REVIEW ARTICLE

Biology of the Rap proteins, members of the *ras* superfamily of GTP-binding proteins

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INTRODUCTION

A large superfamily (> 50 members) of monomeric GTP-binding proteins structurally related to the *ras* oncogene proteins has been described in the past few years (Bourne et al., 1991; Grand and Owen, 1991; Hall, 1990; Valencia et al., 1991). These proteins have been implicated in the regulation of a diverse array of cellular processes, utilizing their ability to bind and hydrolyse GTP as a means to regulate the reversible interactions and/or activities of macromolecules involved in these processes. Based upon sequence homology, the *ras* superfamily can be divided into four subfamilies, *ras*, *rho*, *rab* and *arf* (Valencia et al., 1991; Kahn et al., 1992). The *ras* proteins are essential components of receptor-mediated signaling pathways controling cell proliferation and differentiation; the *rho* proteins are involved in cytoskeletal assembly and NADPH oxidase regulation in phagocytes; and the *rab* and *arf* proteins regulate the transport of vesicles between intracellular compartments and/or the plasma membrane (see Hall, 1990).

The mammalian *rap* proteins, which are the focus of this Review, are members of the *ras* family of GTP-binding proteins,and share highly conserved structural motifs with the *ras* transforming proteins. Overall, the *rap* proteins share ~50% sequence similarity with *ras* and, like *ras*, are found in nearly all tissues. Two *rap* families, designated *rap*1 and *rap*2, have been identified to date. Within each family are two members denoted as A and B, i.e. *rap*1A and *rap*1B (see Figure 1). As is the case with the majority of the low-molecular-mass GTP-binding proteins, the actual role(s) of the Rap proteins in cellular function have not yet been elucidated in detail. There is evidence however that Rap proteins exert biological activities in at least two cellular arenas: first in that of cellular growth and differentiation control, and secondly in a phagocyte-specific enzyme system responsible for the generation of microbicidal oxygen radicals. The latter suggests either that Rap can play very specific biological roles in certain cells or, alternatively, that Rap serves a very general function which can involve different proteins and different effector systems depending on the cell involved. In this Review, I examine current knowledge of the biology of the Rap proteins.

CLONING AND ISOLATION OF RAP PROTEINS

The Rap proteins were cloned and/or purified by a number of laboratories within a very short time period by using a variety of strategies. Pizon et al. (1988a) identified the *rap*1A and *rap*2A genes by screening a Raji human Burkitt lymphoma cell library with probes based upon the *Dras*3 gene previously identified in *Drosophila* (Schejter and Shilo, 1985). This *Drosophila ras*-related protein differed from the other members of the *ras* superfamily known at that time in that it possessed a threonine-for-glutamine substitution at residue 61 of the highly conserved DTAGQE sequence found in positions 57–62 of *ras*. *rap*1B was identified very soon afterward by Pizon et al. (1988b) using the same strategy. *rap*1A (termed Krev-1) was isolated by Kitayama et al. (1989) from a human fibroblast cDNA expression library based on its ability to cause reversion of the transformed phenotype of v-Ki-*ras*-transformed DT fibroblasts. Kawata et al. (1988) purified a 22 kDa GTP-binding protein (termed smg p21) from bovine brain, sequenced, and cloned the protein, and found it to be identical to *rap*1B. Takai's laboratory has subsequently purified Rap1B from human platelets (Ohmori et al., 1989) and bovine aortic smooth muscle (Kawata et al., 1989a). Rap1A has also been purified, sequenced, and cloned from human neutrophils (Bokoch et al., 1988; Quilliam et al., 1990). The identification of a fourth member of the *rap* family, *rap*2B, emerged through molecular cloning from a platelet cDNA library (Ohmstede et al., 1990).

*rap*1 and *rap*2 proteins are 70% identical at the amino acid level (Figure 1). *rap*1A and 1B differ by only nine out of 184 amino acids (95% identity), with the sole region of substantial nonidentity being between positions 171–189 of the C-terminus. Similarly, *rap*2A and 2B differ by 18 out of 183 amino acids (90% identity), with the major area of divergence at residues 170–183 of the C-terminus.

POST-TRANSLATIONAL MODIFICATION OF RAP PROTEINS

Isoprenylation

It has been established that a diverse group of yeast and mammalian proteins are modified post-translationally by the covalent addition of an isoprenoid group (reviewed by Maltese, 1990; Cox and Der, 1992). This event is usually signalled by a C-terminal CAAX motif (C, cysteine; A, aliphatic; X, any residue) present in the proteins. The sequence of events involved in post-translational processing of the low-molecular-mass GTP-binding proteins includes the addition of a C_{15} (farnesyl) or a C_{20}

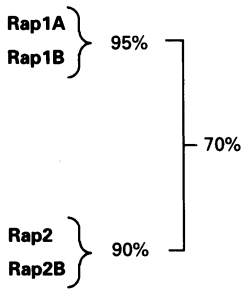

Figure 1 The Rap proteins, members of the *ras* superfamily

Four *rap* subtypes have been identified, as described in the text. The percentage of sequence similarity at the amino acid level between the indicated proteins is shown.

Abbreviations used: GTPγS, guanosine 5′-[γ-thio]triphosphate; GAP, GTPase-activating protein; GDS, GDP/GTP dissociation stimulator. Note that Rap2 is denoted as Rap2A in most of the literature cited.

(geranylgeranyl) isoprenyl group to the cysteine residue of the CAAX motif via a thioether linkage, proteolytic cleavage of the AAX residues, and carboxylmethylation of the now terminal and isoprenylated cysteine. While the specific contribution of isoprenoid modification to protein function is not known, it is apparent that such modifications are critical for the biological activities of isoprenylated proteins.

Like many of the other members of the *ras* superfamily, the Rap proteins contain a C-terminal CAAX consensus motif which directs post-translational isoprenylation (Maltese, 1990). Rap1A, ending in CLLL, has been shown to be modified by a geranylgeranyl group at Cys-181, with proteolytic truncation and subsequent carboxylmethylation also shown to take place (Buss et al., 1991). Rap1B and Rap2B, ending in CLQL and CVIL, appear to undergo similar modifications (Kawata et al., 1990; Winegar et al., 1991). Carboxylmethylation of Rap1B (Huzoor-Akbar et al., 1991) and Rap1A (Quilliam and Bokoch, 1992) has been found to be stimulated by guanosine 5′-[γ-thio]triphosphate (GTPγS); the significance of this interesting observation in terms of Rap modification and function *in vivo* is unknown, but suggests the possibility of regulatory significance for this modification.

It appears to be a general rule that isoprenylated proteins terminating in leucine are geranylgeranylated, while those ending in serine or methionine are farnesylated (Moores et al., 1991; Cox and Der, 1992). It is of note that Rap2B, which terminates in CVIL, is geranylgeranylated while the very closely related Rap2A, which terminates in CNIQ, is farnesylated (Farrell et al., 1992). These two structurally similar forms of Rap may thus partition into different membranes within cells, perhaps reflecting distinct biochemical activities. The concept that farnesylation versus geranylgeranylation of a low-molecular-mass GTP-binding protein may impart specific functions and/or subcellular localizations to that protein has been explored to a limited extent. A chimaeric protein containing the N-terminal half of oncogenic H-Ras linked to the C-terminal half of Rap1A was geranylgeranylated and retained the ability to transform cells (Buss et al., 1991). Cox et al. (1992) found that switching the C-terminal CAAX sequences of H-Ras and Rap1A did not prevent oncogenic Ras activity, nor the ability of Rap1A to antagonize Ras transforming activity. Farnesyl and geranylgeranyl moieties are thus functionally interchangeable for these biological activities. However, in this same study the expression of moderate levels of geranylgeranyl-modified normal Ras inhibited the growth of untransformed NIH 3T3 cells. These findings suggest that normal Ras function may specifically require protein modification by a farnesyl isoprenoid. Whether this reflects any changes in the subcellular localization of the chimaeric proteins however was not examined in this study.

The reported subcellular distribution of the Rap proteins seems to be distinct from that of Ras (see Grand and Owen, 1991). Both Beranger et al. (1991a) and Kim et al. (1990) observed Rap1 to be associated with subcellular fractions devoid of Ras. Beranger et al. (1991a) showed by immunofluoresence labelling and co-fractionation with Golgi-specific markers that Rap1 was associated with a Golgi-like structure in Rat-1 fibroblasts and HEP2 epidermoid carcinoma cells, while Ras was clearly detected in plasma membrane. Similarly, Rap2 was observed to associate with a low density structure that morphologically overlapped with the endoplasmic reticulum in Rat-1 cells (Beranger et al., 1991b). The structural and/or functional basis for this differential subcellular localization of Rap and Ras has not yet been determined. Rap1 and Rap2 proteins have been localized to both the plasma membrane and specific granules of human neutrophils (Maridonneau-Parini and de Gunzburg, 1992; Quinn et al., 1992a). This may relate to the role of Rap1 in the NADPH oxidase system of phagocytic cells (discussed below).

Phosphorylation

After the purification of Rap1A and Rap1B, these proteins were soon found to serve as substrates for phosphorylation by cyclic AMP-dependent protein kinase *in vitro* (Bokoch and Quilliam, 1990; Hoshijima et al., 1988; Kawata et al., 1989b; Lerosey et al., 1991; Quilliam et al., 1991). Both Rap proteins incorporate phosphate to a level of 1 mol/mol, suggesting a single site of phosphorylation. The site at which phosphorylation occurs has been shown to be Ser-180 in Rap1A (Quilliam et al., 1991). This is contained within the sequence KKKPKKKSC, which is similar to consensus cyclic AMP-dependent protein kinase phosphorylation sites (Kemp and Pearson, 1990). Rap1B is phosphorylated solely on Ser-179 within the sequence GKARKKSSC, again similar but not identical to "classical" cyclic AMP-dependent kinase motifs (Hata et al., 1991). In contrast, the Rap2 proteins lack such (potential) consensus motifs for phosphorylation near the C-terminus and do not serve as substrates for phosphorylation by cyclic AMP-dependent, or any known, protein kinases.

Phosphorylation is not influenced by whether Rap is in a GDP-bound versus GTP-bound state, and stoichiometric phosphorylation of Rap1A or 1B *in vitro* has no effect on the guanine nucleotide-binding or hydrolysis properties of the two proteins, nor the responsiveness to Rap-GAP (Bokoch and Quilliam, 1990; Hoshijima et al., 1988). The phosphorylation sites of both Rap1A and Rap1B are adjacent to the cysteine residue which is geranylgeranylated. Possible influences of the phosphorylation on post-translational processing of either Rap protein remain to be investigated. A role for Rap1 phosphorylation in regulating the interaction of Rap1 with other macromolecules will be discussed below.

Phosphorylation of Rap1A has been shown to occur in intact HL-60 cells which had been differentiated into neutrophil-like cells in response to dibutyryl cyclic AMP, forskolin, prostaglandin E_1 or isoprenaline (Quilliam et al., 1991). Phosphorylation of Rap1B occurs in human platelets in response to prostaglandin E_1 or the prostacyclin analogue iloprost (Kawata et al., 1989b; Lapetina et al., 1989; Siess et al., 1990). A form of Rap1 has also been found to be phosphorylated in intact fibroblasts by exposure to 8-bromo cyclic AMP (Lerosey et al., 1991). The possibility that Rap may mediate some of the cellular effects of cyclic AMP will be considered later in this Review.

The Rap proteins clearly do not serve as substrates for protein kinase C, myosin light chain kinase or insulin/EGF receptor tyrosine kinases *in vitro* (Bokoch and Quilliam, 1990; Hoshijima et al., 1988; Kawata et al., 1989b; Quilliam et al., 1991). Recently however, Rap1B has been reported to be phosphorylated by cyclic GMP-dependent protein kinase (Miura et al., 1992) and a neuronal calcium/calmodulin-dependent protein kinase (Sahyoun et al., 1991) *in vitro*. Both kinases modified the same serine residue in Rap1B that is phosphorylated by cyclic AMP-dependent kinase. However, phosphorylation of Rap by such kinases has not been demonstrated to occur in intact cells.

RAP1 REGULATORY PROTEINS

It is thought that the low-molecular-mass GTP-binding proteins, like the heterotrimeric signalling GTP-binding proteins, are regulated by a cycle of GTP binding and hydrolysis. The Rap proteins have clearly been shown to bind and hydrolyse GTP

(Bokoch and Quilliam, 1990; Kawata et al., 1988; Lerosey et al., 1991). The rate of GTP binding to Rap is limited at physiological concentrations of Mg^{2+} by the dissociation of GDP from the protein, suggesting that such exchange must be catalysed *in vivo* by other protein(s). Hydrolysis of GTP to GDP also occurs at a very low rate on the Rap proteins (0.0005–0.010 mol/min), and would be too low to terminate the action of Rap–GTP effectively without some means to enhance GTP hydrolysis. GTPase-activating proteins (GAPs) which stimulate the rate of GTP hydrolysis by Rap have been identified and are described below. A second regulatory protein, capable of stimulating the dissociation of GDP/GTP from Rap (Rap GDS), has also been purified and cloned (see below).

GTPase-activating proteins (GAPs)

Several GAP activities that appear to be specific for Rap1 have been detected in the plasma membrane and cytosol of a number of cell types. Polakis et al. (1991) purified a membrane-associated 88 kDa GAP from HL-60 cells. This GAP was subsequently cloned and shown to be a unique protein which did not exhibit similarity to any of the GAPs specific for Ras (Rubinfeld et al., 1991). This form of Rap-GAP was not ubiquitously expressed, and was most abundant in fetal tissues and certain tumour cell lines. Interestingly, the expression of the 88 kDa Rap-GAP was decreased in HL-60 cells which had been induced to differentiate by dimethyl sulphoxide.

Two chromatographically resolvable peaks of Rap-GAP activity have been observed in human platelets (Ueda et al., 1989) and bovine brain (Kikuchi et al., 1989) cytosol. One such cytosolic GAP was purified (Nice et al., 1992) as a 55 kDa protein. Limited amino acid sequence information indicates that this 55 kDa GAP is very closely related, if not identical, to the 88 kDa GAP purified by Polakis et al. (1991). It is thus not clear how many distinct forms of Rap-GAP exist and what the significance of the cytosolic versus membrane localization of this protein might be. The changes in Rap-GAP expression upon HL-60 cell differentiation and the identification of multiple, phosphorylated forms of this GAP (Polakis et al., 1992) suggest that Rap-GAP may be regulated in a very specific manner in order to control Rap activity.

GDP/GTP dissociation stimulator (GDS)

A guanine nucleotide exchange protein which is active on Rap1 has been identified and purified from bovine brain cytosol (Yamamoto et al., 1990). This 53 kDa protein has been cloned (Kaibuchi et al., 1991) and shown to be a unique protein with limited amino acid sequence similarity to the CDC25 and SCD25 proteins which may regulate the GDP/GTP exchange reaction of the yeast Ras 2 protein. Rap-GDS appears to interact with Rap1A and 1B as a 1:1 stoichiometric complex (Kawamura et al., 1991a). This interaction involves, at least in part, the C-terminal portion of Rap1, as indicated by: (a) the requirement for the post-translationally processed form of Rap1 for GDS binding and activity (Hiroyoshi et al., 1991) ; (b) the demonstration that proteolytic removal of an ~ 1000 Da fragment of the C-terminus prevents binding and activation of Rap1 by GDS (Hiroyoshi et al., 1991); (c) geranylgeranylated synthetic peptides representing the C-terminus of Rap1 inhibit GDS action (Shirataki et al., 1991); and (d) phosphorylation of Rap1B at the C-terminus enhances its interaction with GDS (Hata et al., 1991; Itoh et al., 1991). It is of interest that this "Rap1-GDS" is also able to catalyse GDP/GTP exchange for other post-translationally processed low-molecular-mass GTP-binding proteins, including K-Ras, Rho A and Rac1 (Mizuno et al., 1991). These proteins have in common a lysine/arginine-rich cationic region at their C-terminus which may enable them all to interact with the GDS.

A number of anionic phospholipids have been reported to antagonize the GDP/GTP exchange activity of Rap-GDS, and to reduce markedly the ability of GDS to stimulate Rap1B GTP binding (Kawamura et al., 1991b). The effect of these lipids was reduced when Rap1B was phosphorylated by cyclic AMP-dependent protein kinase (Itoh et al., 1991). Takai and associates (Itoh et al., 1991; Kawamura et al., 1991b) have suggested that Rap1B may bind to anionic lipids in the plasma membrane through the polycationic C-terminal domain, suppressing GDS action. Phosphorylation of Rap1B by cyclic AMP-dependent protein kinase would decrease the ionic interaction of the lipids with Rap1B in this region, sensitizing Rap1B to the action of the GDS. GDS binding to Rap1B is associated with the release of Rap1B from the membrane as a GDS complex (Kawamura et al., 1991a) and this may occur *in vivo* as a consequence of Rap1B phosphorylation (Lapetina et al., 1989). The operation of such a mechanism would implicate cyclic AMP as a critical regulator of Rap activity and could provide a link between this important hormonally-regulated second messenger system and biological activities regulated by Rap.

BIOLOGICAL ACTIVITIES OF RAP1 PROTEIN

Antagonism of Ras by Rap1

The isolation of *rap*1A by Noda and colleagues (Kitayama et al., 1989) as a cDNA which was able to suppress transformation of NIH3T3 cells by v-Ki *ras* led to the hypothesis that the Rap1A protein might directly antagonize Ras by competing for a common downstream target. Such a mechanism was postulated based upon the conservation in Rap1A and 1B of the putative "effector" domain region (amino acids 32–44) found to be crucial for the transforming activity of Ras and for GAP binding (Adari et al., 1988). It was possible however that Rap might antagonize Ras action by other mechanisms, such as directly activating a pathway which regulates cell growth and/or differentiation in a negative manner, or by indirect means, such as activation of other enzymes [such as kinases (Labadia et al., 1992) or phosphatases] able to inhibit Ras growth signals (see Figure 2).

In support of the "competition" hypothesis, Kitayama et al. (1990*a*) showed that an Asp-38 → Ala or Asn point mutation in the effector domain of *rap*1A markedly inhibited the ability of *rap*1A to cause phenotypic reversion of *ras*-transformed cells. Mutations at position 12 (Gly → Val) and at position 59 (Ala → Thr), which are thought to activate *rap*1 by maintaining it in the GTP-bound form (by analogy with *ras*), substantially enhanced its reversion-inducing activity. The latter observation suggested that *rap*1A was more "active" in the GTP-bound form.

More direct evidence that such a competitive model of Rap action was possible came from studies by Hata et al. (1990) and Frech et al. (1990). Both groups used purified components *in vitro* to demonstrate that Rap1A (Frech et al., 1990) or 1B (Hata et al., 1990) was able to compete with Ras for binding to Ras-GAP. Binding to Ras-GAP was more effective when Rap1 was bound with GTP than with GDP, with Frech et al. (1990) reporting that the GDP form of Rap1A had an affinity of at least 100-fold less than the GTP form, while Hata et al. (1990) only found a 2–3-fold difference between the two forms of Rap1B. Ras-GAP did not stimulate GTP hydrolysis by Rap1, as described in Quilliam et al. (1990), suggesting that the association of Rap1A with GAP produced a catalytically-inactive complex.

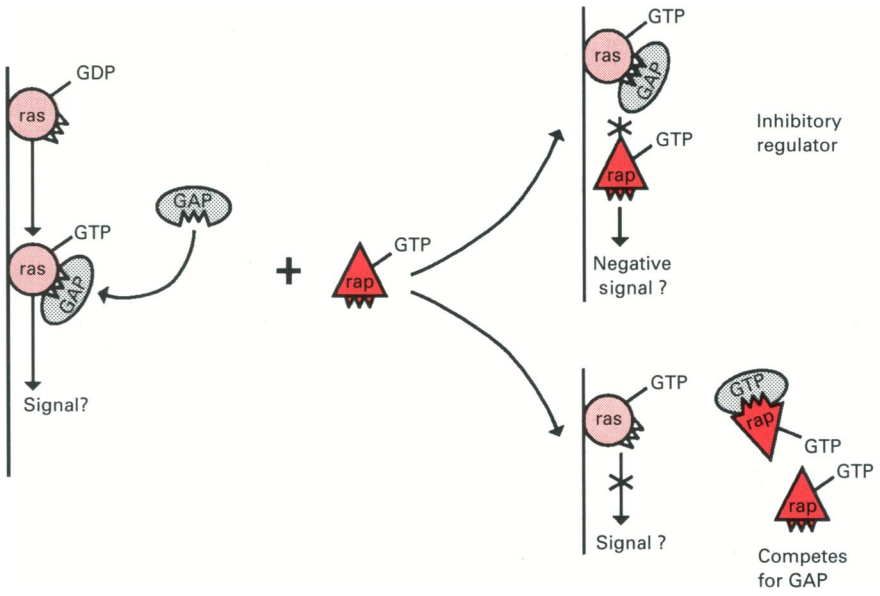

Figure 2 Hypothetical mechanisms through which Rap might antagonize Ras downstream signalling

▲▲▲ denotes the "effector" domain of Ras/Rap. GAP refers to Ras-GAP.

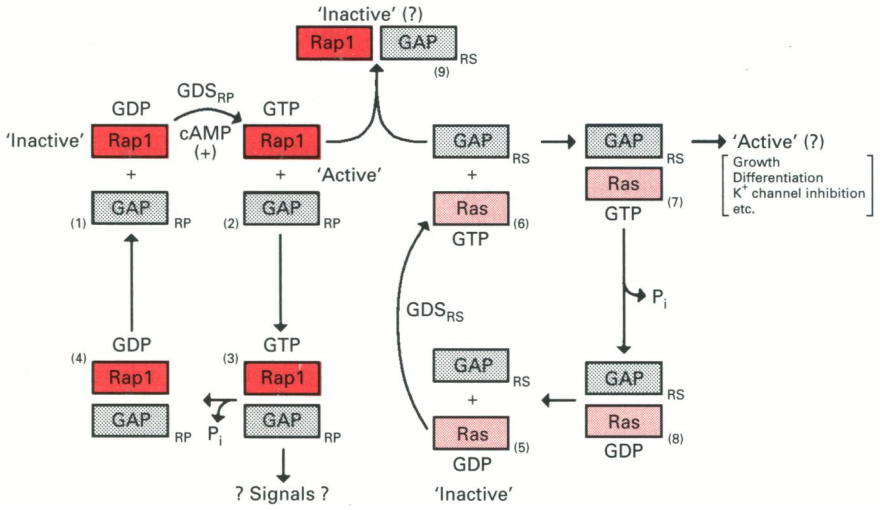

Figure 3 Model of Rap/Ras regulatory cycles

This Figure describes biochemical states of Ras and Rap1 and how the two GTP-binding proteins might interact based upon the concept that Rap acts as to bind Ras-GAP in a competitive manner, as described in the text. (1) In the basal or "inactive" state, Rap1 exists as the GDP form which is not associated with Rap GAP (GAP_{RP}). (2) Under the influence of a Rap-active GDS (GDS_{RP}), an interaction which may be regulated by cyclic AMP, GTP is exchanged for GDP on Rap1, producing the "active" form of Rap1. This active Rap1 may exert biological activities of its own, or may act in concert with GAP_{RP} to pass on downstream signals. The Rap1-GTP form has a high affinity for Ras-GAP (GAP_{RS}), effectively binding to GAP_{RS} to form a presumably inactive complex. This interaction would account for the anti-oncogenic action of Rap1 toward Ras. (3) Rap1-GTP binds to GAP_{RP}, which could represent an active signalling complex or (4) which catalyses the hydrolysis of Rap1-GTP to GDP, causing Rap1 to dissociate from GAP_{RP} and return to the "inactive" state. (5) Ras with GDP bound also represents an "inactive" state which (6) is acted on by a Ras-active GDS (GDS_{RS}) to exchange bound GDP for GTP, producing a biologically "active" Ras. (7) Ras-GTP binds to GAP_{RS}, leading to the generation of signals which result in the biological manifestations of Ras activity. (8) The action of GAP_{RS} converts Ras to the GDP form, which releases GAP_{RS} and returns to the initial "inactive" state.

Interestingly, the affinity of Rap1A–GTP for Ras-GAP was 50–100-fold greater than that of Ras–GTP. Since the concentration of Rap1 in cells such as platelets (Ohmori et al., 1989) and neutrophils (Quilliam et al., 1991) seems to be at least 10 times greater than that of Ras, this suggests that Rap1A might be able to limit the amount of GAP available for interaction with Ras *in vitro*.

The ability of Rap1A to antagonize Ras action by a competitive mechanism in an intact membrane system was demonstrated by Yatani et al. (1991). This study utilized the M_2 muscarinic receptor-regulated K^+ channel of atrial membranes, which is inhibited by the action of Ras and Ras-GAP acting in concert (Yatani et al., 1990). Using a patch-clamp technique to manipulate the levels of Rap1A, Ras and GAP in the system, it was

shown that: (1) Rap1A antagonized the effect of Ras-GAP on channel opening in a manner that was inversely proportional to the level of GAP added; (2) antagonism was dependent on an intact effector domain in Rap1A; (3) the inhibitory effect of Rap1A could be overcome by the addition of exogenous Ras; and (4) Rap1A did not antagonize a form of GAP (GAP32) whose ability to inhibit M_2 muscarinic receptor-regulated K^+ channels was independent of Ras. These results indicated that Rap1A was acting by a competitive mechanism, and that such inhibition could occur at picomolar concentrations of Rap1A, levels which are likely to occur in normal cells. The ability of Rap1A to suppress Ras-GAP action in this system, and also the antagonistic activity of Rap1B in *Xenopus* oocytes (Campa et al., 1991), indicates that this biological activity of Rap1 is not limited to the pathway leading to cell transformation by Ras. These studies also indicate that the antagonism of Ras action by Rap1 occurs via a competitive mechanism involving the "effector domain" of Rap1. A number of studies have localized the suppressive activity of Rap1 to the N-terminal portion of the molecule (Buss et al., 1991; Kitayama et al., 1990b; Zhang et al., 1990), and have identified residues 26, 27, 30, 31 and 45 as crucial in this regard (Marshall et al., 1991; Nur-e-Kamal et al., 1992). It is of interest then to note that both of the Rap2 proteins, as well as R-Ras, also contain an "effector domain" identical to Ras, yet do not suppress the ability of Ras to transform cells (Schweighoffer et al., 1990; Jimenez et al., 1991). Although R-Ras differs from Rap1 in the crucial amino acids adjacent to the "effector domain", Rap2 is identical to Rap1 in these positions. Consideration of these facts indicates that additional structural components need to be accounted for in order to understand antagonism of Ras by Rap1.

The physiological significance of the inhibition of Ras action by Rap1 is still uncertain. There is as yet little data to indicate that Rap1 plays such a regulatory role in normal cells. In fact, in some cell models of Ras action, Rap1 has been reported not to inhibit the actions of Ras (Schweighoffer et al., 1990). In yeast, mammalian *rap* genes can *stimulate* some of the same effector pathways as does H-*ras* (Xu et al., 1990; Ruggieri et al., 1992). It should be pointed out that most models we have discussed here to explain Rap suppression of Ras activity are based on the idea that Ras-GAP serves a downstream signalling function that can be disrupted by Rap (see Figure 3). If, in fact, Ras-GAP acts solely as a negative regulator of Ras activity in certain cells by keeping it in a GDP-bound state, then disruption of the binding of GAP to Ras could actually result in increased Ras activity!

It is tempting to speculate that, if Rap1 is indeed a physiological suppressor of Ras function, modulation of the guanine nucleotide state of Rap1 would play an important role in regulating the transduction of growth and differentiation signals via Ras (see Figure 3). One could thus envisage that changes in the activity of Rap1 GAPs and GDSs, etc., would also produce marked effects on Ras activity indirectly through their ability to regulate Rap1–GTP formation and thus binding to Ras-GAP(s). Mutations in Rap1 or in Rap1-associated regulatory components might then play a significant role in the pathogenesis of mammalian tumours. A marked decrease in the levels of expression of *rap*1 mRNA was reported in several types of tumours not normally associated with *ras* mutations (e.g. salivary gland fibrosarcomas and adenocarcinomas) by Culine et al. (1989). *rap*1B has been mapped to a chromosomal location near breakpoints associated with a number of malignant and benign neoplasms (Rousseau-Merck et al., 1990). Several investigations of Rap1 levels (Hong et al., 1990; Hsu and Gould, 1991) and loss of heterozygosity (Young et al., 1992) in certain types of tumours have proven negative. However, Kyprianou and Taylor-Papadimitriou (1992) reported that Rap1 levels in azatyrosine-induced revertants of *ras*-transformed human mammary epithelial cells were significantly increased. Clearly, further studies along such lines are warranted.

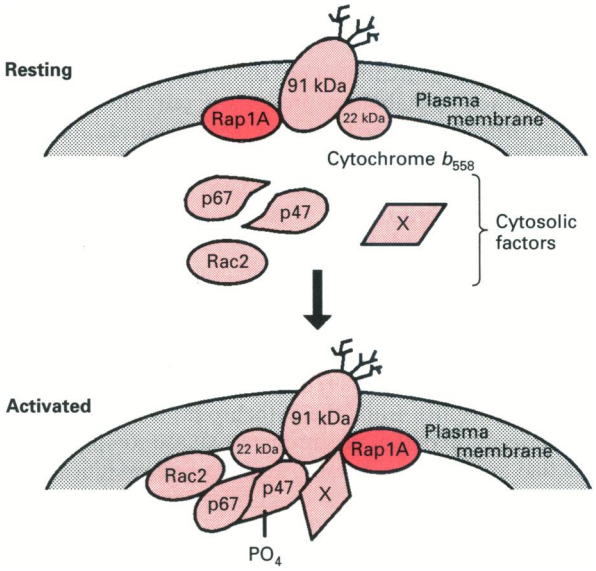

Figure 4 The phagocyte respiratory burst oxidase

Two GTP-binding proteins, Rac2 and Rap1A, appear to be involved in this enzyme system. 91 kDa and 22 kDa represent the subunits of the cytochrome b_{558}. Changes in the relative positions of these two subunits in the Figure are intended to represent schematically the potential conformational changes occuring in the cytochrome upon oxidase activation. p47, p67 and X are the cytosolic components of the NADPH oxidase. PO_4 indicates the known incorporation of phosphate groups into p47 during activation of the oxidase.

Interaction of Rap1A with the phagocyte NADPH oxidase

The question of whether Rap1 serves as a physiological anti-oncogene remains an unsettled issue. The abundance of Rap1 in a number of untransformed cells, such as platelets and neutrophils, the existence of multiple Rap-specific GAPs which can potentially serve unique effector pathways, and the possible regulation of Rap1 and Rap-GAP by phosphorylation, all suggest that Rap may regulate other functions normally in cells. In support of this hypothesis, there is evidence that Rap1A may play some role in regulation of the NADPH oxidase system of human neutrophils.

Human neutrophils and other phagocytic cells respond rapidly to contact with opsonized micro-organisms by undergoing a "respiratory burst" in which molecular oxygen is reduced to form superoxide anion (O_2^-). O_2^- is subsequently converted to other toxic oxygen metabolites (Clark, 1990). This respiratory burst is catalysed by the NADPH oxidase, a multicomponent enzyme composed of at least four known proteins (see Figure 4). A cytochrome b_{558}, consisting of 91 kDa ($gp91_{phox}$) and 22 kDa ($gp22_{phox}$) subunits, resides in the plasma membrane and membrane of neutrophil-specific granules where it serves as the terminal electron carrier of the oxidase. Two additional cofactors, $p47_{phox}$ and $p67_{phox}$, are cytosolic and may exist as preformed complexes which translocate to the plasma membrane upon phagocyte activation, becoming integral parts of the active oxidase (Clark, 1990; Curnutte, 1992).

Guanine nucleotides, specifically GTP, have been shown to be absolutely required for NADPH oxidase activity in cell-free assay systems (Peveri et al., 1992; Uhlinger et al., 1991),

supporting the idea of a GTP-binding protein being involved in oxidase regulation (Bokoch, 1990). Bokoch and Prossnitz (1992) have also shown that treatment of HL-60 cells with drugs able to block isoprenoid metabolism prevents a respiratory burst response in these cells after they have been differentiated into a neutrophil-like form by dimethyl sulphoxide. This isoprenoid requirement is presumably due to the need for isoprenoids in the post-translational processing of a low-molecular-mass GTP-binding protein. Indeed, Rac2 (Knaus et al., 1991) and possibly Rac1 (Abo et al., 1991) have been identified as important stimulatory regulators of the NADPH oxidase in human neutrophils. There are indications though that Rap1A must also play some modulatory role in this system. The first suggestion of this came when Quinn et al. (1989) observed the co-isolation of a 22 kDa protein distinct from the cytochrome *b* 22 kDa subunit during purification of the cytochrome *b* oxidase component. This protein had an N-terminal amino acid sequence identical to that of Rap1. Evidence that this co-purification was not merely fortuitous (since Rap1 could be separated from the cytochrome at a final sucrose gradient step) was provided by their observation that Rap1 present in neutrophil extracts would also bind to anti-cytochrome 91 kDa or 22 kDa antibody columns.

These observations were confirmed and extended by *in vitro* studies using a pure cytochrome preparation and purified baculovirus-Sf9 cell recombinant Rap1A or Rap1A purified from human neutrophils (Bokoch et al., 1991). Complexes between the two purified proteins were detected by gel filtration analysis. Formation of complex appeared to occur through the 1:1 association of Rap1A with the cytochrome. These results demonstrated that Rap1A binds directly to the cytochrome itself. The ability of Rap1A to interact with the cytochrome when it was in a GDP versus GTPγS-bound state was also examined. Rap1A bound to cytochrome in both the GTP and GDP forms; it appeared that the interaction was more stable when Rap1A was complexed with GTPγS, however, since the ability to observe the Rap1A–GDP–cytochrome complex was variable.

Agonists which elevate cyclic AMP in neutrophils are able to attenuate the respiratory burst in these cells (Rivkin et al., 1975; Sha'afi and Molski, 1988; Mueller et al., 1988). Since Rap1A can be phosphorylated by cyclic AMP-elevating agents in intact HL-60 cells (Quilliam et al., 1991), and stoichiometric phosphorylation of Rap1A *in vitro* does not affect its ability to bind or hydrolyse guanine nucleotides, nor to respond to a cytosolic Rap-GAP (Quilliam et al., 1990; Bokoch and Quilliam, 1990), the possibility of effects on Rap1A–cytochrome binding was examined. Bokoch et al. (1991) observed that the ability of phosphorylated Rap1A–GTPγS to form complexes with cytochrome *b* was markedly reduced. A potential mechanism by which elevations in neutrophil cyclic AMP could inhibit the respiratory burst response, therefore, is by modulation of Rap1A–cytochrome interactions.

It is of note that Rap1B phosphorylation has been shown to enhance its ability to bind and be stimulated by a GDS (Hata et al., 1991; Itoh et al., 1991). It is possible that Rap1 phosphorylation serves a general regulatory role for this low-molecular-mass GTP-binding protein. Thus, the interaction of Rap1A and 1B with other macromolecules at the C-terminus is postulated to occur, and to be modulated, either positively or negatively, by Rap1 phosphorylation. Such regulation could also extend to K-Ras, which has a similar cationic region at its C-terminus and which has been reported to undergo C-terminal phosphorylation as well (Ballester et al., 1987).

While it has been established that Rap1A can interact with the NADPH oxidase-associated cytochrome *b*, the significance of this interaction is not yet clear in terms of NADPH oxidase function. Eklund et al. (1991) have reported that an antiserum against a synthetic peptide corresponding to the effector region of Rap1A (amino acids 31–43) was able to totally inhibit oxidase activity in a cell-free system and that activity could be restored by addition of recombinant Rap1A. Similar experiments have proven unsuccessful in our laboratory (G. M. Bokoch, unpublished work). Recently, Mizuno et al. (1992) have also reported that Rap1 was inactive in supporting oxidase activity in a cell-free system. The reasons for these discrepancies are not yet clear. Several laboratories (Maridonneau-Parini and de Gunzburg, 1992; Quilliam et al., 1991; Quinn et al., 1992a) only find Rap1 in neutrophil membrane fractions when localized by specific immunblotting, in contrast to Eklund et al. (1991), who report Rap1 to be cytosolic and to translate from cytosol to membrane upon neutrophil activation by phorbol myristate acetate. The functional role of Rap1A in the NADPH oxidase system is thus not clear. It is possible that Rap1A (unlike Rac2) may play a more subtle role in the system which may not be evident in studies using cell-free assays, which clearly rely on non-physiological activators in a structurally disrupted system. For example, based upon studies of the BUD1 (or RSR1) Rap1A homologue in *Saccharomyces cerevesiae* which indicate it is crucial for positional information relevant to bud site selection (Ruggieri et al., 1992) and upon data which indicate an association of Rap1B with the platelet cytoskeleton upon thrombin activation (Fischer et al., 1990), it is possible that Rap1A in the neutrophil might direct translocation of cytochrome *b* to specific membrane sites upon cell activation or might mediate interactions between oxidase components and the cell cytoskeleton. Such interactions are thought to be important for oxidase activation and deactivation *in vivo* (Clark, 1990; Heyworth et al., 1991; Nauseef et al., 1991) and would be undetectable in normal cell-free oxidase assays. Recently, Quinn et al. (1992b) reported that the ability of partially purified Rap1-associated cytochrome *b* to reconstitute NADPH oxidase activity in CGD patient membranes lacking the cytochrome was decreased when the cytochrome was purified to essential homogeneity. This may reflect a requirement for Rap in order to allow the reconstitutive capacity of the cytochrome to occur. Other scenarios are also possible, as discussed in Quilliam and Bokoch (1992).

CONCLUSIONS AND FUTURE PROSPECTS

There are a number of inferences that can be drawn from the information just presented with regard to the biology of Rap. While it has not yet been shown that the ability of Rap1 to antagonize Ras action is of significance *in vivo*, such an action of Rap does occur *in vitro* and seems likely to be relevant to physiological growth regulation and/or tumourigenesis. Interactions of the Rap1 and Ras proteins at the biochemical level are depicted in Figure 3. The fact that Rap1A and 1B serve as excellent substrates for cyclic AMP-dependent protein kinase, as well as a number of other kinases, and that this phosphorylation can regulate the macromolecular interactions that Rap can undergo *in vitro*, also implicates Rap in cellular regulation. Activation of these kinases via cyclic AMP generation, etc. in intact cells is likely to exert significant effects then on the GTP state of Rap (via GDS) and, therefore, its suppression of Ras function. Hopefully, studies directed at such regulatory pathways involving Rap in intact cells will be forthcoming in the near future.

It is known that in some cell types elevations in cyclic AMP levels can lead to tumourigenesis (Dumont et al., 1989; Vallar et al., 1987), but the mechanism of this effect has not been established. A possible role for Rap activity in this process

deserves investigation. Stimulation of Rap1 GTP binding via the activation of Rap GDS caused by increases in cyclic AMP would be expected to influence biological actions of Rap in other cellular systems as well. Rap may mediate some of the effects of cyclic AMP in human neutrophils (Quilliam et al., 1991), where cyclic AMP inhibits activity of the NADPH oxidase, and in human platelets (Lazarowski et al., 1990), where activation of phospholipase C is blocked by cyclic AMP. Finally, there are indications that Rap activity may be regulated by hormones *in vivo* through actions on its GAP (Marti and Lapetina, 1992). The concept that the activity of Rap and other low-molecular-mass GTP-binding proteins may be regulated by the seven-trans-membrane-spanning-domain receptors classically linked to the heterotrimeric G-proteins and/or that Rap might regulate the activity of these receptors in turn (Yatani et al., 1991) is an exciting one, with widespread implications for the integration of cell functions.

The association of Rap1A with the NADPH oxidase cytochrome *b* component in human neutrophils is more difficult to assess. Unlike oxidase regulation by Rac2, a GTP-binding protein which is expressed solely in cells of haematopoietic lineage (Didsbury et al., 1989), Rap1A is widely expressed and would seem unlikely to be solely a regulatory component for a phagocyte-specific enzyme. One hypothesis is that Rap1 plays a more general function in the cell, and that this function involves different protein components in different cells. Regulation of cytoskeletal interactions or protein–protein interactions in general are possibilities that are consistent with the known functions of other low-molecular-mass GTP-binding proteins. This hypothesis predicts similar roles for Rap1 in other cells, but the biological consequences of its action would differ completely. Suppport for this hypothesis will require further work on the biochemical actions of Rap in a variety of cell types.

While at this time we do not fully understand the interrelationships between biological events regulated by various members of the *ras* superfamily, there are indications that there may be a substantial amount of cross-talk between individual low-molecular-mass GTP-binding proteins. This is indicated by the recent identification of a variety of proteins which appear to contain multiple functional domains capable of GTP binding, GAP, or guanine nucleotide exchange activities directed against more than one GTP-binding protein (described in Hall, 1992; Marx, 1992). It is apparent from yeast genetic studies (Chant and Herskowitz, 1991) that the yeast homologue of Rap1A (BUD1) may interact with CDC42 to co-ordinate bud site selection and assembly. CDC42 is a low-molecular-mass GTP-binding protein which is closely related to Rac (Shinjo et al., 1990; Didsbury et al., 1989). It is very suggestive then that both Rap1A and Rac are associated with regulation of the NADPH oxidase system, which may involve very similar processes of site-directed protein assembly at the membrane. Hopefully, continuing progress in yeast genetic studies and in biochemical investigations of Rap1A, Rac, Ras, and CDC42Hs function in mammalian cells will provide us with a much clearer view of the complicated biological interactions involving these proteins and their associated regulatory proteins.

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