Minireview

In search of a function for the Ras-like GTPase Rap1

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Abstract Rap1 (Krev-1) is a small GTPase first identified as a transformation suppressor of K-ras. This GTPase is very similar to Ras, particularly in the effector region, but its function is still elusive. Recent progress in the search for Rap1 function has come from the development of a novel assay to measure Rap1 activation. Using this assay activation of Rap1 was observed in human platelets and neutrophils after stimulation with various agonists. We speculate that Rap1 plays a role in one of the specialised functions of these cells.

Key words: Rap1; Activation; GTPase

1. Introduction

To identify proteins involved in oncogenic Ras transformation, Noda and coworkers introduced cDNA libraries into v-K-ras-transformed fibroblasts and selected for flat-revertants of the transformed phenotype. One of the clones obtained (Krev-1) encoded a protein which showed great homology with the small GTPase Ras [1]. In particular, the similarity with the effector domain of Ras was striking (Fig. 1). At the same time low stringency hybridisation of various cDNA libraries with Ras cDNA revealed the same protein, which is now commonly called Rap1 [2,3]. Rap1 consists of two isoforms, Rap1a and Rap1b, which differ mainly at the C-terminus. Since most experimental approaches did not discriminate between the two we will refer to both as Rap1. Characteristic features of Rap1 are its geranylgeranyl modification at the C-terminus, which is responsible for membrane attachment, and a threonine residue at position 61. In most other GTPases this residue is a glutamine. Substitution of threonine for glutamine in Ras resulted in a weakly oncogenic activation. Indeed, Rap1 has a 10-fold lower intrinsic GTPase activity than Ras, but is still sensitive to RapGTPase activating proteins (RapGAPs) (for review see [4]).

The striking similarity of the effector domains of Ras and Rap1 suggested that Rap1 may bind to the same effectors as Ras. For Ras several effectors have been identified, most notably (i) the serine-threonine kinase Raf1, (ii) phosphatidylinositol 3-kinase and (iii) Ral guanine nucleotide exchange factors (RalGEFs). For both Raf1 and RalGEF it has been shown that they indeed interact with active Rap1. Importantly this interaction does not seem to lead to activation of the effectors, providing a simple model for the antagonistic effect of Rap1 on Ras signalling [5,6].

The hypothesis that Rap1 functions in a pathway which is antagonistic to Ras signalling was further developed by reports that cAMP can both interfere in Ras signalling at the level of Raf1 [7] and phosphorylate [8] or activate Rap1 [9]. However, little evidence exists that indeed cAMP-mediated inhibition of Raf1 is mediated by Rap1 activation. Furthermore, we should be aware that thus far little solid evidence exists that any antagonistic effect on Ras signalling is mediated by Rap1, questioning the physiological relevance of this effect.

2. Rap1 expression and localisation

Recent results in specialised cell types indicate that Rap1 has additional functions apart from being an antagonist of Ras signalling. A first clue for a specialised function of Rap1 came from expression and localisation studies. Although Rap1 is ubiquitously expressed, it is particularly abundant in human platelets [10]. Platelets are anuclear cell fragments that adhere to sites of injury in a blood vessel and aggregate to form a blood clot. To perform this function platelets are activated by a variety of platelet agonists, of which thrombin is the most potent. During activation three main processes are induced: (i) secretion of a variety of components, including ADP and fibrinogen (ii) cytoskeletal rearrangements and (iii) adhesion and aggregation by integrin activation (predominantly $\alpha IIb\beta 3$). In platelets Rap1 is mainly localised in the plasma membrane or in the membrane of the open canalicular system. Some localisation is observed in the membranes of α-granules [11].

Rap1 is also abundant in neutrophils. Neutrophils are phagocytic cells that are induced to migrate into the extravascular tissue by, for instance, chemoattractants produced by bacteria. In these cells Rap1 is also located mainly in the plasma membrane and in specific granules [12,13].

Interestingly, both in platelets and neutrophils Rap1 translocates from the granules to the plasma membrane after simulation, but whether this indicates a function of Rap1 in secretion or whether this is the consequence of secretion is unclear. In contrast, in fibroblasts Rap1 is mainly localised in either the mid-Golgi compartment or the early and late endosomes [14,15].

3. Rap1 activation

Until recently little information was available on the signalling pathways in which Rap1 may be involved. This was due to the absence of suitable antisera to precipitate Rap1 to measure activation, i.e. an increase in the GTP-bound state after stimulation. However, a novel type of assay to identify active GTPases was recently developed. This assay makes use of activation-specific probes, which only recognise the GTP-bound form of a particular GTPase. For Rap1 the activation-

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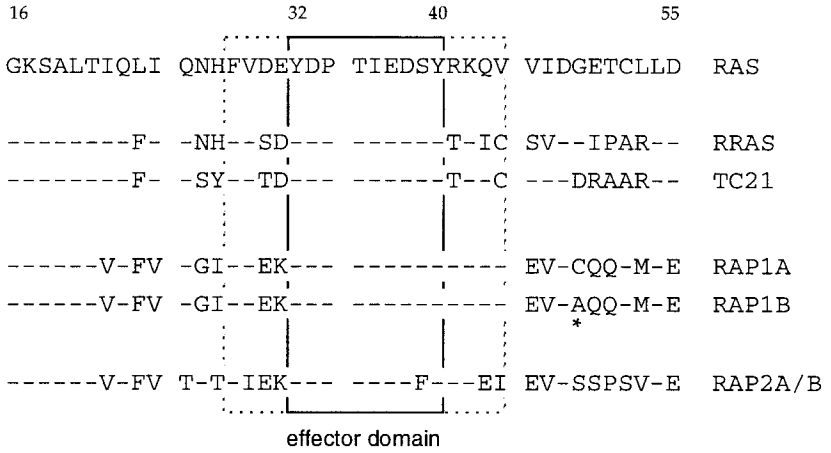

Fig. 1. Comparison of the effector domains of Ras and Rap1. Indicated are the regions between residues 16 and 55. The core effector domain of Ras spans the residues 32–40. Mutations in this region affect signalling from active Ras, without affecting guanine nucleotide binding. The asterisk indicates a difference between Rap1A and Rap1B. The other differences are at the C-terminus.

specific probe is the Rap binding domain (RBD) of RalGDS [16]. This 97 amino acid domain binds to the GTP-bound form of Rap1 with an in vitro affinity of 10 nM, whereas the affinity for RapGDP is three orders of magnitude lower [16,17].

In platelets Rap1 is activated by thrombin and by most if not all other platelet agonists. After thrombin stimulation more than 50% of Rap1 is activated within 30 s [16]. In combination with the abundant expression of Rap1 this indicates that a massive activation of Rap1 occurs. Also after stimulation of neutrophils with the chemoattractant fMLP, Rap1 is activated, but less pronouncedly so than after platelet stimulation (M'Rabet, Coffer, Zwartkruis, Franke, Koenderman and Bos, manuscript in preparation). In contrast, in the A14 fibroblast cell line we did not observe any increase in Rap1 activation after stimulation with thrombin, LPA, or peptide growth factors, such as EGF, PDGF, or insulin. However, in A14 cells the basal level of the GTP-bound form was clearly higher than in platelets or neutrophils (van Triest, Franke, Zwartkruis and Bos, unpublished observation). It could well be that in fibroblasts Rap1 is involved in a 'housekeeping' process, whereas in platelets and neutrophils Rap1 is involved in specialised processes. Surprisingly, cAMP also did not result in any increase in Rap1 activation in NIH-3T3 cells and Rat1 fibroblasts (van Triest, Franke, Zwartkruis and Bos, unpublished observation). This result is in apparent contrast to the finding of Altschuler et al. [9] showing activation of ectopically expressed epitope-tagged Rap1 in Ras-transformed fibroblasts by cAMP. Perhaps the Rap1 activation system in fibroblasts can be induced by oncogenic Ras.

4. Rap1 in platelets

In human platelets, thrombin-induced Rap1 activation is mediated by the activation of phospholipase C and the increase in intracellular calcium concentration (Fig. 2). This activation occurs prior to and is independent of secretion, cytoskeletal rearrangements and integrin activation [16]. By which mechanism calcium activates Rap1, however, is unclear. It could be by activation of a GEF, or by inactivation of a GAP. The only known specific RapGEF is C3G, a Crk SH3 domain-binding guanine nucleotide-releasing factor, but information on its involvement in calcium-induced activation of Rap1 in platelets awaits further experiments [18]. Several Rap1GAPs have been identified, i.e. a p88Rap1GAP [19], the inositol 2,3,4,5 P4 receptor Gap^{IP4} [20], tuberin [21] and Spa1 [22]. But their function in the control of Rap1 activity is also largely elusive.

After stimulation with thrombin, Rap1 is active for at least 10 min. However, for this sustained activation calcium may not be necessary, since the elevated level of calcium declines rapidly. Surprisingly, in Glanzmann patients, who lack functional αIIbβ3 integrins, Rap1 is rapidly downregulated, following the decline of calcium. This intriguing observation suggests that functional integrins are necessary for sustained activation, and may point to a positive feedback loop in the activation of Rap1 (Franke, van Triest, van Willigen, Akkerman and Bos, manuscript in preparation).

Rap1 activation is inhibited by the platelet antagonist PGI_2 [16]. PGI_2 inhibits platelet function by increasing the levels of cAMP and the subsequent activation of protein kinase A. Previously it was shown that Rap1 is a substrate for protein kinase A and indeed after PGI_2 treatment of platelets, Rap1 is phosphorylated stoichiometrically [8,16]. However, this phos-

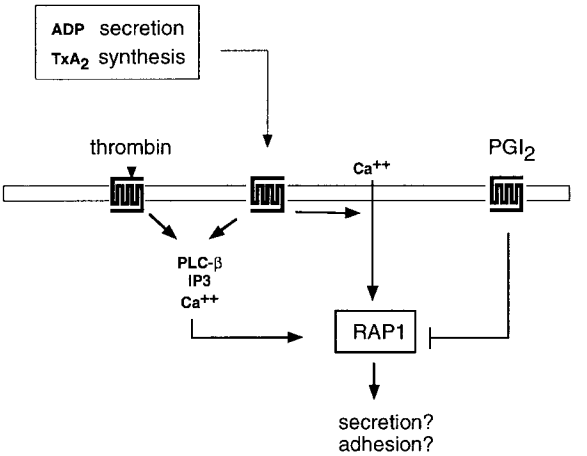

Fig. 2. A simplified model of Rap1 activation in human platelets. For explanation see text.

phorylation occurs much later than Rap1 inactivation and phosphorylation of Rap1 per se did not inhibit thrombin-induced activation of Rap1 [16]. These results indicated that phosphorylation of Rap1 by PKA is not responsible for its inactivation. Phosphorylation of Rap1 has been implicated in the translocation of Rap1 to the cytoplasm, but details of this process are lacking [23]. Alternatively, activation of Rap1-GAP may be responsible for the inhibition of Rap1, since this protein is also phosphorylated by protein kinase A [24].

After thrombin stimulation Rap1 also associates with the cytoskeleton [25]. This association does not interfere in the activation of Rap1, as shown by inhibition studies with cytochalasin D, an inhibitor of actin polymerisation. This inhibitor prevents cytoskeletal association of Rap1 but not activation [16]. Perhaps cytoskeletal association of Rap1 reflects a downstream event induced by Rap1 activation. However, several other agonists that result in Rap1 activation and platelet activation, such as ADP and thromboxane A_2, do not lead to this strong cytoskeletal association.

5. Rap1 in neutrophils

A clue for the function of Rap1 in neutrophils was obtained by the finding that Rap1 associates with flavocytochrome *b*, a component of the NADPH-oxidase system [26]. Although in vitro Rap1 is not necessary for the assembly and the functioning of the oxidase system, in vivo both a dominant negative mutant of Rap1 and a constitutively active mutant of Rap1 inhibited oxidative burst in differentiated HL60 cells and in a EBV-transformed B-cell line [27,28]. These results were interpreted that cycling of Rap1 between the GDP- and GTP-bound states is necessary for regulated oxidative burst. Compatible with a role of Rap1 in the regulation of the oxidative burst is our observation that Rap1 is rapidly activated by the chemoattractant fMLP, a strong inducer of oxidative burst in primed neutrophils. However, a similar fMLP-induced activation of Rap1 is observed in unprimed neutrophils, where fMLP is unable to induce oxidative burst (M'Rabet, Coffer, Zwartkruis, Franke, Koenderman and Bos, manuscript in preparation). This indicates that Rap1 activation alone is probably not sufficient to induce burst.

6. Downstream targets of Rap1

In principle all proteins that interact with the GTP-bound form of Rap1 and not with the GDP-bound form are putative effector proteins of Rap1. As mentioned above, $Rap1^{GTP}$ does interact with Ras effector molecules, but for Raf1 and for RalGEFs these complexes seem to be inactive. Association of $Rap1^{GTP}$ with RafB, a close relative of Raf1 [18], and with protein kinase C [29] leads to activation of these kinases in vitro. Whether this activation also occurs in vivo awaits further experiments. Other putative effector molecules are the already mentioned Rap1GAPs, since they also specifically bind to the GTP-bound form of Rap1. Unfortunately, for none of these putative effector molecules it is clear whether they mediate Rap1 function.

7. A specific function of Rap1

Although the function of Rap1 in platelets and in neutrophils is still elusive, it is tempting to speculate that Rap1 plays an important role in one of the specialised functions of these cells. Interestingly, in the budding yeast *Saccharomyces cerevisiae*, the Rap1 homologue RSR1 (BUD1) plays a role in the selection of the bud site. In addition, RSR1 interacts with CDC24, a GEF for the small GTPase CDC42, which in yeast is involved in bud site assembly and in mammalian cells in the formation of actin-rich cell surface protrusions (filopodia) [30]. All these processes are highly localised. Perhaps Rap1 serves a similar function in mammalian cells, i.e. the selection of sites where protein complexes have to be formed. In platelets these complexes could be focal adhesion type of complexes responsible for cell adhesion, or fusion sites for regulated secretion [16]. In neutrophils it could be the formation of phagosomes, responsible for oxidative burst [31]. A common denominator in these events is the proper connection of the various complexes to the cytoskeleton and perhaps Rap1 is involved in this process. Maybe Rap1 mediates signals that induce the formation of these complexes and executes this specialised function by using similar effectors as Ras but in a specialised cellular environment. Alternatively, Rap1 may be directly involved in the assembly of the complexes, for instance by association with components of a complex such as flavocytochrome b of the NADPH oxidase complex [26].

With the development of activation-specific probes to identify signals that activate Rap1 and the availability of specific dominant negative mutants, the secrets of Rap1 will be unravelled rapidly.

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