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Minireview

Ras–effector interactions, the problem of specificity

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Abstract Ras plays the role of a molecular switch in many cellular signalling pathways. The Raf-kinase has been identified as the direct target molecule of Ras in mammalian cells. However, in recent reports other proteins have been characterised as putative Ras effectors which have neither a functional nor a structural relationship to each other. In addition it has been shown that also other members of the Ras family like Rap and R-Ras can interact with some of these proteins. To address the problem of specificity and of biological relevance of the interactions, they have to be carefully quantified and the cellular localisation of the proteins involved taken into account.

Key words: Ras; Raf; PI(3)-kinase; RalGEF; Effector

1. The functional cycle of GTP-binding proteins

GTP-binding proteins function as molecular switches, which cycle between the GDP-bound inactive state and the GTP-bound active state. Without external activation they are found predominantly in the inactive form since GDP is tightly bound and the rate of dissociation is correspondingly slow. Activation occurs when a so-called guanine-nucleotide exchange factor GEF accelerates nucleotide release from the protein. Since in the cell there is much more GTP than GDP the protein becomes predominantly loaded with the guanosine triphosphate and thus active. Once activated, GTP-binding proteins can now interact with so-called effector molecules and this interaction creates some form of a chemical signal. Effector molecules are operationally defined as proteins which interact more strongly with the GTP-bound state. Return to the inactive state is due to the fact that GTP-binding proteins are also GTPases which hydrolyze GTP, albeit slowly, and create inorganic phosphate and the GDP-bound state, thus interrupting the signal. The GTPase reaction is accelerated by GTPase-Activating Proteins (GAPs), which are either purely regulatory proteins or can themselves be effectors. General reviews dealing with the general aspects of GTP-binding proteins have appeared [1,2].

Ras-related GTP-binding proteins constitute a superfamily of proteins with approximately 50–60 members in mammals, which based on sequence and functional homology can be grouped into the Rac/Rho, Rab/Ypt, Ran, Arf, Rad and the actual Ras subfamilies [3]. The Ras family consists of Ras, Rap, R-Ras and Ral with various isoforms. Ras itself is a central switch in many signal transduction pathways which control proliferation, differentiation and metabolism. Its guanine nucleotide state is tightly controlled by the two regulatory types of molecules Ras-GEF and Ras-GAP. Three different GEF proteins called Ras-GRF or $Cdc25^M$, Sos^M and C3G and three Ras-specific GAPs called p120-GAP, neurofibromin and $Gap1^M$ have so far been described for mammalian systems (Fig. 1). These regulators are multifunctional proteins that are potentially involved in a number of interactions since they contain many different modular domains, some of which connect these proteins to events at the plasma membrane or to other small GTP-binding proteins such as Rac/Rho. Reviews covering these interactions have appeared [4–7]. We will thus confine ourselves to the description of the interactions of Ras with its downstream effectors.

2. Effector Residues of Ras

Despite an intensive effort to find it, the effector of Ras has been elusive for quite some time. However, it had been shown by mutational analysis that residues 32–40 of human Ras and similar residues of the yeast *Saccharomyces cerevisiae* RAS were involved in the interaction with effectors since mutations of these residues interrupted the Ras-signalling pathway (summarized by [8,9]). For example, if mutations such as D38A (and many others) are introduced into oncogenic Ras these Ras mutants are no longer transforming [10–12]. It was postulated that the Ras effector would bind via these residues and that the binding was necessary to transmit the signal for transformation and proliferation. In support of this concept it was found that similar mutations in the RAS gene of the yeast *S. cerevisiae* interrupt the interaction with the yeast RAS effector molecule adenylyl cyclase [10]. Since adenylyl cyclase is not the effector of mammalian Ras and since mammalian Ras can nevertheless complement the loss of the yeast RAS genes, these experiments also showed for the first time that there are different effectors of Ras and that the interactions between yeast RAS and adenylyl cyclase and mammalian Ras and its effector are via similar structural motifs.

The structure of the GTP-bound form of Ras showed that these residues are located in loop L2 and β-strand 2 and are highly exposed to the solvent [13,14]. The structural comparison of the GDP-bound and GTP-bound state also showed that there are only two regions of structural difference between the active and inactive state, called switch I and II, and that the effector region is one of them (switch I) [14–16]. This was the first structural verification of how the conformational change is triggered by the loss of the γ-phosphate on GTP hydrolysis and that these structural changes somehow change the affinity towards the effector molecule.

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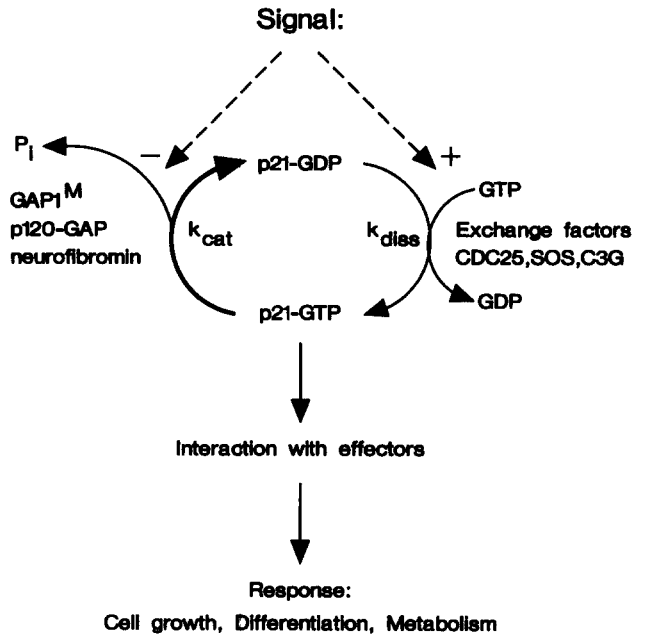

Fig. 1. Schematic view of signal transduction from the cell surface to the nucleus via the regulatory cycle of the Ras gene product p21. Exchange factors catalyse the activation of Ras in transferring it into the GTP-bound form. Deactivation by intrinsic GTP hydrolysis of Ras can be accelerated by GTPase activating proteins. Only the GTP-bound Ras is able to interact strongly with effector molecules which somehow transmit the signal to produce a biological readout.

3. Raf as effector

The first suggestion that Raf is downstream of Ras came from microinjection experiments where it was shown that the monoclonal anti-Ras antibody Y13–259 inhibited the signalling via the PDGF receptor, the transforming activity of v-scr and v-ras, but not of v-Raf but that an anti-Raf antibody could inhibit the transforming activity of v-Ras [17]. Likewise antisense Raf RNA and dominant negative Raf mutants were shown to inhibit ras oncogene mediated signalling [18], and many other experiments in different genetic systems implicated Raf and Ras in receptor tyrosine kinase (RTK) mediated effects. Experiments to prove the direct interaction between Ras and Raf by coimmune precipitation with the widely used antibody Y13–259 were unsuccessful due to the fact that Raf and Y13–259 interact with the same region on Ras, which is not surprising in hindsight since Y13–259 inhibits the biological activity of Ras, which again is mediated by Raf. With the use of another antibody Y13–238, the arrival of the two-hybrid system and the widely used GST-fusion protein constructs, the story of Raf as the direct effector protein and the direct proof of their interaction was then made independently by several laboratories [19–24]. They showed that the regulatory region of the c-Raf-1 protein kinase binds tightly to mammalian Ras, that this interaction is dependent on GTP and can be interrupted by mutations in the effector region, such as D38A, and that there is a strong correlation between the biological activity of effector mutants and their ability to bind to Raf.

Vojtek et al. [24] were able to isolate via the two-hybrid screen many overlapping fragments of Raf which were able to bind to Ras. The only common fragment of these clones consisted of amino acids 51–131 which have then been shown to constitute an independent domain, the Ras-Binding-Domain (RBD) [25,26] though other elements of the regulatory region of Raf have also been implicated in binding [22,27]. The affinity of RBD for $p21^{ras}$-GTP is in the order of 10–20 nM, and the affinity to the GDP-bound form is 1000 fold lower [26]. In vivo the binding of Raf kinase to Ras leads to the translocation of the kinase to the plasma membrane where Raf gets activated by a process that has not been fully elucidated but seems to involve the members of the 14–3–3 family of proteins [28]. Raf may become fully activated by either autophosphorylation or by the action of either Ser/Thr- or Tyr-specific protein kinases [29]. The activated Raf kinase is the start of a kinase cascade where the next-in-line kinase is activated by phosphorylation and then itself activates the next downstream kinase. Raf activates MEK which in turn activates ERK which gets translocated to the nucleus and activates either other kinases such as Rsk or transcription factors such as Elk. The biological role of Ras may thus be rather boring, translocating Raf to the plasma membrane and presenting it to the activation machinery [30,31], an anticlimactic end to a furious search.

It has been shown that the rate of GTP hydrolysis in the Ras-RafRBD complex is the same as that of Ras alone [22,24–26] and that GAP is unable to stimulate hydrolysis of Ras in the Ras–Raf complex [22,23,25]. Therefore it seems reasonable to assume, as proposed before, that the intrinsic GTPase rate of Ras is important for signal termination [26,32]. It is possible that after activation of Raf, possibly by phosphorylation, Ras is released from the complex in order to activate another Raf molecule.

The three-dimensional structure of the RBD domain has been determined to be similar to that of ubiquitin structure [25,33] and the structure of the complex between Rap1A in the triphosphate form and RBD has also recently been solved by X-ray crystallography [34]. Since it involves residues on Rap1A which are identical to Ras, the structure is probably similar to that of the corresponding Ras–RafRBD signalling complex. It is the first atomic view of the interaction between a GTP-binding protein and its effector. It shows that this interaction is indeed mediated by the effector residues of Ras/Rap, part of which form an apparent antiparallel β-sheet with RBD.

4. PI(3)kinase

Since the discovery of Raf as an effector or downstream target of Ras, other possible effectors have since been found, either by using the two-hybrid system or by measuring the direct interaction of isolated proteins. One of them is PI(3)kinase which consists of two subunits, p85, which contains SH2 domains involved in receptor interaction, and p110 which is the catalytic subunit. Ras has been shown to bind to both the α and β type p110 subunits [35,36]. The dissociation constant of this complex is 500 nM, thus the interaction is weaker than the Ras–Raf interaction, but it is again dependent on the presence of GTP and effector residues such as D38 and is inhibited by the Y13–259 antibody. Although it is not quite clear which signals are created by this interaction, it apparently leads to a modest increase in the PI(3)kinase activity [35,36], thus increasing the concentration of 3′-phosphorylated inositol lipids which are themselves bona fide signal transduction molecules or are involved in their generation. Neither the only moderate in-

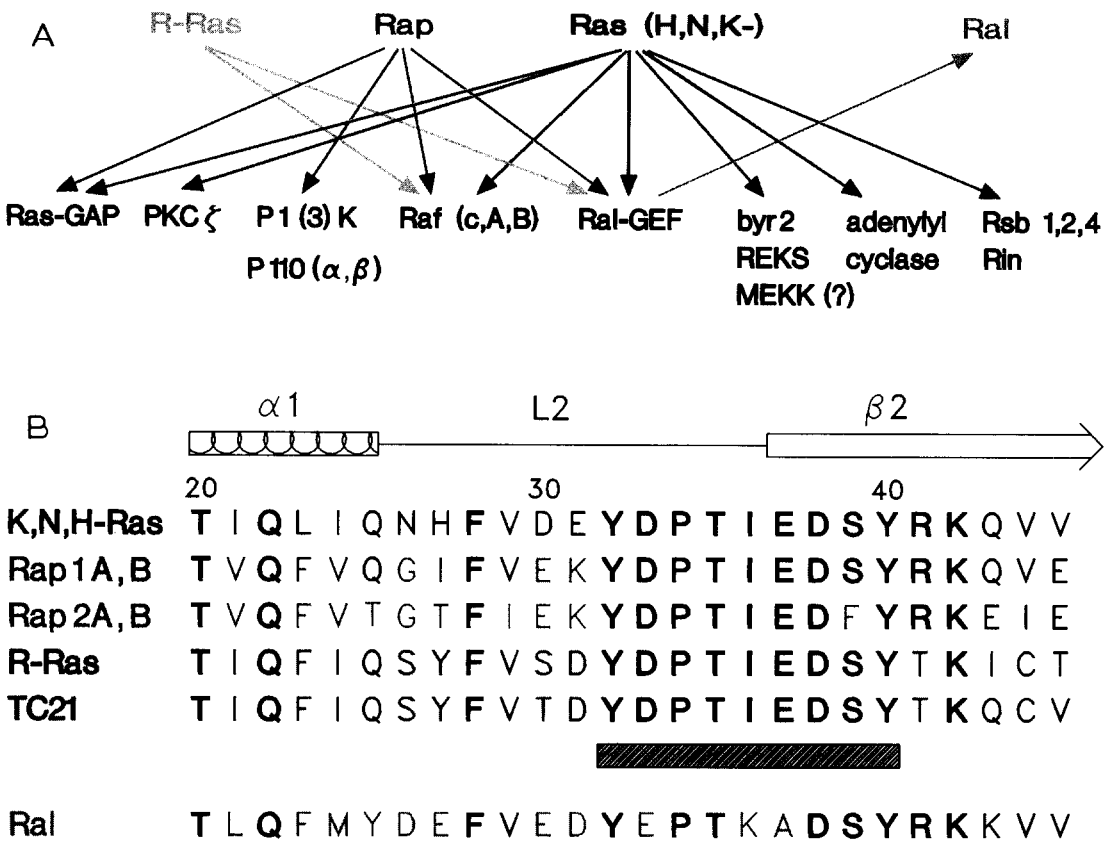

Fig. 2. (A) A schematic presentation of the promiscuity of the Ras family members. All direct interactions of the members of the Ras subfamily sofar reported in the literature (see text) are indicated by arrows. Particularly for Ras and Rap several possibilities for mutual antagonistic action by competition for the same effector seem to exist. (B) Sequence alignment of effector residues (aa 20–45 for K-Ras) of the Ras family proteins. Secondary structure elements are given on top. There is an almost complete identity in the core effector region (part of L2 and β2, see hatched bar) which in Ras/Rap mediates the interaction with Raf [34]. Ral does not show high homology to the other family members.

crease in enzymatic activity nor the weak affinity between $p21^{ras}$ and p110 are necessarily arguments against the biological significance of this interaction, since such types of protein/protein interactions may be much more pronounced on a membrane surface where both the higher local concentrations in a 'two-dimensional solution' and their proper juxtaposition may enhance the biological readout. That PI(3)kinase is not simply a downstream target molecule of Ras comes from the observation that constitutively active enzyme acts upstream of Ras and that its effect is inhibitable by N17Ras [37] and from its role in Rac activation [38,39].

5. Other candidate effectors

Recently the case for the existence of further Ras effectors was strengthened by the finding that different effector-site mutations block different Ras–effector interactions and that two such mutations can complement each other in producing a biological readout [27]. Other proteins have indeed been found to bind to Ras in a GTP-dependent manner which, according to the definition given, qualifies them as potential Ras effectors or targets (Fig. 2A). These are Ral-GEF, the guanine nucleotide exchange factor for Ral, another protein of the Ras family, and molecules that have a high homology to Ral-GEF and have been labelled RGL, RalGEF-like [40–42]. These molecules contain a homologous independently folding domain which is between 80 and 100 amino acids long (Herrmann, unpublished). The interaction of this domain with Ras in vitro is also mediated by effector residues, and it may somehow modify the enzymatic activity of the RalGEF and thus the nucleotide state of Ral. However, since nothing is known about the function of Ral, its activation does not help us to explain the function of Ras.

In *Saccharomyces pombe*, the protein kinase byr2 has been defined genetically to be situated downstream of Ras [43] and does in fact bind very tightly with nM affinity to $p21^{ras}$ in a GTP-binding manner [44]. Mammalian homologues of byr2 termed MEK kinases have been identified, which are serine-threonine protein kinases, at least one of which seems to be activated in a ras-dependent way, demonstrated by the inhibition through Ras(S17N) [45]. However, direct binding between Ras and MEKK has so far not been demonstrated. An apparent Ras-dependent MEKK called REKS (for Ras-dependent ERK kinase stimulator) has been described biochemically, which is immunogenically distinct from Raf, but has not yet been cloned molecularly [46]. GTP-dependent binding of Ras has also been found for the ζ isoform of PKC and this interaction seems to be stimulated by PDGF in fibroblasts [47]. As discussed earlier [4] it is also possible that p120-GAP, which contains many signal transduction modules for protein–protein

interactions, may also be a Ras target molecule. It should be added that its site of interaction is at least overlapping with the effector region of Ras [8,9].

In two recent reports further Ras-binding (Rsb) proteins have been identified by using the two-hybrid screen and even less is known about these proteins and what effect they might have in the biological function of Ras [48]. The human protein Rin (Ras interaction/interference) has also been identified as a protein that can bind to H-Ras and yeast Ras2 in a manner similar to the Ras–Raf interaction, the function of this protein being similarly mysterious [49]. Together with the bona-fide effectors of Ras in yeast, adenylyl cyclase in *S. cerevisiae* and byr2 in *S. pombe*, we notice a large number of possible Ras effectors, which have at least overlapping binding regions on Ras (Fig. 2A). However, so far no sequence similarity has been detected between any of these interacting sequences and it will be necessary to investigate the structure of these proteins and their complexes with Ras to find out whether they have anything in common with the Ras-Binding Domain of Raf. It will also be necessary to find out whether these Ras-effector interactions feed into different signal transduction pathways. Already there is enough evidence to suggest that Ras branches off into two different pathways which lead to the activation of Rac and MAP kinase, both of which are necessary for cellular transformation [39].

6. Promiscuity of the Ras-family

The situation is, however, even more complicated by the promiscuity of the Ras family members since it has been shown that the other proteins of the Ras subfamily of Ras-related GTP-binding proteins, Rap1 (A and B) and R-Ras/Tc21, but not Ral, also bind to some of the presumed effectors of Ras [40–42,48,50,51] which is shown schematically in Fig. 2A. The reason for this promiscuity seems obvious from the sequence alignment of the effector residues of the Ras family members (Fig. 2B). The sequence of the β2 region, which contains many of the reported effector residues and mediates the Ras-Raf interaction as shown in the 3D structure of the complex [34], is identical for most of these proteins. The different subtypes of the different effectors such as three isoforms of Raf and at least three isoforms of the RalGEF motif create even more possible interactions.

The task will be to sort out if all of these multiple possible interactions are biologically relevant. As a case in point we have carefully measured the affinities of different Ras and Ras-like proteins with Raf-1 and find that the affinity of Rap1A is about 100fold weaker than that of H-Ras. This by itself seems to suggest that Rap1A is unable to function as a antagonist of the Ras signal transduction pathway, as suggested by its ability to suppress K-Ras induced transformation and Ras-dependent activation of ERK-1 and ERK-2 [52,53]. Measuring the affinities of these interactions alone may, however, not be sufficient to explain biological behaviour. It is becoming increasingly evident that signal transduction pathways depend on the relocalization of proteins away and onto their place of action [54] and that protein–protein interactions are modified by the membrane environment in which they take place. The rate of the GTP hydrolysis may be another modulator to be considered. In the case of Rap and Ras it has already been found that they are located on different membrane compartments and have vastly different GTPase rates [55,56]. Thus the question of who does it with whom may have to be extended to the question of where and for how long in order to sort out the biological significance and specificity of effects mediated by Ras and its family members.

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