Cellular functions of the Rap1 GTP-binding protein: a pattern emerges

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Summary

Rap1 belongs to the Ras subgroup of small GTP-binding proteins. Whereas its early history has focused on its biochemical homology to Ras and the alleged functional antagonism between these two small GTPases, recent cellular evidence suggests that endogenous Rap1 plays a unique, Ras-independent role in eukaryotic cells. Activated by virtually all receptor types and second messengers, Rap1 controls adhesion-related functions such as phagocytosis, cell-cell contacts and functional activation of integrins through inside-out signalling. Whereas the precise mechanism by which its downstream effectors exert these diverse functions is unknown, Rap1 seems to fulfil the evolutionarily conserved function of patterning the eukaryotic cell, thus enabling it to respond to its environment, in particular through cytoskeletal remodelling.

Key words: Rap1, Adhesion, Integrin, Small GTP-binding proteins

Introduction

The Ras superfamily of GTP-binding proteins comprises over 100 members, all functioning according to a unique biochemical paradigm. Activated in response to upstream signals, small GTPases act as molecular switches (ON when GTP-bound, otherwise OFF) and control a variety of essential cellular processes in eukaryotic cells. Active, GTP-bound Ras proteins interact with specific downstream effectors, that is, signalling or structural proteins that are ultimately responsible for mediating their cellular effects. Small GTP-binding proteins are generally grouped into five subfamilies of structurally and functionally related proteins named after their prototypical member: Ras, Rho, Ran, Rab and Arf. Each subfamily is thought to control a key cellular process (e.g. cytoskeletal organization for the Rho subfamily and vesicular transport for the Rab subgroup) (Takai et al., 2001).

Rap1 belongs to the Ras subfamily of small GTP-binding proteins, which is considered to control cell growth, differentiation and survival. The Ras subfamily consists of 19 members, the best characterized of which are further divided into the classic Ras (H-Ras, K-Ras, N-Ras), R-Ras, Ral and Rap groups. The Rap subgroup contains two pairs of quasi-identical GTP-binding proteins (Rap1A and Rap1B; and Rap2A and Rap2B), which differ by only a few residues (95% and 90% identical at the amino acid level, respectively). By contrast, Rap1 and Rap2 proteins are only 60% identical to each other, showing noticeable variations at their C-terminus (Reuther and Der, 2000; Takai et al., 2001). This could impact on their subcellular localization and also explain their different sensitivities to RapGEFs (Rap guanine-nucleotide-exchange factors) or their distinct profiles of downstream targets (e.g. Van der Berghe et al., 1997; Janoueix-Lerosey et al., 1998).

Historically, our understanding of the cellular function of individual Ras subfamily members has been shaped mainly by comparisons with H-Ras or K-Ras. Rap1 is no exception, and it took about 15 years to dissociate the cellular functions of Rap1 and Ras. Three or four major findings have led to our current – incomplete – understanding of Rap1 function. Human *RAP1* was originally cloned by virtue of its homology with the *Drosophila RAS3* allele (Pizon et al., 1988), later renamed *Drosophila RAP1*. The following year, *Rap1* was found to be identical to *Krev-1*, a cDNA able to revert the phenotype of *K-Ras*-transformed cells (Kitayama et al., 1989). One hypothesis advanced at the time to explain this major finding was that the sequence-related, 53% identical, Rap1 and Ras compete for downstream targets. Overexpressed Rap1was soon found to be unable to activate Raf-1, contrarily to Ras (Cook et al., 1993). Seemingly confirming the original postulate, this result quickly led to the persistent view that Rap1 is merely a Ras antagonist that forms non-productive complexes with Ras effectors. Nevertheless, recent biochemical, cellular and developmental evidence unambiguously reveals that Rap1 has at least one Ras-independent function in a variety of cellular systems: the control of adhesion-related events.

Rap1 biochemistry, as well as the trail of discoveries leading to the recognition of a positive, Ras-independent role for Rap1 and some of the persisting ambiguities in the field have been well analyzed recently (Bos et al., 2001). Here, I only briefly summarize the essential biochemical features of Rap1, focusing instead on its specific cellular functions. A variety of key findings obtained in the past three years suggests a conserved role for Rap1 in integrating extracellular signals to allow the organization of cell architecture.

Rap1: a close relative of Ras, but with a distinctive character

The cellular function of a protein reflects its biochemical

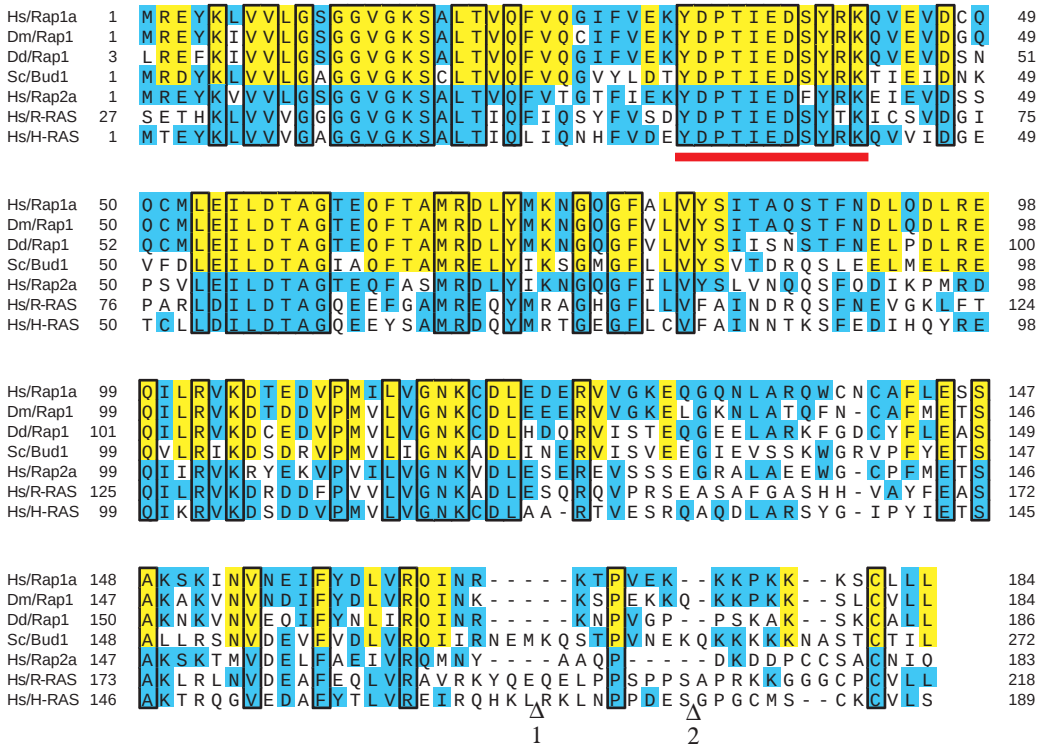

Fig. 1. Amino-acid sequence of the human (Hs) Rap1a GTP-binding protein compared to the primary structures of its *Drosophila melanogaster* (Dm), *Dictyostelium discoideum* (Dd) and *Saccharomyces cerevisiae* (Sc/Bud1) homologs and three other human Ras subfamily members: Rap2a, R-Ras and H-Ras. Boxes define regions of identity between all sequences, whereas the overall homology is shown in blue. Notice the total identity between Rap1 homologs and Ras in the main effector-binding region, delineated by the red bar (amino acids 32-42). Also of note is the high degree of conservation in primary sequence amongst the 4 Rap1 homologs, with homology indicated in yellow. Significant homology is defined here as a score of over 85% in the Dayhoff PAM250 scale. For commodity, several stretches of sequence have been deleted from this alignment. These include the two N-terminal residues in Dd Rap1, the first 26 amino acids in R-Ras and two regions unique to Bud1. Arrowheads indicate the position of these latter two deletions, which correspond respectively to amino acids 171-226 (1) and amino acids 236-258 (2).

properties and its subcellular distribution, both of which combine to determine the range of molecules it interacts with in vivo. As shown in Fig. 1, Ras and Rap1 are extremely similar at the amino-acid level, which at first glance suggests that they have the same biochemical properties and related cellular functions. The regions of highest sequence similarity correspond to the switch 1 and switch 2 regions, that is, the two regions that adopt different conformations when bound to GDP or GTP. Remarkably, the so-called effector region is absolutely identical in the two molecules, which suggests that Ras and Rap1 share the same effectors. Although qualitative studies (e.g. yeast two-hybrid analysis) have seemed to validate this hypothesis, the in vitro affinities of Rap1 and Ras for a given target (e.g. Raf-1 or RalGDS) differ dramatically (Vetter et al., 1999). Moreover, exquisite FRET experiments using RAICHU (Ras GTPase and interacting protein chimeric unit) constructs recently revealed that growth-factor-induced activation of Rap1 and Ras occurs at distinct subcellular locations. Whereas Ras is converted to the GTP-bound form at the plasma membrane, Rap1 activation takes place in the perinuclear region both in PC12 and COS-1 cells (Mochizuki et al., 2001). These data are in agreement with earlier findings showing distinct cellular distributions for Ras and Rap1 (Béranger et al., 1991). Therefore, despite the strong homology between Ras and Rap1, both their different affinities for downstream effectors and their subcellular distributions argue for distinct biological functions.

The number of reports on Rap1 has increased exponentially in recent years, leading to the realisation that a considerable variety of stimuli acting through the whole spectrum of signalling pathways activate Rap1 (Fig. 2). The latest additions to this list include adhesive surfaces (Ohba et al., 2001), ephrin and ephrin kinases (Prevost et al., 2002), adenosine diphosphate (Woulfe et al., 2002; Lova et al., 2002), the NMDA neurotransmitter (Zhu et al., 2002), hyperosmotic and cold stresses (Kang et al., 2002), interleukin-1 (Palsson et al., 2000), stromal cell-derived factor-1 [SDF-1 (McLeod et al., 2002)], CD98 crosslinking (Suga et al., 2001), CD31 crosslinking (Reedquist et al., 2000), lipopolysaccharide (Caron et al., 2000; Schmidt et al., 2001) and antigen-loaded antigen-presenting cells [APCs (Katagiri et al., 2002)]. R-Ras, another member of the Ras subfamily of small GTP-binding proteins that has been implicated in the control of integrin-mediated adhesion (Zhang et al., 1996), was recently linked to Rap1. Although it remains to be established whether active R-Ras (or an elusive R-Ras activator) is able to activate Rap1 in cells, RapGAP expression blocks R-Ras-induced effects in several cell types, which suggests that Rap1 acts downstream of R-Ras (Self et al., 2001). The parallel discovery of numerous, evolutionarily conserved RapGEFs able to relay signals from extracellular stimuli and second messengers to Rap1 (reviewed in Bos et al., 2001; Gao et al., 2001), as well as specific RapGAPs (Polakis et al., 1991; Kurachi et al., 1997; Chen et al., 1997; Pak et al., 2001), reinforced the possibility that Rap1 has a central function in signal transduction processes.

Rap1 has multiple cellular roles, most of which are adhesion related

Rap1 fulfils an essential function in eukaryotes, as proven by the embryonic lethality of loss-of-function *RAP1* alleles in

Drosophila (Hariharan et al., 1991) and mice lacking C3G (a Rap1 exchange factor) (Ohba et al., 2001). This crucial role is mirrored at the cellular level, and Rap1-deficient mammalian and slime mold cell lines are impossible to establish (Ribeiro-Neto et al., 2002; Kang et al., 2002). Most of the several functions proposed for Rap1 relate to cell adhesion (see Fig. 3). There are, however, some Rap1 functions that do not appear to be related to adhesion. Whether these will prove to be dependent on some form of adhesion or consequences of an elusive core role on which all Rap1 functions depend remains an unanswered question.

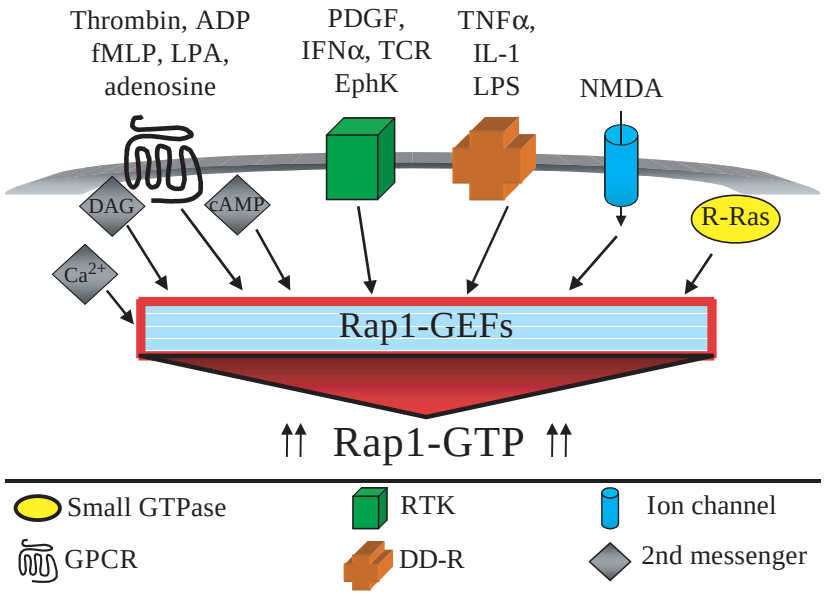

Fig. 2. Many receptors and second messengers are coupled to the activation of Rap1 guanine nucleotide exchange factors (Rap1GEFs), and an increase in the cellular levels of active, GTP-bound Rap1. Abbreviations used in this figure: ADP, adenosine diphosphate; fMLP, formyl-methionine leucine phenylalanine; LPA, lysophosphatidic acid; DAG, diacyl glycerol; PDGF, platelet-derived growth factor; IFN-α, alpha-interferon; TCR, T cell receptor; EphK, ephrin kinase; TNFα, tumor necrosis factor alpha; IL-1, interleukin 1; LPS, lipopolysaccharide; NMDA, N-methyl-D-aspartate; GPCR, G-protein-coupled receptor; RTK, receptor tyrosine kinase (including kinase-associated receptors); DD-R, death-domain-associated receptor. The corresponding bibliographic references are given in the text body.

Adhesion-independent functions

Rap1 signalling has contrasting effects on cell growth and proliferation and activation of the ERK (extracellular-signal-regulated kinase) MAP kinase pathway. Rap1 activation potentiates the response to mitogenic stimuli in thyroid follicular cells and antigen-challenged thymocytes (Ribeiro-Neto et al., 2002; Sebzda et al., 2002), and links cyclic AMP signalling to the regulation of ERK activity and cell proliferation (Schmitt and Stork, 2001; Ribeiro-Neto et al., 2002; Lou et al., 2002; Laroche-Joubert et al., 2002). Whether Rap1 activates (e.g. in neuronal and neuroendocrine cells), inhibits or has no effect on Erk activation, however, seems to depend on the cell type and the experimental conditions used (Bos et al., 2001; Schmitt and Stork, 2001; Sebzda et al., 2002; Klinger et al., 2002; Laroche-Joubert et al., 2002).

Another seemingly adhesion-independent function of Rap1 is exemplified by yeast cells, where the Rap1 homolog Bud1p/Rsr1p controls bud-site selection and interacts with upstream regulators of the actin cytoskeleton (Gulli and Peter, 2001). Bud1p is localised on internal membranes and at the plasma membrane, where it is enriched at sites of polarized growth and budding (Park et al., 2002). Local enrichment at the plasma membrane could be specified by Bud5p, the yeast RapGEF, which is recruited to the bud site in a cell-cycle-dependent way. Bud5p recruitment itself is driven by other proteins, noticeably by Axl2p/Bud10p, a transmembrane protein and Bud5p-binding partner thought to act as an internal spatial landmark (Kang et al., 2001).

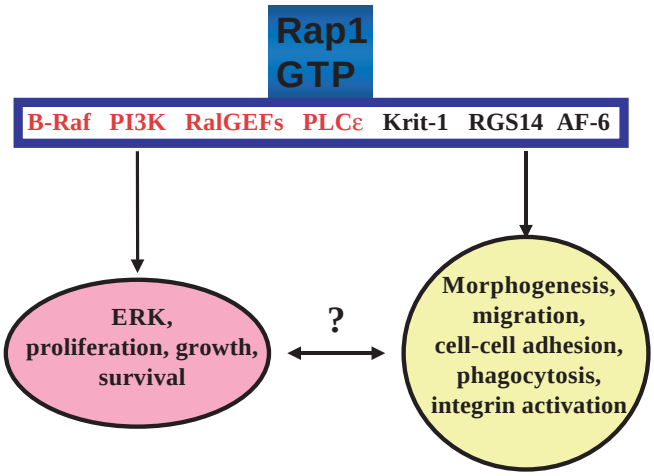

Fig. 3. Rap1 downstream effectors and proposed roles in mammalian cells. Adhesion-dependent and -independent functions are separated for simplicity but could prove related, as discussed in the text. The Rap1 targets indicated in red have been ruled out from playing a role in Rap1-mediated integrin activation.

Adhesion-related functions

The evidence implicating Rap1 in the control of cell adhesion is compelling and extends from *Dictyostelium* to mammalian cells. Krev-1 was first characterized as a cDNA inducing 'flat', that is, morphologically non-transformed, strongly adherent revertants in K-Ras-transformed cells (Kitayama et al., 1989), and it is now well established that interfering with Rap1 GTP cycle has profound effects on a wide range of adhesive processes: morphogenesis, phagocytosis, cell-cell adhesion, cell migration and spreading.

Overexpression of membrane-targeted RapGEFs or active forms of Rap1 induces cell spreading in 293T cells, whereas low levels of active Rap1, achieved through overexpression of either RapGAP or the dominant-negative mutant N17Rap1 cause cell rounding (Tsukamoto et al., 1999). Similarly, the activated V12Rap1 mutant induces spreading of *Dictyostelium*

cells (Rebstein et al., 1997) and the integrin-dependent spreading of a variety of haematopoietic cells (see below). As expected, Rap1 has opposite effects on spreading and cell motility, and mouse embryo fibroblasts derived from C3G-knockout animals show delayed cell spreading and increased motility, both of which are suppressed by overexpressed active Rap1 (Ohba et al., 2001).

The profound influence of Rap1 signalling on cell morphology is also evident in vivo: Rap1 mutations disrupt normal cell shape and morphogenesis in the eye, ovary and wing of *Drosophila* embryos (Hariharan et al., 1991; Asha et al., 1999; Knox and Brown, 2002). The most recent data suggest that Rap1 regulates the position of adherens junction markers (DE-cadherin, ZO-1 and canoe/AF-6, a downstream target of Rap1) at the apical face of the epithelium lining the wing. Remarkably, in Rap1-mutant cells, the cell junction proteins concentrate on one side of the cell, whereas Rap1-GFP is enriched at the apical junctions in wild-type cells. Interestingly, Rap1GFP is also preferentially recruited to the boundary between the two daughter cells after cytokinesis; this is reminiscent of the *S. cerevisiae* situation, where the Rap1 exchange factor Bud5p is enriched at the cell division site (Kang et al., 2001). Finally, Rap1 controls several specialized types of cell-cell adhesion in immune cells, such as the immunological synapses that form at the interface between T cells and APCs (Katagiri et al., 2002) and integrin-dependent macrophage phagocytosis (Caron et al., 2000). Two additional facts regarding Rap1 and phagocytosis are worth noting: first, Rap1 also regulates phagocytosis of bacteria and latex beads in *Dictyostelium* (Seastone et al., 1999); second, in mammalian cells, Rap1 is found associated with maturing phagosomes (Pizon et al., 1994; Garin et al., 2001).

Rap1 controls inside-out signalling to integrins

Given its effects on cell spreading and motility, Rap1 was postulated some time ago to be involved in integrin function; however, confirmation of this required alternative cellular models. Leukocytes represented such a model, since the positive effect of GTP-bound Rap1 on integrin-mediated adhesion is easier to see: in blood cells, in contrast to the more traditional, fibroblast and epithelial, adherent cell systems, integrins are normally kept inactive. Upon inside-out signalling elicited by various agonists, leukocyte integrins can rapidly and transiently be converted to a functionally active, ligand-binding state able to trigger the classic outside-in signalling to Rho-like GTPases (Harris et al., 2000; Schoenwaelder and Burridge, 1999).

Rap1 regulates functional activation of several integrin heterodimers: α4β1 (VLA-4), α5β1 (VLA-5), αLβ2 (LFA-1, CD11a/CD18), αMβ2 (CR3, CD11b/CD18) and αIIbβ3 (Reedquist et al., 2000; Caron et al., 2000; Katagiri et al., 2000; Arai et al., 2001; Sebzda et al., 2002; Bertoni et al., 2002). Active Rap1A is sufficient to induce integrin function in T-cell- and macrophage-like cell lines (Reedquist et al., 2000; Caron et al., 2000; Katagiri et al., 2000; Schmidt et al., 2001), as well as in primary T-cells and megakaryocytes (Sebzda et al., 2002; Bertoni et al., 2002), even in the absence of extracellular agonists. Conversely, expression of Rap1GAP or Rap1(N17) abolishes the ability of integrins to bind to their ligands, even in agonist-stimulated cells. In the various models, all the agonists that elicit inside-out signalling increase the levels of endogenous GTP-bound Rap1 in the absence of integrin ligand. None of the Rap1 effects are attributable to measurable changes in integrin expression. Finally, most studies used read-outs that exclude an impact of Rap1 signalling downstream of ligand-bound integrins. The studies mentioned above used the binding of a soluble ligand (Katagiri et al., 2000; Bertoni et al., 2002; Sebzda et al., 2002), a small phagocytic target (Caron et al., 2000) or a conformation-specific antibody (Reedquist et al., 2000; Katagiri et al., 2000; Bertoni et al., 2002), rather than mere adhesion to a substratum, which potentially results from both inside-out and outside-in signalling. Altogether, these data establish a general role for Rap1 in the control of inside-out signalling to integrins.

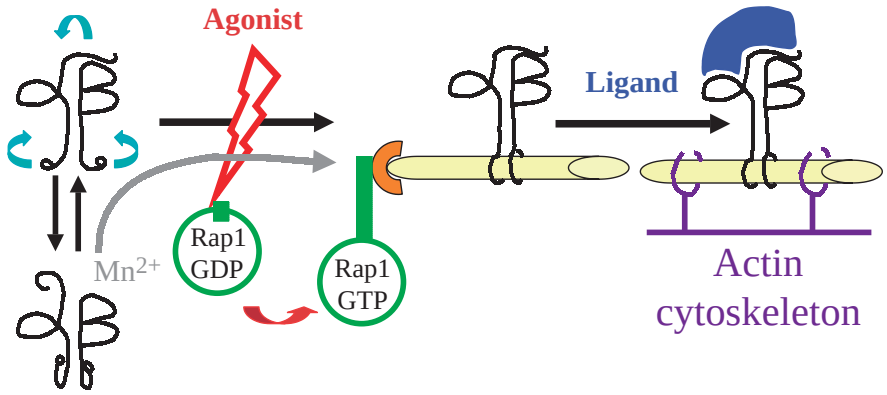

Fig. 4. A speculative model for how Rap1 governs the functional activation of integrins. In resting blood cells, most integrins are kept inactive, possibly owing to conformational constraints in the cytoplasmic tails (bottom left). A small proportion of the integrin dimers display the thermodynamically unfavourable, active conformation and can bind their ligand (top left). However, if Rap1-GTP levels remain low, structural constraints (blue arrows) force the equilibrium towards the inactive form. Upon agonist stimulation, Rap1 is transiently converted to the active GTP-bound form (red arrows), exposing the effector-binding region(s), and one of its downstream targets (depicted in orange) directly or indirectly (through the yellow rod-shaped molecule) maintains the integrin in its active conformation. By contrast, Mn^{2+} treatment (grey arrow) does not activate Rap1, although endogenous levels of active Rap1 still control Mn^{2+}-induced integrin activation. Rap1 activity is therefore required in all cases for ligand binding and outside-in signalling to take place, as suggested in this figure by the anchoring of the ligand-bound integrin to the actin cytoskeleton.

The molecular mechanism underlying this spectacular effect of Rap1 remains unknown. First, the exact meaning of functional activation, that is, whether inside-out signalling induces changes in integrin affinity, avidity or both, is controversial (Hughes and Pfaff, 1998; van Kooyk and Figdor, 2000). The studies on Rap1 and integrin activation have failed to answer this question, because changes in integrin affinity (Katagiri et al., 2000; Reedquist et al., 2000) and avidity (Sebzda et al., 2002) were observed in the case of the LFA-1 integrin. Second, the downstream target mediating the effect of Rap1 on inside-out signalling remains

unknown. Several candidates (shown in red on Fig. 3) have been ruled out, on the basis of either the inability of specific inhibitors of the ERK MAP kinase, PI3K and PLC signalling pathways to inhibit or the ability of active RalGEF to mimic Rap1-induced effects (Self et al., 2001; de Bruyn et al., 2002). An exciting new finding, however, is that activation of integrins in Jurkat T cells by Mn^{2+} or activatory anti-LFA-1 or anti-VLA-4 antibodies is still Rap1-sensitive despite being unable to elevate Rap1 GTP levels (de Bruyn et al., 2002). These results led the authors to suggest that Rap1 controls the availability of active integrins for productive binding. One possible model for how Rap1 governs both the activation and functional availability of integrins is depicted in Fig. 4. In resting blood cells, most surface-expressed integrins are inactive, therefore unable to bind their ligands. Functional activation converts the inactive integrin into a ligand-binding heterodimer, a spontaneously unfavourable process that involves major conformational changes in the extracellular domains of both α and β chains (Xiong et al., 2001; Beglova et al., 2002) and probably also in their cytoplasmic domains (Haas and Plow, 1997; Vinogradova et al., 2002). Accordingly, the three-dimensional conversion of the extracellular domains into a ligand-binding unit is elicited either directly by Mn^{2+}, that is, from the outside the cell, or after agonist stimulation, by the combination of inside-out signalling, structural changes to the integrin cytoplasmic domains and propagation of these changes to the extracellular domains. Remarkably, the presence of active, GTP-bound Rap1 is required in the two activation scenarios, which suggests that Rap1 activity is necessary to stabilize the integrin in an 'active' conformation. Rap1 could promote the interaction of the integrin cytoplasmic domains with an undefined binding partner. Whether the postulated integrin-binding partner is a direct Rap1 effector remains to be clarified.

Concluding remarks

The small GTP-binding protein Rap1 has found itself an identity in recent years. First considered as a mere antagonist of Ras signalling, Rap1 is now recognized as exerting essential regulatory roles in all eukaryotic cells. Ironically, integrin activation, which is probably the most spectacular Ras- and ERK/MAP-kinase-independent role of Rap1 in mammalian cells, is known to be negatively regulated by Ras (Hughes et al., 1997). The complex relationships between the two small GTPases will take some time to understand!

Two immediate challenges in *Drosophila* and in mammals are to identify the effector mechanisms mediating the various Rap1 functions and to understand how Rap1, which is activated in the perinuclear region, exerts most of its effects at the cell surface. A striking common feature of the various Rap1 functions described here is their presence upstream of a Rho GTPase signalling pathway. This is certainly true for bud selection in yeast (Gulli and Peter, 2001), for β_2-dependent phagocytosis (Caron and Hall, 1998; Caron et al., 2000), for integrin signalling (Schoenwaelder and Burridge, 1999) and probably also for cadherin-based adhesion (Braga, 2000; Knox and Brown, 2002; Magie et al., 2002). By analogy with the situation in yeast, one might speculate that Rap1 is needed to recruit a GEF for a Rho GTPase at sites of cell-cell or cell-matrix contacts and thus enables the stabilisation of adhesive structures. Understanding just how far these similarities extend will be a major task for the coming years.

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