation of the cell cycle kinase inhibitor p21WAF1 [147]. Preclinical data suggest that efficacy of farnesyltransferase inhibitors may be maximized by combinatorial application with signal transduction inhibitors that affect integrin signals. Finally, this knowledge should allow us to design more specific drugs against hematopoietic malignancies.

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