REVIEW ARTICLE

The biochemistry of *ras* p21

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INTRODUCTION

If ever there was a structure in search of a function it is *ras* p21. Since their discovery as the cellular homologues of the 21 kDa (thus p21) transforming proteins of the Harvey and Kirsten murine sarcoma viruses (*H-ras* and *K-ras*), *ras* proteins have been the subject of exhaustive study to such an extent that we now know their primary structures, three-dimensional structure (to 1.35 Å), post-translational modifications, features in common with a number of related polypeptides, subcellular localization, and the mechanism of guanine nucleotide binding and hydrolase (GTPase) activity. The sum of our knowledge, in biochemical and biophysical terms, of p21 is immense and yet its function within the cell remains virtually unknown. It seems an appropriate time, therefore, to take stock of all this accumulated structural data, together with the results of a large number of more biological studies, and see whether they provide any significant clues to the role of p21 in the normal cell as well as to examine how a single amino acid substitution (at one of a very limited number of sites) produces an 'activated' protein which, even though its ability to bind GDP is normally unchanged, is capable of transforming cells *in vitro* and possibly plays a major part in tumourigenesis *in vivo*. By analogy with classical G-proteins, Ras polypeptides are considered to be in an active (possibly signal transducing) 'on conformation' in the presence of GTP and 'off' in the presence of GDP.

In this Review we have tried to examine these themes, amongst others, and to give an overview of the biochemistry of mammalian *ras* p21. We have, however, paid relatively little attention to other related, well-characterized members of the GTPase family (such as elongation factor Tu or the α subunits of heterotrimeric G proteins) or to the RAS proteins in yeast. Readers requiring a more detailed consideration of these aspects are referred to excellent recent reviews by Bourne *et al.* (1990, 1991) and Broach & Deschenes (1990).

STRUCTURE OF *ras* p21

The structure of p21 is known in considerable detail. The amino acid sequences of different Ras and Ras-related proteins from a number of sources have been determined indirectly by DNA sequencing. Recently, the three-dimensional structures of wild type and mutant H-Ras proteins have been described based on crystallographic and n.m.r. studies. In both cases the Ras polypeptides show considerable similarity to other well-characterized G protein α chains and *Escherichia coli* EF-Tu (Leberman & Egner, 1984; Masters *et al.*, 1986; Jurnak, 1985).

The primary structures of *H-ras*, *K-ras* A and B and *N-ras* (a gene with no viral equivalent, first identified in a human neuroblastoma cell line) proteins are very similar within species (for example, human N, H and K-Ras have more than 80 % of their amino acid sequence in common) with the N-terminal

halves of the proteins being virtually identical (detailed amino acid sequence data are given by Barbacid, 1987). Similarly, *ras* genes are highly conserved between species; thus the human and rat H-*ras* p21s are identical and there are only four amino acid substitutions when comparing these sequences with that from chicken. Ras equivalents have also been identified in more primitive organisms such as insects, molluscs, slime moulds, plants and yeast (reviewed by Barbacid, 1987). All of these sequences are most similar in the N-terminal 80 and in the final four amino acids. The primary structures of *ras* proteins are least similar in the C-terminal region from residues 165 to 185 (based on the mammalian sequence) and this part of the molecule has been termed the 'hypervariable region'. It has been suggested that it is involved in the interaction with other unique cellular components, but unequivocal supportive evidence is not yet available. The C-terminal four residues comprising the CAAX box are conserved in all *ras* proteins (see below) and are involved in post-translational modification and membrane localization. On the basis of mutational analysis and crystallographic studies, various regions in p21 have been assigned as interaction sites for either nucleotide and/or other proteins. Thus, the guanine base of GDP and GTP is bound at around amino acids 120 and 145 and the phosphate groups bind around amino acids 12–20, 32–35 and 60. The GTPase activating protein (GAP) binds in the 'effector domain', amino acids 32–40. These binding sites are discussed in detail below.

As well as the *bona fide ras* genes a large number of Ras-related proteins have been identified (reviewed by Hall, 1990a); these are discussed in more detail below. Briefly, however, it is convenient to divide these polypeptides into three groups on the basis of their similarity to mammalian H-Ras and to each other. Firstly, those with greater than 50 % similarity, the *ras* family; secondly, the *rho* family with about 30 % similarity; and thirdly, the *rab* group which is also about 30 % similar to Ras (see below). All of these proteins appear to bind GTP and probably exhibit common structural features which are likely to be involved in nucleotide binding and/or hydrolysis.

From X-ray crystallographic studies of human H-*ras* protein, it has been possible to give a detailed description of the three-dimensional structure of both wild-type and mutant polypeptide chains in the presence of GDP. Also, by using p21 crystallized with p^3 -1-(2-nitro)phenylethylguanosine 5'-O-triphosphate (caged GTP) at the active site it has been possible to examine the GTP-bound form as well as the conformational changes occurring on GTP hydrolysis (Schlichting *et al.*, 1989, 1990a,b).

On the basis of reports from two laboratories, the overall backbone structure of p21 (Figs. 1 and 2) (de Vos *et al.*, 1988; Tong *et al.*, 1989a, 1991; Pai *et al.*, 1989, 1990; Milburn *et al.*, 1990) can be described. The protein as crystallized comprises five α -helices, one six-stranded β -sheet and ten interconnecting loops (Figs. 1 and 2). The β -sheet may be divided into two three-stranded subsets which are approximately at right angles to each

Abbreviations used: GAP GTPase activating protein; GNRP, guanine nucleotide release protein; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; PL, phospholipase; CSF, colony-stimulating factor; PKC, protein kinase C; ARF, ADP-ribosylation factor; GDI, GDP dissociation inhibitor; NGF, nerve growth factor; FGF, fibroblast growth factor; DAG, diacylglycerol.

Fig. 1. Cartoon of the three-dimensional structure of H-ras p21 in the presence of GTP

The structure shown is based broadly on the crystal structure of the *N*-terminal 161 amino acids of p21 as determined by Pai *et al.* (1989, 1990), and represents a truncated form of the protein (residues 1–166). β -sheets are shown as broad arrows, loops as narrow lines and α -helices as helical structures. Residue numbers are shown. Areas of the protein involved in GTP binding are shown in red and the effector loop that is involved in the interaction of p21 with GAP by hatching.

other. Of the six strands, five are anti-parallel and one parallel. There are some discrepancies, however, in the precise dimensions of the different sheet, loop and helix regions as described by the two groups of workers (Pai *et al.*, 1990; Tong *et al.*, 1991), but overall there is general agreement on the secondary structure of the protein.

Only three hydrophobic amino acids appear to be exposed on the surface of p21 (A121, A122 and L171), the remainder being buried inside (Tong *et al.*, 1991). Conversely, only two charged amino acids (K16 and D57) are buried in the interior, the remainder being located on the surface. Many of these polar residues are involved in salt bridge formation (Tong *et al.*, 1991). K16 and D57 are two of the amino acids which are involved in GDP and GTP binding. These, and the other residues which form hydrogen bonds with the nucleotide, are summarized in Fig. 3 (taken from Pai *et al.*, 1990).

Two areas of the protein comprising amino acids 116–119 and 145–147 are highly conserved in all *ras* molecules and are involved in binding to the purine base of GDP (Pai *et al.*, 1990; Tong *et al.*, 1991). Thus, hydrogen bonds are formed with N116 and D119 and S145 and A146 (Fig. 3). N116 appears to play a central role in determining the conformation of the GDP-binding site because, not only does it form a hydrogen bond with the purine ring but also with T144 and the main chain O of V14, orienting the three elements of the nucleotide-binding site (Pai *et al.*, 1990). Hydrophobic interactions take place between the guanine ring and the phenyl group of F28 and the alkyl group of K117. These amino acids (F28, N116, K117, D119, S145, A146 and K147) form a 'deep narrow groove' which has great specificity for the guanine base (Tong *et al.*, 1991). There are a large number of interactions formed between the phosphate groups of GDP or GTP and p21 (Fig. 3). The α phosphate is hydrogen-bonded to the main chain amide of A18 and weakly to the similar group of S17 (Pai *et al.*, 1990). The paucity of hydrogen bonding to the α -phosphate compared to the β and γ groups

explains why K_a for GMP is approximately six orders of magnitude less than for GDP (John *et al.*, 1990). The β -phosphate group is hydrogen-bonded to the main chain amino groups of residues 13–17 [all of which point towards the phosphate group, creating a polarized local electrostatic field (Pai *et al.*, 1990)]. The ϵ amino group of K16 also interacts with oxygen atoms in both the β and γ phosphate groups (Fig. 3). The γ -phosphate is also extensively hydrogen-bonded to T35 (both main chain amino and hydroxyl groups), Y32 and G60. The extent of hydrogen bonding to the nucleotide presumably accounts for the considerable increase in stability of the GDP-bound state over the apoprotein. A single Mg^{2+} ion is bound to *ras* p21 which is essential for GTPase activity (J. John, unpublished data quoted in Pai *et al.*, 1990) and for conformational stability (Grand & Grant, 1989). The six Mg^{2+} ligands are co-ordinated in an octahedral arrangement. There is some contradiction in the reports of the precise nature of the Mg^{2+} -binding site (Milburn *et al.*, 1990; Pai *et al.*, 1990; Tong *et al.*, 1991). Pai *et al.* (1989a, 1990) have suggested that, in the GTP-bound form, the metal ion is co-ordinated to oxygen atoms from the β and γ -phosphates, hydroxyl groups of S17 and T35 and two water molecules which are, in turn, hydrogen-bonded to D57 and D33 (Pai *et al.*, 1990). In this model in the GDP-bound form the interaction with the γ -phosphate O is replaced by a direct co-ordination to the carboxyl group of D57. T35 is presumably replaced in the co-ordination sphere by a water molecule (Schlichting *et al.*, 1990). An alternative structure for the Mg^{2+} binding site has been suggested by Milburn *et al.* (1990) and Tong *et al.* (1991) for the GDP·p21 in which the Mg^{2+} ion is co-ordinated by O atoms from the β -phosphate of GDP, S17 and four water molecules. In the triphosphate-bound form one of the water molecules is replaced by the OH side chain of T35 (Tong *et al.*, 1991), in agreement with the observations of Pai *et al.* (1990). However the weight of crystallographic evidence appears to favour the structure proposed by the 'Heidelberg group' since they have determined the

Fig. 2. Diagrammatic representation of the secondary structure of H-*ras* p21

Amino acid numbers are shown and those residues involved in α -helices, β -sheets and loop regions are enclosed in $\diamond$, $\square$ and $\circ$ respectively. Hydrogen bonds are shown by arrows and the five α -helices, six β -sheet regions and ten loops are indicated. Data adapted from Pai *et al.* (1990) and reproduced by kind permission of Oxford University Press.

crystal structure at higher resolution, but it should be noted that data from e.p.r. investigations of Mn(II)GDP·Ras complexes indicates that four water molecules co-ordinate to the metal ion together with an O from the β -phosphate group and one O from the protein (Smithers *et al.*, 1990).

For future investigations of the mode of action of p21 it is essential to understand in detail the interaction with GAP and for this it is necessary to have as much structural information as possible about the binding site (amino acids 32–40, termed the 'effector region' or 'switch I' region). This area, which mainly comprises loop 2, is exposed on the surface of p21 and is therefore available for binding to the large GAP polypeptide chain (de Vos *et al.*, 1988) (Fig. 1). A comparison of the GDP- and GTP-bound states of Ras indicates that differences between the two proteins are most marked in this area (Schlichting *et al.*, 1990a; Milburn *et al.*, 1990), in particular Y32, D33, P34, T35 and I36 all have different orientations (Schlichting *et al.*, 1990a; Milburn *et al.*, 1990; Pai *et al.*, 1990). The implication from these observations is that GAP binding to p21 will be determined by the conformation of this area which will, in turn, be dependent on the nucleotide status of Ras.

Differences in structures of the GDP- and GTP-bound states of p21, other than in the effector region, have also been observed. The Mg²⁺ ion is co-ordinated to the carboxyl group of D57 directly in the presence of GDP (at least in the structure proposed by Schlichting *et al.*, 1990a) rather than to a water molecule. There are also changes in the phosphate-binding site; in particular, K16 no longer interacts with the β -phosphate group and, of course, the hydrogen bonds between the oxygen atoms of the γ -phosphate (to G60, Y32 and T35) are absent. Differences have also been observed in the loop 4 region (residues 60–65, which is part of the switch II region; Milburn *et al.*, 1990), which has a well-defined structure in the presence of GDP but not GTP. This change in conformation in this area may be partially explained by the absence of the hydrogen bond between the γ -phosphate group and the main chain amino group of G60 (Schlichting *et al.*, 1990a; Milburn *et al.*, 1990).

Based on the refined crystal structure (1.35 Å) of p21 in the presence of caged GTP, a mechanism for GTP hydrolysis has been proposed (Pai *et al.*, 1990; Krenkel *et al.*, 1990). It has been suggested that a water molecule (Wat175), hydrogen-bonded to the carbonyl O of T35, is activated for a nucleophilic substitution

Broken lines indicate hydrogen bonds ($< 3.4 \text{ \AA}$). Distances between donor and acceptor atoms (in $\text{\AA}$) are indicated next to the labels except where one residue makes several bonds, in which case the distances are given next to the dotted lines. Wat, water molecule; MC, main chain. Data taken from Pai *et al.* (1990) and reproduced by kind permission of Oxford University Press.

A conformational change by p21 after GTP binding is probably the rate-limiting step in the hydrolysis reaction (Neal *et al.*, 1990; discussed in detail below) and it has been suggested that the movement of loop 4, bringing Q61 into the correct orientation, fulfils this requirement (Pai *et al.*, 1990; Krengel *et al.*, 1990).

Whilst it may be rather premature to consider the crystal structure described above as totally correct, particularly in the light of past retractions and changes made to published work (Tong *et al.*, 1989b), it does appear that there is now general agreement on the major features of the three-dimensional structure of p21 (Figs. 1 and 2). However, there are differences in detail (for example in the Mg²⁺ binding site) between the crystal structures that have been determined in the two laboratories. The implications of these discrepancies for the proposed scheme of GTP hydrolysis which is described above are not clear at present, as a precise mechanism based on the structure proposed by Tong *et al.* (1991) has not yet been described.

ras genes with point mutations are found in approximately 30% of human tumours and are therefore the most common oncogenes associated with human carcinogenesis (Barbacid, 1987; Bos, 1988, 1989). A study of mutational analysis of *ras* falls into two categories, firstly the identification of *ras* mutations in natural or induced malignancies, and secondly mutation *in*

in vitro of cloned *ras* genes followed by assays for transforming ability. The first approach identifies mutations only in amino acids 12, 13 and 61, with residue 59 implicated from studies with viral *ras* analogues [Reddy *et al.*, 1982; Tabin *et al.*, 1982; McGrath *et al.*, 1984; Manne *et al.*, 1985; Der *et al.*, 1986a; Temeles *et al.*, 1985; Bos *et al.*, 1985; Trahey *et al.*, 1987; Barbacid, 1987 (and references therein); Bos, 1988, 1989 (and references therein)]. However, extensive use of the second approach has revealed many other sites where mutations give rise to transforming ability. These mutants fall into three main biochemical categories: (i) mutants with decreased nucleotide binding capacity, (ii) mutants with decreased GTPase activity, and (iii) effector region mutants. In the light of the recent exciting advances in the elucidation of the high-resolution structure of p21 in both the GDP- and GTP-bound forms (as detailed in the previous section), it has become possible to explain many activating mutations in molecular terms. Indeed, many such mutations group into functionally distinct domains of the Ras proteins.

Mutants that have a decreased affinity for nucleotides are frequently found as transforming mutants. These proteins exchange their GDP faster than the wild type and are therefore likely to go through more activating conformational changes due to GTP binding. In essence, these mutants appear to have made superfluous the need for a guanine nucleotide release protein (GNRP; see below), utilized by the wild-type protein to increase the normally slow GDP off-rate. These mutations are found in those residues involved in nucleotide binding (see Fig. 3). D116, K117 and D119 form hydrogen bonds with the guanine ring, while the amide protons of D116 and K117 stabilize the nucleotide binding site by hydrogen-bonding with G13 and V14. Mutation at any of these three sites results in a significant reduction in nucleotide binding (10–5000 fold) so ‘activating’ the protein (Der *et al.*, 1986a; Feig *et al.*, 1986; Sigal *et al.*, 1986; Walter *et al.*, 1986; Clanton *et al.*, 1986). However, mutations

that totally abolish nucleotide binding have no transforming ability, as the mutant proteins can never enter the active conformation (for signal transduction) (Clanton *et al.*, 1986, 1987). The nucleotide-binding site is also stabilized by amino acids 144–147 (see Fig. 3). Mutation of T144, which interacts with N116, has been shown to have a similar phenotype to that described for mutations in residues 116–119 (Feig *et al.*, 1986).

Substitution of N for K at residue 16 results in a decrease in nucleotide affinity but not specificity (Sigal *et al.*, 1986). The ϵ -amino group of K16 is seen to interact with the β - and γ -phosphates of GTP, thus explaining a loss in affinity without affecting specificity, as K16 does not interact with the guanine ring. However, mutation of residue 16 has further implications. As the ϵ -amino group is co-ordinated to both β - and γ -phosphate groups it is ideally situated to aid hydrolysis of GDP, by increasing hydrophilicity of the γ -phosphate and the acidity of the leaving GDP (Pai *et al.*, 1990), as discussed in the previous section. K16 is also of overall importance in the structure of p21, as it is one of only two interior charged residues (see the previous section) and hydrogen bonds with residues 10 and 11 to stabilize secondary structure of the protein (Tong *et al.*, 1991). Substitution of this residue often leads to gross instabilities in the polypeptide chain (our unpublished work). Substitutions at K16 can also have steric effects on S17, which hydrogen-bonds with the α - and β -phosphates to stabilize the binding site. This effect of substitution of different side chains applies equally well to many mutations in different regions of the molecule. The other residue critical for nucleotide binding is F28, which binds the guanine base through aromatic–aromatic interactions. F28 is itself held by a similar interaction with K147. Mutation at either of these sites can lead to a decrease in nucleotide binding and produce a ‘transforming mutation’. Similarly, substitutions in any of the residues involved in stabilizing the nucleotide binding site (Fig. 3), or holding the nucleotide could cause a decrease in nucleotide affinity and therefore be transforming.

Many groups have reported a decrease in GTPase activity associated with oncogenic variants of p21 (McGrath *et al.*, 1984; Temeles *et al.*, 1985; Manne *et al.*, 1985; Colby *et al.*, 1986; Trahey *et al.*, 1987). Mutations that fall into this group include those found in malignancies at residues 12 and 61 (Seeburg *et al.*, 1984; Fasano *et al.*, 1984; Bizub *et al.*, 1988). Most can be explained by considering the molecular model for GTP hydrolysis by p21 outlined above.

Hydrolysis is mediated by a nucleophilic attack by Wat175, the carbonyl group or the amide nitrogen of the carbamyl group of Q61 activating the water molecule assisted by the carbonyl group of T35. This immediately explains the activating ability of mutations at amino acids 35 and 61. Oncogenic substitutions of L and H at codon 61 cannot activate Wat175 due to side chain differences (Krengel *et al.*, 1990); specifically, the side chains of L61 are unable to activate the nucleophile and H61 cannot orientate correctly, occupying the position of Wat175 itself. This is also reflected in the GTPase activity of EF-Tu which is lower than that for p21, possibly because of a histidine residue at the equivalent position to p21 residue 61. Analysis of Rap proteins, which have a lower GTPase activity than Ras, also shows the presence of a threonine at position 61; mutation of this to glutamine increases GTPase activity to the level of Ras (Frech *et al.*, 1990). Mutational analysis of various substitutions at amino acid 61 has been undertaken (Der *et al.*, 1986b) and 17 mutations giving rise to a decrease in GTPase activity have been identified. Interestingly, E61 is not an activating mutation, presumably because this amino acid can provide the necessary groups for polarization of the attacking water molecule (Milburn *et al.*, 1990).

The other commonly found activating mutations at position

12 can also be explained by this GTP hydrolysis model. Crystal structures of both R12 and V12 mutants have been determined (Krengel *et al.*, 1990). In the former the guanidinium group of R12 displaces Wat175, not allowing hydrolysis to occur, whereas the large hydrophobic branched side chain of V12 pushes Q61 and Wat175 too far apart for them to be able to interact. The model also explains why proline is the only non-activating substitution at codon 12, its presence causing no distortions of the protein molecule in this position. Mutations at residue 13 have also been found to be activating (Bos *et al.*, 1985). The rigidity of loop 1 means the replacement of either glycine residue at positions 12 or 13 with any substitution other than proline leads to structural distortions that prevent hydrolysis (Tong *et al.*, 1991). G13 is also involved in stabilizing the nucleotide binding site through a hydrogen bond with K117, which itself interacts with the ribose group, thus explaining why codon 13 mutations also can lack GTP binding activity (Clanton *et al.*, 1987), although these are not activating as discussed previously. Deletions or insertions in the region around codons 12 and 13 produce transforming proteins, presumably due to distortions causing non-alignment of Wat175 with the γ -phosphate (Chipperfield *et al.*, 1985).

Other activating mutations can be explained on the basis of their involvement in maintaining correct orientations of residues involved in hydrolysis. Both Schlichting *et al.* (1990a) and Pai *et al.* (1990) note different possible conformations for the side chain of Q61, only one of which can act to activate Wat175. E63 is postulated to hydrogen bond with Q61, stabilizing its active configuration and so aiding hydrolysis (Pai *et al.*, 1990). Substitution of K at codon 63 has been found to be an activating mutation which substantiates this role for E63 (Fasano *et al.*, 1984). Activating mutations also occur at positions 59 and 60. G60 is thought to hydrogen bond to the γ -phosphate of GTP, so substitution here could lead to the misalignment of the substrate in the hydrolysis reaction. This amino acid is also highly conserved in many GTPases, emphasising its functional importance. Amino acid 59 is found mutated in the viral *ras* analogues, thus becoming the site of autophosphorylation (see below). In an A-to-T substitution at amino acid 59, the side chain of threonine comes within 1 Å of Wat175 and so can attack the γ -phosphate itself. However, as this reaction occurs at a reduced rate in comparison to the wild type GTP hydrolysis reaction, it would explain the activating properties of this mutation (allowing the active GTP form of p21 to persist) (John *et al.*, 1988).

The Mg^{2+} -binding site comprises Wat172 and 173, S17, T35 and the γ -phosphate of GTP primarily, but is further stabilized by interactions with D57, D33 and the α -phosphate (Pai *et al.*, 1990). Thus, mutations in any of these amino acids could again decrease GTPase activity and lead to activation (because of the absolute requirement for Mg^{2+} for GTP hydrolysis). Certain of these residues also lie in the effector or switch 1 region (the GAP binding site).

Originally, it was felt that the decrease in GTPase activity of various mutants was the reason for their oncogenic activity. However, decrease in GTPase activity does not correlate well with transforming ability. It is therefore more probably due to an inability of these mutants to have their GTPase activity stimulated by GAP, only the GAP-stimulated GTPase activity being biologically significant. The precise molecular contribution of GAP to GTPase activity has not yet been determined, although some clues from the secondary structure of Ras and its mutants are available.

Although T35 is involved in stabilizing the nucleotide-binding site through interactions with the γ -phosphate, it also contacts the Mg^{2+} ion, and is involved in activating Wat175 in conjunction with Q61. T35 is located in the effector region and its con-

formation is dependent on the presence of GDP or GTP, dictating whether an interaction with GAP can take place (see the previous section). In A35 mutants, GAP can no longer interact with p21, as A cannot interact with either the γ -phosphate or the Mg^{2+} ion and so dictate the correct conformation of the effector region. Mutations at both positions 36 and 38 also abolish GAP interactions (Adari *et al.*, 1988; Calés *et al.*, 1988). Mutations in this region, however, are not always transforming because, although they effectively act to maintain Ras in the active GTP-bound state, changes in the effector region prevent interaction of Ras with downstream factors. D38, for example, prevents GAP stimulation of GTPase activity but is non-oncogenic (Krengel *et al.*, 1990). GAP could either exert its effects by stabilizing the active conformation of Ras by helping to align all necessary residues, or could introduce an attacking moiety of its own. Milburn *et al.* (1990) reported binding of Y32 to the γ -phosphate. However, Pai *et al.* (1990) found this amino acid to derive from a second Ras molecule in the crystal, indicating the accessibility of the γ -phosphate to other proteins, possibly GAP. GAP and effector mutations will be discussed further below.

GUANINE NUCLEOTIDE BINDING AND HYDROLYSIS

In the study of the biochemical properties of ras p21 over the past decade attention has focused primarily on the protein's ability to bind and hydrolyse guanine nucleotides (Scolnick *et al.*, 1979; Shih *et al.*, 1980; Sweet *et al.*, 1984; Gibbs *et al.*, 1984b; McGrath *et al.*, 1984). When purified after expression in bacteria in the presence of Mg^{2+} (or from eukaryotic cells by immunoprecipitation), wild type Ras contains 1 mol of strongly bound nucleotide per mol of protein (Poe *et al.*, 1985). Detailed analysis has shown the nucleotide component of this complex to be a heterogeneous mixture containing primarily rGDP but also small amounts of dGDP, rGTP and dGTP (Feuerstein *et al.*, 1987a). N.m.r. studies have shown that in solution the guanosine moiety of GDP is in an *anti*-conformation about the *N*-glycosidic bond with the ribose ring in the C2' endo form (Ha *et al.*, 1989;

alternative explanations, but essentially they concluded that binding involves a two-step mechanism in which a rapid first step (in which the protein interacts weakly with GTP or GDP) is followed by a slow 'quasi-irreversible' second isomerization reaction (John *et al.*, 1990). Binding constants for GDP and GTP at 25 °C in the presence of Mg^{2+} for the first step are $6 \times 10^4 \text{ M}^{-1}$ and $1.3 \times 10^5 \text{ M}^{-1}$ respectively and the rate constant for the second step approximately 15 s^{-1} (25 °C).

Whilst p21 is normally found complexed to GDP, the apo-protein is also capable of weakly binding GMP and guanosine [overall affinity constants at 25 °C of $3.5 \times 10^4 \text{ M}^{-1}$ and $6.5 \times 10^3 \text{ M}^{-1}$ respectively (John *et al.*, 1990)], both of which have a stabilizing effect on p21. However, it is unlikely that either of these interactions will be of physiological significance because of the much higher affinity for GDP and GTP.

The rate of exchange for p21 of GDP for exogenous GTP in the presence of Mg^{2+} is very low, with a half-life for p21 · GDP of about 20 min (Hall & Self, 1986). In the absence of Mg^{2+} ($< 0.5 \mu\text{M}$) there is an increase in dissociation and association rate constants for the reaction of GDP with p21 (by about 10- and 2-fold respectively; Feuerstein *et al.*, 1987). The net result of this is a dramatic decrease in the half life of p21 · GDP to less than 30 s (Hall & Self, 1986).

In vivo it is unlikely that the intracellular concentration of Mg^{2+} drops below 1 mM. Thus, rapid exchange of GTP for bound GDP is not favoured. Recently, however, proteins that stimulate guanine nucleotide exchange have been identified in human placenta and rat brain (Downward *et al.*, 1990a; West *et al.*, 1990; Wolfman & Macara, 1990). Incubation with these reduces the half-life of GDP · p21 and GTP · p21 complexes by at least 5-fold without impairing the ability of the apoprotein to bind fresh nucleotide (see below).

The hydrolysis of GTP by p21 is extremely well documented, but very few studies have considered the kinetics of the GTPase reaction in detail. Neal *et al.* (1988, 1990) have suggested a minimal scheme for the mechanism of GTP hydrolysis (Scheme 1):

Schlichting *et al.*, 1990b). In the absence of nucleotide, p21 is relatively unstable, being much more susceptible to thermal denaturation (Feuerstein *et al.*, 1987a) and proteolytic degradation (Grand & Grant, 1989).

Binding of both GDP and GTP to the apoprotein is very rapid, with association rate constants of approx. $1.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively (Feuerstein *et al.*, 1987a) at 0 °C. These are typical values for association between proteins and nucleotides. Association rate constants for *ras* proteins containing mutations at residue 12 were found to be somewhat higher than for the wild type (Feuerstein *et al.*, 1987b; Neal *et al.*, 1988). Dissociation rate constants, measured in the presence of excess Mg^{2+} , have been found to be very low [approx. 10^{-4} s^{-1} for GTP · p21 and 10^{-5} s^{-1} for GDP · p21 (Feuerstein *et al.*, 1987b; Neal *et al.*, 1988)]. The calculated binding constants for Mg^{2+} -GDP and Mg^{2+} -GTP are of the order of 10^{10} – 10^{11} M^{-1} (Feuerstein *et al.*, 1987a,b; Neal *et al.*, 1988; John *et al.*, 1990), explaining why the protein is isolated in a complex with bound GDP. Using fluorescent GDP and GTP analogues it has recently been shown that the binding of nucleotides to apo-p21 is not as simple as had been previously supposed. John *et al.* (1990) have considered a number of kinetic schemes that could account for their observations. Lack of space precludes a detailed discussion of their

Rate constants for steps 2, 3 and 5 in the forward and reverse directions have been determined for wild type and a mutant p21 (Neal *et al.*, 1988) and are listed in Table 1. Step 2 involves a conformation change and this probably controls the rate of cleavage of GTP (Neal *et al.*, 1990); thus no direct data are available for k_3 . [It is not clear at which point in the scheme the original conformation of p21 is reformed but it may well occur in the nucleotide-free protein (Neal *et al.*, 1990). Since no evidence is available, however, this step has been omitted from the Scheme.] Interestingly, the conformational change (step 2) cannot be observed for mutant (G12→D) N-Ras and it has been concluded that the protein is permanently in a 'GTP-like conformation' (Neal *et al.*, 1990). This is consistent with observed small structural differences for these proteins (Grand *et al.*, 1989).

Hydrolysis of GTP at step 3 occurs with inversion at the γ -phosphorus (Feuerstein *et al.*, 1989), indicating that the most likely mechanism is a single-step in-line transfer of the terminal phosphate from GTP to water without a phosphoenzyme intermediate as has been observed for EF-Tu (Eccleston & Webb, 1982). Steps 2 and 5 are considered to contribute most to the steady state rate of hydrolysis and the major intermediates present are p21 · GTP and p21 · GDP; thus the steady-state GTPase rate is given by $k_{+2} \cdot k_{+5} / (k_{+2} + k_{+5})$ (Neal *et al.*, 1988).

Table 1. Rate constants of N-*ras* p21 GTPase as outlined in Scheme 1 at 37 °CValues are taken from Neal *et al.* (1988).

	Wild type	Mutant (G12→D)
k_{+1} (M ⁻¹ ·s ⁻¹)	1.4×10^8	4.8×10^8
k_{-1} (s ⁻¹)	1.0×10^{-4}	5.0×10^{-4}
k_{+3} (s ⁻¹)	3.4×10^{-4}	1.5×10^{-4}
k_{-3} (s ⁻¹)	$< 0.17 \times 10^{-4}$	$< 0.08 \times 10^{-4}$
k_{+5} (s ⁻¹)	4.2×10^{-4}	2.0×10^{-4}
k_{-5} (M ⁻¹ ·s ⁻¹)	0.51×10^8	0.7×10^8

Many of the 'activating' mutations found in p21 are known to cause a reduction in the GTPase activity either partially or totally (see above); this is consistent with the observation that k_{+2} and k_{+5} are reduced for the D12 protein (Table 1).

The GTPase activity of p21 is generally low, but the addition of GAP (see below) results in an appreciable increase in the rate of nucleotide hydrolysis [approx. 100–200-fold (Trahey & McCormick, 1987; Gibbs *et al.*, 1988)] of wild-type protein. It is not apparent at present where GAP exerts its influence in the hydrolysis scheme postulated above, but an investigation of its effect on the various rate constants should go some way towards elucidating its mode of action.

POST-TRANSLATIONAL MODIFICATIONS AND SUBCELLULAR LOCALIZATION OF p21

The elucidation of the post-translational modifications that occur in the p21 system has taken almost a decade, so only now do we begin to understand the complexity and sophistication of the changes that take place after synthesis of the protein. Similarly,

the significance of these modifications to the functioning of *ras* proteins both in the normal cell and during *ras*-induced transformation is just becoming apparent.

Post-translational modifications of the C-terminus of p21

One of the earliest important observations of the biochemistry of p21 was the fact that it could be labelled with [³H]palmitate and that this tightly bound lipid could be removed after incubation with mild alkali (Sefton *et al.*, 1982), suggesting attachment through a thioester or ester linkage. Mutational analysis indicated that the substitution of a serine residue for C186 abolished palmitoylation, membrane localization and the transforming ability of activated Ras (Willumsen *et al.*, 1984*a,b*). It was therefore concluded, and accepted for some time, that the palmitate moiety was bound through C186 and that this modification was sufficient to determine the subcellular fate of the polypeptide. Hancock and Magee, in an elegant series of experiments, have however shown these inferences to be erroneous and have demonstrated a number of other post-translational modifications that are summarized in Fig. 4. These changes can be essentially divided into two sets. In set one, three irreversible modifications occur that are apparently triggered by the presence of a C-terminal CAAX sequence (in which C is cysteine, A any aliphatic amino acid and X any amino acid) similar to that originally identified in yeast mating factor (Anderegg *et al.*, 1988). The cytoplasmic pro-p21 is converted to an intermediate form by (i) farnesylation of C186 (Hancock *et al.*, 1989); (ii) cleavage of the three C-terminal amino acids (Gutierrez *et al.*, 1989); and (iii) carboxymethylation of the α -carboxyl group of the newly exposed C-terminal cysteine (186) (Clarke *et al.*, 1988; Gutierrez *et al.*, 1989). In set two, either or both of the cysteine residues close to the C-terminus (residues 181 and 184 in H-Ras) are palmitoylated by an acyltransferase (Hancock *et al.*, 1989).

The first set of processing reactions are similar to those that have recently been observed for a number of other proteins

Fig. 4. Post-translational modifications at the C-terminus of the H-*ras* proteins

The amino acid sequence of the C-terminal nine amino acids is shown. Enzymes involved in the modifications are also indicated.

containing the CAAX C-terminal sequence (reviewed by Glomset *et al.*, 1990), for example yeast mating factor a (Anderegg *et al.*, 1988), the nuclear lamins A and B (Holtz *et al.*, 1989) and the γ subunit of transducin (Lai *et al.*, 1990; Fukada *et al.*, 1990). An enzyme that may be responsible for farnesylation of *ras* p21 has been purified to homogeneity from rat brain by affinity chromatography using a peptide equivalent to the C-terminus of K-Ras B (TKCVIM) (Reiss *et al.*, 1990). This protein farnesyltransferase has a molecular mass of about 50 kDa by SDS/PAGE and is presumed to exist as a dimer *in vivo*. It has an absolute requirement for divalent metal cations but is relatively indiscriminating having equivalent activity in the presence of Mg^{2+} , Mn^{2+} or Zn^{2+} (Manne *et al.*, 1990; Reiss *et al.*, 1990; Schaber *et al.*, 1990). The enzyme appears to be located in the cytoplasm of the cell and is very widely distributed, being present in all mammalian cell lines and tissues examined (Manne *et al.*, 1990). The donor species for the farnesyltransferase is farnesyl pyrophosphate, which is an intermediate in the biosynthesis of squalene and isoprenic acid (reviewed by Goldstein & Brown, 1990). Inhibition of the pathway may be achieved by the addition of mevinolin, lovastatin or compactin, all of which act by inhibiting HMG-CoA reductase, an enzyme of central importance to the pathway. In the presence of these compounds no farnesylation of p21 occurs (Hancock *et al.*, 1989; Kim *et al.*, 1990b; Leonard *et al.*, 1990). Whilst the farnesyltransferase is cytoplasmic, the enzymes responsible for proteolysis and carboxymethylation are located on intracellular membranes (Stephenson & Clarke, 1990; Hrycyna & Clarke, 1990; Hancock *et al.*, 1991). It is interesting to note that carboxymethylation and cleavage are necessary for efficient membrane binding of the K-*ras*-B p21 and that farnesylation alone is probably not sufficient (Hancock *et al.*, 1991). Studies on the protease and methyltransferase have confirmed the order of set 1 modifications (Hrycyna & Clarke, 1990; Schafer *et al.*, 1989).

Knowledge of the mechanism of palmitoylation of p21 is far more sketchy due to the difficulty in purifying an active palmitoyltransferase. However, the modification itself is relatively common, occurring on a number of viral and eukaryotic proteins (reviewed by Grand, 1989; Schmidt, 1989). So far no consensus amino acid sequence has been identified and it may be that there exists within the cell a number of acyltransferases, each having considerable specificity. Palmitoylation of *ras* proteins is a dynamic process, with a very high rate of turnover of the lipid [the half-life of palmitate is 20 min compared with a half-life for the protein of about 24 h (Magee *et al.*, 1987)]. In a preliminary study, Magee (1990) has suggested that acylation and deacylation are both enzymically modulated, with the acyltransferase located in the Golgi and the deacylating enzyme found primarily in a plasma membrane fraction. It is obvious that the dynamic nature of palmitoylation must have considerable implications for the functioning of p21 as a signal transducer, but these are certainly not clear at present.

Subcellular localization and post-translational modifications of p21

Within any eukaryotic cell a large proportion of the *ras* proteins are localized to the inner surface of the plasma membrane (Willingham *et al.*, 1980) although it clearly cannot be considered a conventional membrane protein. The remainder of the p21, at least in cells expressing the protein at high level, is cytoplasmic (Magee *et al.*, 1987). Mutational analysis has shown that the first set of modifications outlined above (farnesylation, cleavage and carboxymethylation) is not sufficient to ensure membrane localization of H-Ras and N-Ras (Hancock *et al.*, 1989, 1990). A proportion of *ras* proteins that are not palmitoylated (because they lack C181 and C184) associate weakly with a membrane

fraction although they can be displaced by 0.25 M-NaCl (Hancock *et al.*, 1990). It appears, therefore, that farnesylation creates a C-terminus on p21 which is hydrophobic enough to ensure weak interaction with the plasma (or other) membranes, but is insufficient to produce a permanently or strongly bound protein. In these experiments considerable use has been made of the K-*ras*-B protein which is not palmitoylated *in vivo* because it has no available cysteine residues in the C-terminal portion of the molecule after farnesylation, but is as firmly anchored in the plasma membrane as are the N and H-Ras homologues (Hancock *et al.*, 1989, 1990; Casey *et al.*, 1989). This localization has been attributed to the extremely basic region, comprising six lysine residues, just upstream of the C-terminus (Hancock *et al.*, 1990). In this polypeptide, set one processing is essential, but also sufficient, for membrane localization (Casey *et al.*, 1989; Hancock *et al.*, 1989; Jackson *et al.*, 1990). It therefore is likely that within the hypervariable region of all the *ras* polypeptides there resides a signal sufficient to ensure membrane localization. It is tempting to propose that the different signals (none, one or two cysteines in K-Ras-B, N-Ras and K-Ras-A or H-Ras respectively, together with alternate upstream amino acids sequences in the hypervariable region) target the different proteins to specific sites in the membrane where they fulfil slightly different requirements for the cell. The replacement of the palmitate moiety with a polylysine sequence as a membrane anchor in the K-*ras*-B protein implies that this polypeptide, unlike H-Ras, N-Ras and K-Ras-A, is not in dynamic equilibrium between cytoplasm and plasma membrane, and once post-translationally modified by the 'set one enzymes' is permanently locked into the membrane. Whether this is indicative of a unique function for the K-*ras*-B protein remains to be seen.

It was concluded from the original observation that mutation of C186 produced a *ras* protein which was unpalmitoylated and cytoplasmic (Willumsen *et al.*, 1984a,b) that this amino acid was the site of palmitoylation. However, a second important conclusion from this work was that this amino acid substitution negated the transforming ability of the 'activated' protein. It has since become clear that it is probably not membrane localization *per se* that is essential for transformation but rather the post-translational modification of the CAAX sequence, triggered by C186 (Casey *et al.*, 1989; Hancock *et al.*, 1990; Jackson *et al.*, 1990). Thus, mutation of C181 and C184 of 'activated' H-Ras reduces the transforming capability 2–5-fold whereas mutation of C186 renders the protein completely inactive in the transformation assay (Hancock *et al.*, 1990). In both cases the polypeptide remains in the cytoplasm. Similarly, removal of the polylysine region from K-Ras B only reduces the transformation frequency 2–3-fold whilst producing a cytoplasmic protein. Mutation of C186 in this polypeptide gives the same subcellular localization (i.e. the cytoplasm) but abolishes the transforming potential (Hancock *et al.*, 1990; Jackson *et al.*, 1990). The observations by Hancock *et al.* (1990) are, of course, difficult to explain since it has always been assumed that whatever precise role Ras plays in the cell (either normal or transformed) it has been involved, in some way, in signal transduction at the plasma membrane (see below). If it does not localize to the membrane, as is the case for the H-*ras* and K-*ras*-B proteins described above, it must have an activity within the cytoplasm. Whether this is related to what happens to wild type p21 when the palmitate moiety is turned over is not clear at present, but it is tempting to suggest that this is evidence in favour of *ras* proteins having both membranous and cytoplasmic functions. Alternatively, it is possible that farnesylated but unpalmitoylated p21 may still weakly associate with membranes such that it can transform the cells but is displaced during subcellular fractionation. Certainly, the observation by Buss *et al.* (1989) that myristoylation of both

wild type and activated p21 is sufficient to produce a protein with transforming potential would lend support to the view that membrane localization is very important in transformation by p21.

Phosphorylation of p21

The *ras* oncogene product of Harvey sarcoma virus (v-H-*ras*) is autophosphorylated *in vivo* at T59 with GTP acting as phosphate donor (Shih *et al.*, 1979, 1980, 1982). This modification does not occur, however, in mammalian *ras* proteins since T is replaced by an A. If this polypeptide is mutated to resemble the viral homologue the autophosphorylation reaction takes place (Gibbs *et al.*, 1984a). The kinetics of this reaction have been investigated in detail by John *et al.* (1988). Three mutant proteins, all containing T59, were examined: V12, G12 and R12. Rate constants for GTPase activities were similar (about one-fifth of that found for the wild type protein) but the rate constants for autophosphorylation varied considerably (V12 5.8×10^4 , G12 3.8×10^4 and R12 $2.3 \times 10^4 \text{ min}^{-1}$). These observations were taken as evidence of changes in conformation of the protein due to substitutions at position 12. The autophosphorylation reaction is irreversible, first order with respect to protein concentration and intramolecular (John *et al.*, 1988).

Apart from the autophosphorylation of mutant p21, there is some data to suggest that K-*ras*-B protein is phosphorylated in high-expressing lines after treatment with phorbol ester and *in vitro* after incubation with protein kinase C (Ballester *et al.*, 1987). Phosphorylation of H-Ras by protein kinase C *in vitro* has also been reported (Jeng *et al.*, 1987). The sites of modification by protein kinase C have not been determined unequivocally but are on serine residues (Ballester *et al.*, 1987; Jeng *et al.*, 1987). It has also recently been demonstrated that one or more tyrosines in p21 are phosphorylated *in vitro* by insulin receptor tyrosine kinase (Korn *et al.*, 1987; Fujita-Yamaguchi *et al.*, 1989; R. J. A. Grand, L. Ellis & B. A. Levine, unpublished work; see below). It is not clear what, if any, significance can be placed on any of these observations of phosphorylation of p21 since in all cases level of incorporation of phosphate is low and modification appears to have very little effect on either nucleotide binding or hydrolysis (John *et al.*, 1988).

INTERACTION OF p21 WITH OTHER PROTEINS

GTPase activating protein (GAP)

In 1987 Trahey and McCormick reported a cytoplasmic protein in *Xenopus* oocytes that stimulated GTP hydrolysis by wild type p21 at least 200-fold but had no effect on the D12 or V12 p21 oncogenic variants. Thus, this GTPase activating protein (GAP) serves as a negative regulator of p21, catalysing the conversion of GTP·p21 to the inactive GDP-bound form. Its ability to interact with, but not stimulate, mutant forms of p21 further explained their oncogenic potential.

GAP activity was also observed in mouse cells and human peripheral blood lymphocytes and has since been found in many tissue types (Trahey *et al.*, 1988; Adari *et al.*, 1988). Highest expression in mammalian tissues is in brain, testis and placenta with lower levels in kidney, spleen, lung, liver, heart and skeletal muscle (Gibbs *et al.*, 1988). It is also apparent that a family of GAP molecules exist, each interacting with its own target within the *ras* super gene family. Thus RasGAP will bind to Ras and increase its GTPase activity, and it will bind to other Ras-related proteins but not necessarily stimulate their enzymic activity. RhoGAP and RapGAP among others have been isolated, and will be discussed with their related proteins below.

The genes encoding RasGAP have been cloned from two

different sources and show remarkable similarity (Vogel *et al.*, 1988; Trahey *et al.*, 1988). The protein has an apparent molecular mass of 125 kDa, which is in good agreement with the predicted molecular mass of 115762 Da. mRNA studies have identified a similar 6 kb GAP-encoding transcript in a variety of cell sources, and indicated that GAP is probably present as a single copy gene (Vogel *et al.*, 1988).

Cloning the GAP cDNA from human placenta produced a clone which was 96% homologous to that obtained from bovine brain with most of the differences lying in the first 150 amino acids. A second GAP cDNA from human placenta, which has a number of differences to the original clone, has also been isolated. This species of GAP cDNA is found in placental and foetal tissues, but appears to be absent from adults, suggesting that it may be associated with highly proliferating cells. The predicted protein from this second mRNA is a smaller species (100407 Da) lacking the hydrophobic N-terminus, which perhaps directs interactions with other cellular components in particular membrane associations.

The primary structure of GAP falls into several domains (Vogel *et al.*, 1988) (Fig. 5). The N-terminal portion of the protein is highly hydrophobic, and of the first 130 amino acids more than 50% comprise glycine or alanine. This is followed by a proline-rich region, indicating the possibility of a flexible hinge region. Towards the middle of the polypeptide chain (amino acids 513–683) there is another proline-rich region in which there is a repeating proline every 21–24 amino acids, indicative of a repeating structural element. There are two potential tyrosine kinase phosphorylation sites identified at residues 161 and 720; also present are two possible sites for cyclic AMP-dependent protein kinase activity at residues 354 and 662. Interestingly, there is a striking similarity between regions of GAP and the SH2 and SH3 [*src* homology domains 2/3 (Moran *et al.*, 1990)] regions found to be conserved in phospholipase C γ and the non-receptor tyrosine kinase oncogene products; this feature will be discussed later.

The discovery that only the C-terminal third of GAP is required for its GTPase activating ability (Marshall *et al.*, 1989) confirms the suggestion that distinct structural or functional domains lies in the N-terminal two-thirds of the molecule. The most extreme C-terminal 61 amino acids may also contain some structural elements, as their deletion causes the protein to become insoluble.

The effector binding region of Ras has been identified by mutational analysis as amino acids 32–41 (see above) (Sigal *et al.*, 1986). Adari *et al.* (1988) and Calés *et al.* (1988) have identified the GAP binding site in Ras to the same residues comprising the effector binding site. This provides strong evidence for GAP as a downstream effector molecule. However, recent evidence undoubtedly supports its role as a negative regulator as well. Controversy as to whether one or indeed both of these functions for GAP is correct still continues (Hall, 1990b). More evidence for GAP being a downstream effector in the Ras signal pathway came from Yatani *et al.* (1990), who showed that a p21–GAP complex inhibited the signal-transducing G protein G κ which couples muscarinic receptor to K $^{+}$ channels in atrial cells. This action is dependent on both p21 and GAP, and the action can be blocked by antibodies to either protein. Thus, in this situation at least, Ras activity is GAP-dependent. However, no other GAP-dependent Ras functions have been identified at present and the Ras signal transduction pathway downstream of Ras/GAP remains unclear.

It has been known for some time that p21 is necessary for cell growth after stimulation with PDGF or EGF (Mulcahy *et al.*, 1985), but it has recently been observed that binding of PDGF to its receptor causes at least 10% of cytosolic GAP molecules to

Fig. 5. Locations of catalytic and structural domains of GAP

SH2 and SH3 domains refer to *src* homology domains 2 and 3, thought to be involved in phosphotyrosine binding. Possible phosphorylation sites for tyrosine kinase and protein kinase A are included.

complex with the receptor and to be phosphorylated by it. As discussed previously, there are two possible tyrosine phosphorylation sites at residues 161 and 720; intriguingly, residue 720 lies within the proposed Ras binding site (Marshall *et al.*, 1988; R. J. A. Grand, D. Owen & B. A. Levine, unpublished work). The interaction between GAP and the receptor seems to be dependent on the kinase activity of the receptor and the presence of the receptor tyrosine autophosphorylation sites (Kazlauskas *et al.*, 1990; Molloy *et al.*, 1989). This shows a direct link between activated growth factor receptors and the Ras/GAP mitogenic signalling pathway. Association of GAP with PDGF receptor happens concurrently with the receptor complexing with at least three other substrates, which have been identified as 3'-phosphatidylinositol kinase (Auger *et al.*, 1989), phospholipase C- γ (PLC- γ ; Kumjian *et al.*, 1989) and the serine/threonine kinase encoded by the proto-oncogene *raf-1* (Kaplan *et al.*, 1990; Morrison *et al.*, 1989). This implies that PDGF stimulation causes the formation of large multiprotein complexes involved in signal transduction. It has also been reported that in *ras*-transformed cells the association between GAP and PDGF receptor fails to occur, despite the receptor retaining its normal activity and ability to bind to the three other substrates. It is unlikely that this lack of binding is due to the unavailability of GAP, which might occur if it were irreversibly bound to mutant p21, since it has been shown to be in considerable excess over Ras in the cell (Hall, 1990b). While binding of PLC- γ still occurs without GAP, PDGF receptor is then unable to stimulate PLC- γ , implicating GAP in the coupling of PDGF receptor to PLC- γ (Benjamin *et al.*, 1987). It seems that the Ras signal transducing pathway is involved with a response to the PDGF receptor as the active GTP·p21 complex is found at elevated levels in PDGF-stimulated cells (Sato *et al.*, 1990). A similar situation is seen with EGF-stimulated cells when GTP·p21 levels are raised. In this situation the EGF receptor binds GAP when it is itself phosphorylated (Margolis *et al.*, 1990).

GAP is also phosphorylated by cytoplasmic tyrosine kinases *v-src* and *v-fps*, and appears to co-precipitate with two other tyrosine phosphorylated proteins p62 and p190 (these are also seen in immunoprecipitations of EGF-stimulated fibroblasts) (Ellis *et al.*, 1990). GAP and p62 have been shown to bind to and be phosphorylated by the colony-stimulating-factor-1 (CSF-1) receptor, the product of the *c-fms* proto-oncogene. This case seems to be slightly different from that of EGF and PDGF

association as the kinase insert region of the CSF-1 receptor is not required for phosphorylation, and the level of phosphorylation of p62 is higher than that of GAP or p190.

The mechanism by which GAP interacts with all these different protein-tyrosine kinases (both in the receptor and non-receptor families) seems to involve the SH2 and SH3 domains found in the N-terminal region (Fig. 5). These domains, also present in *c-src*, *c-fps*, *c-abl*, *v-crak* and PLC, are distal to the catalytic domains and appear to allow a whole series of proteins to interact with a common set of molecules, possibly at the plasma membrane (Pawson, 1988). It has been suggested that the SH2 region is a phosphotyrosine binding motif (Cantley *et al.*, 1991). GAP only binds to the receptors and protein-tyrosine kinases discussed earlier when they are in their phosphorylated states; thus it seems likely that the SH2 region of GAP binds to phosphorylated tyrosine residues in the receptors and is itself phosphorylated. How this phosphorylation of GAP then affects its functions is still open to question.

Much of the information gathered to date begs the question: is GAP an upstream regulator or a downstream effector of Ras? GAP is clearly a negative regulator for Ras, a property which led to its discovery and has been well documented. Its action on wild-type but not on activated p21 is also consistent with this role. Over-expression of GAP in *ras*-transformed cells acts to suppress transformation by over-expressed *c-ras* but does not inhibit transformation by (activated) *v-ras* (Zhang *et al.*, 1990). GAP activity is also known to correlate with cell density, increasing activity presumably converting Ras to the inactive GDP-bound form and so decreasing cell proliferation signals (Hoshino *et al.*, 1988). Indeed if GAP is the negative regulator for Ras then sequestration of GAP to the PDGF receptor could serve to remove it from the cytosol or modify its action in some way, allowing Ras to remain in its active form. This view has been supported by data showing that the GAP-p190 complex has little GTPase stimulatory activity (Cantley *et al.*, 1991; Moran *et al.*, 1991). Also in *v-src* transformed and EGF-stimulated cells, 40–70% of cytosolic GAP interacts with p190, although the complex is undetectable in normal proliferating cells (Moran *et al.*, 1991), implicating p190 as an inhibitor of GAP which only forms a complex during mitogenesis. On the other hand, binding of GAP to a membrane-bound receptor could result in the juxtaposition of GAP to its target Ras (situated at the plasma membrane), enhancing deactivation.

Thus relocating GAP at the membrane may serve to connect Ras to other proteins complexed with GAP at the receptors e.g. p62, p190, PLC- γ , Raf and 3'-phosphatidylinositol kinase. This could link Ras with many molecules thought to be involved in mitogenic signalling and secondary signal formation. This could resolve the controversy of whether GAP is a negative regulator or effector for Ras, by having the molecule as both, as evidence seems to rule out either simple hypothesis.

Downward *et al.* (1990a) also found GAP to be implicated in the response of T lymphocytes to external stimulation through the antigen receptor. An increase in the level of GTP-bound p21 was caused by a PKC-mediated inhibition of GAP activity. This inhibition was considered to be evidence of an indirect PKC action via phosphorylation of another protein; p190 could fulfil such a role.

A recent intriguing piece of information has brought GAP further into focus. The open reading frame thought to encode the neurofibromatosis type 1 gene (NF1) has been isolated (Xu *et al.*, 1990; Ballester *et al.*, 1990) and shown to be similar to the catalytic domain of GAP. It is even more similar to IRA1 and IRA2, the negative regulators for RAS1 and RAS2 in *S. cerevisiae* (Tanaka *et al.*, 1990). A portion of NF-1 expressed in yeast can complement for defects in IRA and inhibit co-expressed human H-*ras* genes. The data are consistent with NF1 being a tumour suppressor gene, with a recessive action at the cellular level. These observations pose an interesting question however; does this imply that NF1 is a GAP-like molecule interacting with some other Ras-like molecule in a different pathway or could NF1 itself interact with Ras at the cellular level and be involved in some way with the Ras signal transduction pathway?

Interactions with other proteins

As previously discussed, most of the progress made in identifying proteins that interact with p21 has been focused on GAP. Apart from this, however, a GTPase inhibiting protein has been isolated by Tsai *et al.* (1990) from a cytoplasmic fraction by using lipid affinity chromatography. It has an apparent molecular weight of 60 kDa under non-denaturing conditions and obviously, from the purification procedure, binds phospholipids. The phospholipids which bind to GAP, and inhibit its action on Ras, serve antagonistically to activate the GTPase inhibitor action. Thus, the phospholipids have a dual mode of action through the two proteins producing a single result – the increase in the GTP-bound 'active' form of Ras.

As with GAP, there is a direct association between Ras and GTPase-inhibiting protein. However, unlike GAP, it can interact with either GTP or GDP-bound Ras. Further differences between GAP and the GTPase inhibiting protein possibly lie in their mode of action. In assays *in vitro* the GTPase inhibiting protein is active when Ras is in 50-fold excess, perhaps indicating its promotion of a long-acting conformational change and so inhibiting the GTPase activity after dissociation. On the other hand GAP is, in cells at least, in vast excess over Ras and each interaction appears to promote a hydrolysis event (Tsai *et al.*, 1990).

Another activator of Ras activity has been identified by three different groups and is termed a guanine nucleotide release protein (GNRP), catalysing the exchange of guanine nucleotide bound to p21 with cytosolic pools (West *et al.*, 1990; Wolfman & Macara, 1990; Downward *et al.*, 1990b). These polypeptides are thought to work by relaxing hydrogen bonding in the guanine-binding regions (see above) and so promoting GDP release and therefore nucleotide exchange. The activity level of p21 is dependent on whether it is in a GDP- or GTP-bound form and this is controlled by GTPase activity and nucleotide exchange rates. The relatively slow nucleotide exchange for p21 had

indicated the possible presence of a GNRP analogous to that found for other G proteins (Gilman, 1987). West *et al.* (1990) identified a protein of 100 kDa predominantly associated with the plasma membrane which increases the wild-type or activated p21 nucleotide exchange rate 25-fold. The stimulation could be blocked by addition of neutralizing antibody Y13.259, perhaps explaining the observation by Hattori *et al.* (1987) that this antibody specifically blocks the GDP-GTP exchange reaction. Wolfman & Macara (1990), on the other hand, have identified a 100–160 kDa cytosolic protein that promoted guanine nucleotide release from H-Ras. This factor, like that of West *et al.* (1990) and Downward *et al.* (1990b), was specific in its interaction with Ras and did not promote any nucleotide exchange activity in other G proteins tested, indicating perhaps the presence of a series of GNRP's similar to the GAP family. Wolfman & Macara (1990) also noted that their GNRP was rapidly inactivated, possibly by dephosphorylation, suggesting a similar scenario to that seen with eIF-2 where a large cytosolic protein acts as a nucleotide exchange factor, the interaction being controlled by phosphorylation. Downward *et al.* (1990b) reported a much smaller 60 kDa protein, again present in the cytosol. However, they also detected this activity in trypsin-washed membranes, proffering the possibility that their cytosolic protein could be a fragment derived from a larger membrane-bound polypeptide that had been disrupted during the purification process. All these factors appear to promote guanine nucleotide exchange in a reversible fashion, not particularly indicative of a Ras activating protein. However, in normal cells more than 95% of p21 is found in the GDP-bound form (Schramm & Selinger, 1984), although the cytosolic guanine nucleotide pool comprises approximately 95% GTP. Thus, nucleotide exchange in such a situation would almost certainly be GTP for GDP and thus favour the formation of mitogenically active Ras.

Downward *et al.* (1990b) also reported that a C-terminal deletion of p21 interfered with the interaction between Ras and the nucleotide exchange protein. This region of p21 comprises the region in which the four human Ras proteins are most dissimilar and is separate from the catalytic domains. So far no function has been assigned to it, implying that there could be more than one exchange factor. However, in this case, unlike GAP, a different GNRP could be available for each of the Ras types.

Characterization of other Ras-associated proteins has been limited, although a few have been identified by cross-linking experiments. Initially, *in vitro* cross-linking investigations failed to show any Ras-associated proteins, but recently studies *in vivo* have met with some success (de Gunzburg *et al.*, 1989; Lee *et al.*, 1989). However, no function has so far been assigned to any of the associating proteins identified. Interestingly, different proteins were identified for wild type and activated Ras (Lee *et al.*, 1989).

A connection between Ras and a signal transduction pathway has been established by work with the insulin receptor. Purified Ras appears to be phosphorylated by the purified insulin receptor kinase in an insulin-dependent manner (Fujita-Yamaguchi *et al.*, 1989), although previous reports had suggested that this did not occur (Kamata *et al.*, 1987a; O'Brien *et al.*, 1987). This *in vitro* phosphorylation has been shown to be dependent on cofactors such as protamine and poly(L-lysine) for H-Ras. However, for K-Ras B the reaction occurs independently of such factors. This is thought to be due to the presence of an extremely basic domain consisting of residues 172–182. The same domain, which is located within the C-terminal hypervariable region, consists primarily of neutral amino acids in H-Ras and N-Ras. If the different Ras proteins were to respond to different stimuli *in vivo*, it would presumably have to be through interaction with this region. Thus, this work could show a specific interaction of one

type of Ras protein with the insulin receptor in an insulin-dependent manner, the N and H-Ras proteins not containing the necessary basic domain failed to stimulate phosphorylation. It should be noted, however, that a truncated insulin-receptor tyrosine kinase is able to phosphorylate and bind to N-ras p21 *in vitro* (R. J. A. Grand, B. A. Levine & L. Ellis, unpublished work).

RAS AND OTHER ONCOGENES

In this short section we will present a brief summary of data obtained from studies carried out into the way in which transformation of primary cells is effected by *ras* in co-operation with other oncogenes. Readers in need of a more detailed treatment of this topic are referred to a number of excellent reviews which have been published recently (Weinberg, 1989; Ruley, 1990; Hunter, 1991).

The mammalian *ras* oncogenes were originally identified by their ability to transform NIH 3T3 cells in gene transfer (transfection) assays (Shih *et al.*, 1981; Krontiris & Cooper, 1981; Perucho *et al.*, 1981; Pulciani *et al.*, 1982). It has been demonstrated many times over the past decade that the introduction of *ras* genes bearing activating mutations into established cell lines gives rise to a fully transformed population. The *ras* oncogene alone, however, is unable to transform primary cells under normal circumstances, but if it is introduced with another (nuclear) oncogene into rat embryo fibroblasts it produces a fully transformed phenotype (Land *et al.*, 1983; Ruley, 1983). On the basis of co-transfection experiments of this sort using rat embryo fibroblasts, oncogenes have been divided into two functional groups that are determined by their subcellular location (Weinberg, 1985, 1989). Thus, full transformation requires one 'cytoplasmic' (which category includes membrane-bound) oncogene (for example, K-*ras*, H-*ras*, N-*ras*, *src*, adenovirus E1B or polyoma MT) and one nuclear oncogene (for example *myc*, adenovirus E1A, *fos*, *jun*, polyoma LT, SV40 LT, papilloma E7 or mutant p53). Although a number of the nuclear oncogenes can immortalize cells they are unable to produce the gross changes that are normally associated with full transformation (e.g. changes in morphology, growth factor requirements and anchorage status). The cytoplasmic oncogenes alone can give rise to changes in cell morphology and growth factor requirements without immortalizing cells. On the basis of these observations it has been suggested that oncogene co-operation may serve as an *in vitro* model for the study of multistep tumorigenesis. Thus, it has been supposed that changes in at least two cellular oncogenes are required for transformation of cells *in vivo*.

It has also been concluded, on the basis of the categorization, that the nuclear and cytoplasmic oncogenes are responsible for at least two distinct events which are particularly important in the transformation process. The nuclear oncogenes could all be involved in some way in modification to the cell cycle or in transcriptional control. The first possibility has been partially validated by the observation that the DNA tumour virus proteins (SV40LT, AdE1A and HPV E7) are all capable of binding to the retinoblastoma protein Rb1, which appears to play a central role in regulation of the cell cycle. Many of the proteins which co-operate with Ras are thought to be involved in the regulation of transcription. For example, Myc contains leucine zipper and helix-loop-helix motifs common to well-characterized transcription factors (see Collum & Alt, 1990, for a discussion of this point), whilst Fos and Jun have been shown to form part of the AP1 transcriptional complex (Curran & Franza, 1988). The viral nuclear oncogenes (AdE1A, SV40LT and PyLT) are also intimately involved in the regulation of transcription of viral and

cellular genes. Whilst the functions of the cytoplasmic oncogenes remain unknown, it is generally felt that they each play some role in intracellular signalling in its very broadest terms. Thus, transformation can be simplistically envisaged as deregulation of cell cycle and/or changes in transcription by the nuclear oncogenes and changes in growth factor requirements and responses by the cytoplasmic oncogenes to complete the process.

A consideration of oncogene co-operation is particularly relevant to this review because activated *ras* oncogenes have been found in human and murine pre-neoplasias (Balmain *et al.*, 1984; Vogelstein *et al.*, 1988) as well as in a large number of human tumours (reviewed in Nishimura & Sekiya, 1987; Bos, 1988, 1989). From a consideration of these data, Bos (1988, 1989) has concluded that activated *ras* genes are most often found in carcinomas (particularly adenocarcinomas) and haematological malignancies of the myelomonocytic lineage but much less frequently in tumours of neuroectodermal origin and differentiated lymphoid malignancies. It is interesting to note that mutation in a particular *ras* gene seems to correlate, in many instances, with tumour type. For example, in adenomas and adenocarcinomas of the colon K-*ras* is usually mutated, whereas in myeloid disorders N-*ras* mutations predominate (Bos, 1989). The presence of mutant *ras* genes in pre-malignant lesions suggests that this mutation may be of considerable importance in the early stages of the development of a tumour. It is clear, however, that other (genetic and/or environmental) events are essential for tumorigenesis. Based on the idea of 'co-operating oncogenes' it might be supposed that abnormalities in *myc* genes complement the *ras* mutations. However, increased Myc expression in malignancies containing *ras* mutations has been observed only rarely (Weinberg, 1989). Thus, it is likely, if the model is valid, that other genes, analogous to the 'nuclear oncogenes', are responsible for the events which, together with mutated *ras*, produce cell growth irregularities.

One human malignancy in which activated *ras* genes have been implicated is that of colorectal carcinoma. A model for the genetic basis of this neoplasia has been proposed (Fearon & Vogelstein, 1990), which suggests how mutation of *ras*, 'nuclear oncogenes' and other genes are involved in the development of the disease from small benign adenomas to large metastatic carcinomas. At least four separate genetic events have been delineated in this progression. Mutation of K-*ras* at amino acid 12, 13 or 61, mutation or loss of chromosome 5q (the proposed familial adenomatous polyposis gene) and DNA hypomethylation are all early events leading to the production of an adenoma of intermediate size. Later events include the mutation or loss of the DCC gene (chromosome 18q) and p53 gene (17p) and increases in level of expression of c-*myc* and level of activity of a number of tyrosine kinases. The order of these events is not strictly determined but it is clear that more mutations have occurred in carcinomas than in late adenomas and, similarly, more mutations appear to have occurred in late than in early adenomas. With at least four genetic events taking place in the tumour cells it is obvious that the original Weinberg hypothesis is rather simplistic when applied to tumourigenesis *in vivo*. However, it does appear to have been at least partially vindicated in so far as mutations in two cytoplasmic genes (*ras* and DCC) and at least two nuclear oncogenes (p53 and possibly *myc*) may be involved.

RAS-RELATED PROTEINS

It has recently become clear that *ras* genes are part of a large superfamily of genes, which can be subdivided into three main groups. The *ras* family itself contains the three *ras* genes along with R-*ras*, *ral* A and B, *rap* 1A, 1B and 2, and TC21, all of which

Table 2. The Ras superfamily

Abbreviations: Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Dm, *Drosophila melanogaster*; Dd, *Dictyostelium discoideum*; Apl, *Aplysia* sp.

Ras family		Rho family		Rab family		Ras/Gα intermediates
Mammalian	Analogues	Mammalian	Analogues	Mammalian	Analogues	
H- <i>ras</i>	Sc <i>RAS1</i>	<i>rho</i> A	Sc <i>RHO</i> 1	<i>rab</i> 1A	Sc <i>YPT</i> 1	<i>ARF1</i>
K- <i>ras</i> (A + B)	Sc <i>RAS2</i>	<i>rho</i> B	Sc <i>RHO</i> 2	<i>rab</i> 1B	Sc <i>SEC</i> 4	<i>ARF2</i>
N- <i>ras</i>	Sp <i>RAS</i>	<i>rho</i> C		<i>rab</i> 2	Dd <i>Sas</i> 1	<i>SAR1</i>
	Dm <i>ras1</i>	<i>rac</i> 1 (TC25)		<i>rab</i> 3A (<i>smg25A</i>)	Dd <i>Sas</i> 2	
	Dd <i>ras</i>	<i>rac</i> 2		<i>rab</i> 4	Sp <i>YPT</i> 2	
	Apl <i>ras</i>	TC10		<i>rab</i> 5	Sp <i>YPT</i> 3	
<i>rap</i> 1A (Krev1/ <i>smg21</i>)	Dm <i>ras3</i>			<i>rab</i> 6		
	Sc <i>RSR</i> 1			BRL- <i>ras</i>		
<i>rap</i> 1B				<i>smg</i> 25B		
<i>rap</i> 2				<i>smg</i> 25A		
R- <i>ras</i>						
<i>ral</i> A						
<i>ral</i> B						
TC21						

show approximately 50 % similarity to the *ras* genes. The *rho* family consists of *rho* A, B and C, *rac* 1 and 2 and TC10, all of which show about 30 % similarity to the *ras* genes. Finally, the *rab* family also shows approximately 30 % similarity to *ras* genes and consists of *rab* 1–6, BRL-*ras*, *smg* 25A, B and C. This latter family also includes the yeast genes *YPT1* and *SEC4* which have been well characterized and are likely to be a model for the mammalian *rab* genes. A prokaryotic analogue for *ras* has also been reported: the product of the *era* gene in *E. coli* which is 50 % similar to yeast *RAS1* in the N-terminal two-thirds of the protein (March *et al.*, 1988). Members of the superfamily are outlined in Table 2, together with examples of analogues found in different organisms.

Ras family

Within this group the four different *ras* proteins show 85 % similarity to each other and 50 % similarity to the other members of the family. They all have the characteristic CAAX box at their C-terminus (see above) and the other homologous regions always include the four areas involved in GTP binding and hydrolysis, 10–17, 57–62, 113–120 and 143–147 (H-*ras* assignments).

Human R-Ras is a 23 kDa protein comprising 218 amino acids (an extra 26 N-terminal amino acids to H-Ras) with 55 % similarity to human *ras* genes. It is located on chromosome 19, distinct from any of the *ras* genes. A mouse equivalent has been found with 94.5 % amino acid similarity to the human protein (Lowe *et al.*, 1987). Like H-*ras* it is palmitoylated and is membrane-associated (Lowe & Goeddel, 1987), but is non-oncogenic when mutated in analogous positions to activated *ras*. However, making an A-to-T substitution at amino acid 85 (analogous to 59 in H-*ras*) in R-Ras results in GTP-dependent autophosphorylation which suggests that R-Ras functions in a similar manner to Ras with intrinsic binding and hydrolysis activities (Lowe & Goeddel, 1987). RasGAP can interact with R-Ras, causing stimulation of its GTPase activity, which is not surprising as the 32–40 effector region for both the H- and R-Ras is identical (Garrett *et al.*, 1989). The biological effects of the two proteins, however, are quite different (for example, H-Ras can cause transformation and R-Ras cannot) but their abilities to be stimulated by GAP is also attenuated by different phospholipids (Tsai *et al.*, 1989b), which is perhaps indicative of their functioning in different signal transduction pathways.

The Ral proteins are 50 % similar to *ras* p21 and 85 % similar

to each other. Like R-Ras, the regions of similarity are those involved with GTP binding and hydrolysis and with membrane localization. The effector-binding regions, however, show differences. Ral A, which like R-Ras is non-transforming, (Chardin, 1988) consists of 206 amino acids and has a molecular mass of 23.5 kDa. It has eleven extra residues at its N-terminus and six at its C-terminus. No function has yet been assigned to the Ral proteins (Chardin & Tavitian, 1986).

More is known of the *rap* genes in the *ras* family. *rap* 1A (also known as Krev 1 and *smg* 21) was first identified both by its homology to the *Drosophila* D-*ras3* gene (Pizon *et al.*, 1988) and by its GTP-binding properties (Kawata *et al.*, 1988). It comprises 184 amino acids, with a molecular mass of 21 kDa and a 50 % homology to Ras. Its effector region is identical to that of Ras, suggesting, as with R-Ras, that these proteins interact with similar (or the same) targets. GTPase activity of Rap 1A is not, however, stimulated by RasGAP, probably because the Rap proteins have a Q-to-T substitution at amino acid 61, reducing their intrinsic GTPase activity. In fact, mutation of this residue to glutamine in Rap 1A gives a protein with GTPase activity similar to Ras and sensitive to stimulation by RasGAP (Hart & Marshall, 1990). Investigation of genes able to suppress the transforming ability of K-*ras* has revealed *rap* 1A (Noda *et al.*, 1989; Kitayama *et al.*, 1989). Mutagenesis studies have shown Rap 1A to be controlled in a similar fashion to Ras by GTP hydrolysis. The presence of V12 mutations increased the ability of *rap* 1A to suppress transformation, while mutations at amino acids 17, 38, 61, 116 and 181 reduced the degree of transformation suppression (Kitayama *et al.*, 1990). Thus, Ras and Rap 1A are thought to counterbalance each other but via the same mechanism, possibly as antagonists of the same effector. *smg* p21 was first isolated as the bovine equivalent of *rap* 1A, and two GTPase-activating proteins have been found for it (*smg* p21 GAP1 and 2) with molecular masses of 250–400 kDa and 80–100 kDa respectively. These GAPs seem specific to *smg* p21 (Rap 1A) as they increase GTPase activity with *smg* p21 but not with Ras, Rho or *smg* p25 (Kikuchi *et al.*, 1989). The *rap* proteins appear to be ubiquitous and there is some evidence to suggest their abundance in cells with low levels of *ras* p21 (Culine *et al.*, 1989; Kim *et al.*, 1990a). Interestingly, Rap 2 was found to be absent from certain sarcomas, while Rap 1A is expressed only at low level in some adenocarcinomas and fibrosarcomas (Culine *et al.*, 1989).

A yeast analogue has been found for Rap 1 and designated

Fig. 6. Representation of the subcellular localization of various yeast secretory proteins and the mammalian *rab* proteins in the compartments involved in endocytosis and secretion

RSR 1. Mutational studies suggest its involvement in growth processes (Hall, 1990a). Another possible member of this group has recently been reported, the TC21 protein from a teratocarcinoma line, which is 55 % similar to Ras and 89 % similar to R-Ras with a completely conserved Ras effector domain (Drivas *et al.*, 1990).

Rho family

The Rho family of proteins show approximately 30 % similarity to Ras p21. The *rho* genes themselves were first isolated from *Aplysia* (Madaule & Axel, 1985), but counterparts have been detected in yeast, *Drosophila*, rat and man. The human Rho proteins A, B and C are of 193, 196 and 194 amino acids respectively (21 kDa), with homology to Ras in GTP binding regions and the C-terminal CAAX box (Yeremian *et al.*, 1987; Chardin *et al.*, 1988). They differ from Ras in having an alanine at amino acid 15 (13 in H-Ras) instead of a glycine, explaining the reduced GTPase activity since this corresponds to an activating/ oncogenic mutation in Ras (Garrett *et al.*, 1989) (see above). Two *rho* genes have been found in yeast, RHO 1 and RHO 2 (90 % similar to human Rho); RHO 1 is essential for viability whilst RHO 2 is not. The lethality of RHO 1 deletion cannot be suppressed by over-expressing activated RAS 2, suggesting that Rho and Ras function in different pathways (Madaule *et al.*, 1987). RhoC has been found to be ADP-ribosylated by *Clostridium botulinum* exoenzyme C3; this results in the disassembly of actin microfilaments, suggesting that Rho C may be involved in cytoskeletal control (Chardin *et al.*, 1989). Rho A and B are very similar to Rho C and so could be C3 substrates as well and likewise involved in cytoskeletal control. A GAP molecule for Rho A is present in mammalian cells and *Xenopus* oocytes and is of 29 kDa molecular mass (Garrett *et al.*, 1989). Rho does not interact with RasGAP, probably due to E40 (corresponding to D38 in H-Ras) which would destroy a Ras-RasGAP interaction. A GDP dissociation factor has also been reported for Rho (Hall, 1990a). Microinjection of activated Rho into Swiss 3T3 cells leads to a change in cell morphology and prevents GTPase activation by RhoGAP. Ribosylated Rho is

incapable of mediating morphological changes, but can still interact with RhoGAP, suggesting that Rho has other target molecules (Paterson *et al.*, 1990). This observation also further implicates Rho in organization of the cytoskeleton.

Analysis of a teratocarcinoma cell line has revealed two *rho*-like genes TC25 and TC10. TC25 is the gene *rac 1*, which will be described later. TC10 contains a CAAX box and is therefore likely to be lipid-modified and membrane-bound. TC10 has only been found in human and mouse cell lines (Drivas *et al.*, 1990).

Two final members of the *rho* family were reported in 1989, *rac1* and 2 (Didsbury *et al.*, 1989). No definite function can be ascribed to them, but they are ADP-ribosylated by *C. botulinum* C3 in a similar fashion to Rho and are also isoprenylated, localizing them to the particulate cell fractions. Rac is found in large quantities in secreting neutrophils and monocytes. As the Rac proteins are substrates for *C. botulinum* C3 which can block stimulation of exocytosis in neutrophils, they have the potential for a regulatory role in secretory processes (Didsbury *et al.*, 1990). This is consistent with possible functions for *rab* proteins and yeast analogues which will be discussed later.

Rab family

The *rab* gene family is, like *rho*, approximately 30 % similar to *ras*. The areas of similarity lie in the four conserved regions which comprise the GTP binding/hydrolysis site, but differences are seen in this family in the C-terminal regions. The *rab* family do not have the characteristic CAAX box but rather seem to have either CC or CXC as the lipid modification/membrane association signal at the C-terminus.

The first members of this family to be discovered were the yeast genes *YPT1* and *SEC4*. *YPT1* was identified as an essential *S. cerevisiae* gene with 38 % similarity to *ras* p21 and with similar biochemical properties (Gallwitz *et al.*, 1983). It has since been found to be a ubiquitous eukaryotic protein (Haubruck *et al.*, 1987). *SEC4*, encoding a *ras*-like protein, was isolated as a mutation in the yeast secretion pathway, causing a build up of post-Golgi vesicles (Salminen & Novick, 1987). *SEC4* is a 23.5 kDa protein which has 32 % similarity with *ras* proteins.

Fig. 7. Scheme for the role of Ras-related proteins in vesicle trafficking

The Ras-related protein recognizes a 'tagging protein' on a vesicle leaving a donor compartment. This complex then determines attachment to the appropriate acceptor compartment via a specific 'docking protein' on the acceptor membrane. GTP hydrolysis releases the Ras-related protein to be recycled via a GNRP at the donor membrane, leaving the docking and tagging protein complex to ensure vesicle fusion at the acceptor membrane.

YPT1 is now known to function in the early stages of the secretory pathway. It is found associated with Golgi-like structures and is also implicated in microtubule organization (Segev *et al.*, 1988) and Ca^{2+} regulation (Schmitt *et al.*, 1988). *SEC4*, on the other hand, has been localized to the final stages of secretion in yeast and is involved in fusion of vesicles to the plasma membrane (Walworth *et al.*, 1989). It would seem that these Ras-like proteins could control cellular protein movement. Other proteins involved in these processes have been found to be Ras-like, e.g. SAR1, which is proposed to direct endoplasmic reticulum-Golgi protein transport (Nakano & Muramatsu, 1989). SAR1, however, has no C-terminal cysteine and lacks the N-terminal myristoylation motif found in ARFs (discussed below). The membrane-bound protein cofactor for cholera toxin-dependent ADP ribosylation is termed ARF and is a 21 kDa polypeptide with homology to *ras* p21 (Kahn & Filman, 1986). Mammalian analogues have also been found (Sewell & Kahn, 1988). ARF contains the N-terminal myristoylation signal MGXXXS/A/T, found in other G proteins (Buss *et al.*, 1987; Schultz *et al.*, 1987) and is appropriately modified. The protein also shows significant structural similarities to the α subunits of the heterotrimeric G proteins. Possibly, ARF and SAR1 comprise a new family within the *ras* superfamily, being intermediates between the p21 proteins and the heterotrimeric G proteins. ARF protein in yeast is encoded by *ARF1* and *ARF2*; deletion of either one of these genes retains viability but deletion of both is lethal. The protein is localized to the Golgi apparatus in mammalian and yeast cells. A GAP activity has also been identified for ARF in yeast (Stearns *et al.*, 1990).

Many mammalian analogues have now been found to these yeast genes. Originally, four *YPT1* analogues were isolated from a rat brain cDNA library and termed *rab1*, 2, 3 and 4. *Rab1* is in fact the *YPT1* analogue, showing 75% similarity to the yeast protein and is 202–205 amino acids in length (depending on initiation codon use) (Touchot *et al.*, 1987). Human homologues for these four rat genes have now been isolated; H-*rab1*, H-*rab2*, H-*rab3A* and H-*rab4*, along with three additional *rab* cDNAs; H-*rab3A*, H-*rab5* and H-*rab6*, all of which encode proteins of

23–25 kDa (Zahraoui *et al.*, 1989). While all the other *rab* genes show wide transcription patterns, *rab3A* seems to be confined to brain tissue where it is associated with synaptic vesicles and endocrine tissue (Fischer *et al.*, 1990; Burgoyne & Morgan, 1989). It is one of the limited number of *ras*-related proteins to show such specificity.

A picture of the localization of *rab* proteins similar to that of the yeast *YPT1* type proteins is emerging (summarized in Fig. 6). Synthetic peptides comprising the Rab effector domain are potent inhibitors of endoplasmic reticulum to Golgi and intra-Golgi transport (Plutner *et al.*, 1990). A putative effector protein (REP) has also been found for the *rab* proteins, although at present it is not known whether this will be the same for each *rab* protein or will itself constitute a family of related polypeptides (Plutner *et al.*, 1990).

Chavrier *et al.* (1990) have detected similar proteins in canine cells, identifying eleven possible candidates, many of which are localized to different subcellular compartments. *Rab2* is located in the 'salvage' compartment between endoplasmic reticulum and Golgi and is thus involved in the early stages of transport, as is *YPT1*. *Rab5* is found on the cytoplasmic surface of the plasma membrane and early endosomes, while *Rab7* is located on late endosomes, isolating *rab5* and *rab7* to the late stages of endocytosis (see Fig. 6). These clones were from only 30% of a total library, indicating the possible presence of up to 30 such related genes. This correlates with the growing number of yeast *SEC* mutants available. The presence of so many related molecules associated with different membranes which presumably all have their own GAP, GDI and GNRP is all indicative of a very complex series of interactions, as has been proposed by Bourne (1988).

Three different *smg* p25s are also present in bovine brain (Matsui *et al.*, 1988). *smg* p25A is identical to *rab3A*, whilst *smg* p25B and *smg* p25C are of 219 and 227 amino acids respectively. *smg* p25A, B and C are 80% similar and differ only in the C-terminal 32 residues, which is reminiscent of the *ras* proteins. They have higher GTPase activity than Ras due to the presence of serines rather than glycines at positions 12 and 13. A negatively

regulating GDI (GDP dissociation inhibitor) has been isolated for *smg* p25A/Rab3A (two major and two minor species) although it is also found in tissues lacking *smg* p25A, suggesting it could interact with other proteins (Matsui *et al.*, 1990).

BRL-*ras* was isolated from a rat liver cell line, and is a 201-amino-acid protein of 22.8 kDa. It seems to fit into the *rab/YPT1* family as it has a serine at the equivalent of amino acid 12 and a CXC lipid modification motif at the C-terminus. Its effector domain is also similar to that of the *rab* family (Bucci *et al.*, 1988). BRL-*ras* equivalents have been found in many species but no function can be determined for them at present.

It is emerging that the *ras*-related gene family consists of a large number of G proteins, all of which appear to have multiple specific control polypeptides. They are ubiquitous in most cases (*rab3A* being a notable exception) and often absolutely required by the cell. Not only are there many *ras*-related proteins but also analogues of *ras* have been found in all cell types that have been investigated.

There is considerable genetic evidence to suggest that yeast proteins are involved in the secretory pathway. Similarly the mammalian equivalents are involved in intracellular trafficking (Fig. 7). It has been proposed that these proteins control the unidirectional flow through various compartments (organelles) by ensuring that tagged vesicles (i.e. bearing a 'tagging protein') leaving a donor membrane dock with the appropriate acceptor membrane (Walworth *et al.*, 1989; Bourne, 1988). A diagram summarizing this hypothesis is shown in Fig. 7.

p21, GROWTH FACTORS AND SIGNAL TRANSDUCTION

If *ras* p21 plays a role in signal transduction it is reasonable to suppose that there should be changes in the response of *ras*-transfected cells to one or more growth factors. Similarly, if Ras is neutralized in normal cells, through the introduction of a p21 antibody, it is likely that the response to a particular growth factor will be altered.

The essential requirement for Ras in actively dividing normal cells (and NIH 3T3s) has been demonstrated (Mulcahy *et al.*, 1985), as has the ability of wild type or mutant Ras to stimulate cell division (Stacey & Kung, 1984; Feramisco *et al.*, 1984). The protein may be of importance in the action of a number of growth factors since their addition to cells containing neutralizing Ras antibody had no effect on cell division (Mulcahy *et al.*, 1985).

Whilst the details of the role of p21 in growth factor response are not clear, it has been shown to be involved in the pathways by which the cell responds to a number of different stimuli. For example, in the rat pheochromocytoma (PC12) cell line injection of neutralizing antibody to Ras inhibits NGF-induced neurite formation but not neurite outgrowth due to cyclic AMP (Hagag *et al.*, 1986). The effect of NGF is duplicated, however, by the injection of activated p21 or infection with K MSV or H MSV, inducing differentiation (Bar-Sagi & Feramisco, 1985; Noda *et al.*, 1985). More recently it has been shown that Ras in PC12s is involved in the signal pathway by which NGF (or basic FGF) stimulates *c-fos* expression. There is some disagreement, however, over the effects (either up or down regulation) produced by oncogenic p21 in this system (Sassone-Corsi *et al.*, 1989; Thomson *et al.*, 1990; Szeberenyi *et al.*, 1990). Despite this contradiction the majority view appears to be that *c-fos* expression is induced in PC12s and in many other cell types following the introduction (either by microinjection or by means of an inducible promoter) of activated p21 (Stacey *et al.*, 1987; Sassone-Corsi *et al.*, 1989; Cai *et al.*, 1990; Ledwith *et al.*, 1990). This seems to be a manifestation of the mitogenic effects of the *ras*

oncoprotein, although it should be noted that the induction of DNA synthesis requires additional pathways not necessary for an increase in cellular c-Fos level (Cai *et al.*, 1990). The observation that inhibition of *c-fos* expression can negate many of the phenotypic characteristics of *ras*-transformed cells also lends support to the proposition that c-Fos acts downstream of Ras in one or more signal transduction pathways (Ledwith *et al.*, 1990).

The insulin receptor has also been suggested as an initiation point for a signal pathway in which *ras* p21 might be implicated (Korn *et al.*, 1987; Deshpande & Kung, 1987; Sadler & Maller, 1989; Burgering *et al.*, 1989). Certainly, in *Xenopus* oocytes (see below) insulin-induced maturation appears to be mediated through a *ras*-dependent pathway. Additionally, p21 can be phosphorylated *in vitro* by the insulin receptor tyrosine kinase (Fujita-Yamaguchi *et al.*, 1989; R. J. A. Grand, B. A. Levine & L. Ellis, unpublished work; see above) and can bind to that protein (O'Brien *et al.*, 1987; R. J. A. Grand, B. A. Levine and L. Ellis, unpublished work). The significance of all these observations is not clear at present but taken together they strongly suggest that Ras plays a major part in insulin signalling.

The response of mouse 3T3 cells to PDGF involves an increase in GTP-bound Ras that may be important in the transmission of that signal (Sato *et al.*, 1990). This observation, taken in conjunction with the biochemical data, summarized above, points to an intimate relationship between the PDGF receptor, *ras* p21 and GAP. Hopefully, the consequences of these interactions, as well as those seen for the EGF receptor, for signalling pathways will soon be demonstrated.

If p21 were involved in signal transduction it might be expected that it could be shown to exert an influence at one or more points in a particular second messenger system. This view was given considerable encouragement by the demonstration that in *S. cerevisiae* the RAS protein acts as the regulatory subunit of adenylate cyclase (Toda *et al.*, 1985; Kataoka *et al.*, 1985). Unfortunately, however, no evidence has been presented to support the hypothesis that this regulatory function extends to higher eukaryotes (Beckner *et al.*, 1985; Birchmeier *et al.*, 1985).

Since that time, great effort has been invested in attempting to determine precisely where Ras acts in signal transduction pathways. The results obtained have been equivocal and it is still far from clear how or where Ras plays a role (if at all) in various second messenger systems. A number of disparate approaches have been adopted in these studies and they are listed in detail in Table 3. For the sake of convenience, they may be divided into groups – firstly experiments in which Ras is either (a) removed from cells (by means of the injection of neutralizing antibody) or (b) added to cells. This second set may be subdivided using mode of delivery of protein as a criterion. Thus, studies have been performed on cells transfected with *ras* genes (which may or may not be under the influence of an inducible promoter), or micro-injected or 'scrape-loaded' with protein.

The *Xenopus* oocyte system

The general incompatibility of many of the results obtained may be seen by a consideration of the effects of *ras* in a relatively well-defined system, *Xenopus* oocytes. An original study by Birchmeier *et al.* (1985) demonstrated the feasibility of the system by showing that the injection of activated, but not wild type, H-Ras into oocytes induced maturation but not a change in the level of cyclic AMP within the cell. In contrast, it has recently, perhaps more convincingly, been observed that activated Ras stimulates cyclic AMP phosphodiesterase activity (Sadler & Maller, 1989; Sadler *et al.*, 1990) soon after injection. Although the two sets of experiments were performed in rather

different ways and are therefore not directly comparable, this sort of inconsistency is typical of much of the work done on the effects of Ras on secondary metabolism. There is evidence to suggest that microinjection of mutant p21 activates the pathway normally associated with insulin-induced maturation (Deshpande & Kung, 1987; Lacal *et al.*, 1987a; Korn *et al.*, 1987; Sadler *et al.*, 1990), although it has recently been observed that Ras stimulates phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine metabolism (Pan & Cooper, 1990; Lacal, 1990), whereas insulin does not (Pan & Cooper, 1990). Whilst it is possible that the Ras-induced increase in level of phospholipid metabolism could be unrelated to maturation, the close correlation observed between amount of Ras injected, extent of maturation and increase in lipid metabolism has suggested that phosphoinositide-derived second messengers mediate the maturation effects of *ras*. Therefore the *ras*-stimulated mechanism may be somewhat different to that involved in insulin-mediated maturation (Pan & Cooper, 1990).

In the study of the effects of *ras* on mammalian cells one of the areas of relatively widespread agreement is that the introduction of p21 into cells is accompanied by an increase in level of diacylglycerol (DAG). Such observations have also been reported in the oocyte system (Lacal *et al.*, 1987a; Lacal, 1990), although Pan & Cooper (1990) have failed to duplicate these results. Interestingly, the DAG does not appear to be produced by inositol lipid hydrolysis but more probably derives from an increase in phosphatidylcholine (PC) metabolism (Lacal, 1990). Phosphatidylcholine could be hydrolysed by phospholipase C (PLC) producing phosphorylcholine and DAG. Alternatively, phospholipase D (PLD) has been shown to hydrolyse PC to produce choline and phosphatidic acid, which can in turn be converted to DAG by the action of a phosphatidate phosphohydrolase (Liscovitch *et al.*, 1987; Cabot *et al.*, 1988). That the point of action of activated p21 in *Xenopus* oocytes is indeed at some point in the phosphatidylcholine pathway remains to be unequivocally demonstrated, but it is possible that this effect of Ras is not limited to this particular system and is also seen in mammalian cells (see below).

Mammalian cells

Attention has focused on four major areas of the signal transduction system in mammalian cells transfected or injected with p21: the level of DAG, the level of PKC, the effects of changes in the activity of PLC, and the level of inositol lipid metabolism. These areas may be artificially delineated depending on the approach adopted by different groups but are in reality closely interrelated. The level of DAG is increased in cells transformed with *ras* oncogenes or 'scrape loaded' with *ras* oncoprotein (Fleischman *et al.*, 1986; Priess *et al.*, 1986; Lacal *et al.*, 1987b; Wolfman & Macara, 1987; Price *et al.*, 1989), although this probably does not originate from the phosphoinositides that are usually taken to be its source (Lacal *et al.*, 1987b; Price *et al.*, 1989; Lacal, 1990). [Interestingly, an increase in DAG is also seen in *ras*-transformed tissue of transgenic mice (Wilkinson *et al.*, 1989)]. In normal cells DAG is an endogenous activator of PKC (reviewed by Nishizuka, 1988) and therefore it is perhaps not surprising that increase in phosphorylation of an 80 kDa PKC substrate has been observed in *ras*-transformed cells (Wolfman & Macara, 1987; Huang *et al.*, 1988) and in quiescent 3T3s into which activated *ras* protein has been introduced (Morris *et al.*, 1989). Recent evidence also suggests a second link between *ras* and PKC in that transformation of rat embryo fibroblasts and liver epithelial cells by activated H-Ras leads to an increase in level of expression of PKC α and a concomitant decrease in level of expression of PKC ϵ (Borner *et al.*, 1990), as detected at both the mRNA and protein level. This report must,

however, be contrasted with earlier data which had shown a decrease in the level of PKC α (the only isoform present) in *ras*-transfected C3H10T $\frac{1}{2}$ mouse fibroblasts (Weyman *et al.*, 1988). Whilst most evidence suggests that the presence of activated p21 produces an increase in PKC activity, the presence of the enzyme is not necessarily required for *ras*-mediated transformation, nor for *ras*-induced stimulation of *c-myc* mRNA levels (Lloyd *et al.*, 1989; Cai *et al.*, 1990). However, it is clear that the mitogenic effect of the *ras* proteins does require a functional protein kinase C (Lacal *et al.*, 1987b; Lloyd *et al.*, 1989); although protein synthesis and PKC are not required for *ras* transformation *per se* they do appear to be required for maintenance of the transformed phenotype. These results are further evidence of a variety of effects of *ras* in signal transduction in that the protein is capable of producing responses through PKC-dependent and -independent pathways (Cai *et al.*, 1990). Recent data have also implicated PKC in the control of Ras activity in T lymphocytes since stimulation of the kinase results in a decrease in GAP activity and a concomitant rise in the level of GTP-bound p21 (Downward *et al.*, 1990a).

A further enzyme central to phospholipid metabolism is inositol phospholipid-specific phospholipase C (PLC), which hydrolyses phosphatidylinositol 4,5-bisphosphate producing two second messenger molecules, DAG and inositol 1,4,5-trisphosphate. Because of its important role in the production of DAG and the fact that it is capable of inducing transformation of NIH 3T3 cells after microinjection (Smith *et al.*, 1989) PLC activity in *ras* transfectants has been the subject of considerable study. It is clear that *ras* proteins do not directly control the activity of PLC (or phospholipase A $_2$) since the introduction of compounds designed to duplicate the enzymic activity could not produce DNA synthesis in cells injected with an anti-Ras antibody (Yu *et al.*, 1988). Therefore, it is reasonable to conclude, on the basis of these experiments, that the proliferative effect of *ras* proteins is exerted 'downstream' of PLC. Results obtained after microinjection of activated p21 into NIH 3T3 cells are, however, quite different since, in this case, *ras* acts as an 'upstream' effector of PLC activity such that the presence of a neutralizing antibody to p21 has no effect on the stimulation of DNA synthesis produced by the injection of PLC (Smith *et al.*, 1990).

The fourth main area of interest, which is closely linked to PLC activity, is a direct measurement of the absolute level or level of metabolism of inositol lipids and polyphosphates in cells containing exogenous *ras*. Again, the data are conflicting, making it difficult to draw firm conclusions on the effects of either mutant or wild type protein. For example, Seuwen *et al.* (1988) could detect no differences in basal rate of phosphoinositol turnover in hamster cells after transformation by activated *ras* and concluded that the 'transforming potential of activated forms of p21 does not result from persistent activation of phospholipase C'. Similarly, Price *et al.* (1989) found no increase in inositol phosphate metabolism after scrape-loading mutant p21. On the other hand, others have found that the expression of activated, but not wild type, *ras* in NIH 3T3 cells led to an increase in the basal rate of inositol phosphate production (Fleischman *et al.*, 1986; Hancock *et al.*, 1988). An alternative way of examining phosphoinositol metabolism has been to look at the effect of normal and mutant p21 on cell membranes (Kamata & Kung, 1988). In that case, wild type Ras increased the formation of inositol trisphosphate whereas activated p21 had little effect. Whole cell experiments have supported this view that wild type p21 causes an increase in inositol lipid turnover in NRK cells, whereas activated Ras does not (Kamata & Kung, 1988).

As has been observed for the *Xenopus* oocyte system, there is evidence to suggest that activated p21 exerts at least some of its

Table 3. Effect of *ras* p21 on secondary messenger systems

Introduction of p21†	Ras type	Recipient cell	DAG level	Direct measurement of PKC activity	Direct measurement of PLC activity
Microinjection	Activated H- <i>ras</i> (V12)	<i>Xenopus</i> oocyte	ND‡	ND	ND
Microinjection	Activated H- <i>ras</i>	<i>Xenopus</i> oocyte	↑	ND	ND
Microinjection	Activated (V12 T59) H- <i>ras</i>	<i>Xenopus</i> oocyte	ND	ND	ND
Microinjection	Activated H- <i>ras</i>	<i>Xenopus</i> oocyte	↑	ND	ND
Microinjection	Activated (L61) H- <i>ras</i>	<i>Xenopus</i> oocyte	No change	ND	ND
Microinjection	Anti-p21 antibody	<i>Xenopus</i> oocyte	ND	ND	ND
Scrape loading	Activated (V12) H- <i>ras</i>	Swiss 3T3	ND	↑	ND
Scrape loading	Activated (V12) H- <i>ras</i>	Swiss 3T3	↑	↑	ND
Transfection	Activated K- <i>ras</i>	NRK	↑	ND	ND
Transfection	Activated H, K and N- <i>ras</i>	NRK, NIH 3T3	↑	ND	ND
Transfection (<i>ras</i> under inducible promoter)	High level of expression of wt N- <i>ras</i>	NIH 3T3	ND	ND	ND
Transfection	Activated H and K- <i>ras</i>	NIH 3T3, NRK	↑	↑	ND
Transfection	Transforming H- <i>ras</i> constructs	NIH 3T3	↑	ND	ND
Transfection	Activated H- <i>ras</i>	Rat 1	ND	ND	ND
Transfection	v-K- <i>ras</i>	NIH 3T3	↑ In transformed line (DT). No change in <i>ras</i> expressing untransformed line (C11)	↓ in both sets of cells	ND
Transfection	Activated H- <i>ras</i>	CCL39	ND	ND	ND
Transfection	Activated H and N- <i>ras</i>	NIH 3T3 or Cos1 (inducible p21)	ND	ND	ND
Transfection	Activated H- <i>ras</i>	C3H10T½	ND	↓ Level and activity	ND
Transfection	Wt and activated H- <i>ras</i>	Rat 1	ND	ND	ND
Transfection	v-H- <i>ras</i>	208 rat fibroblasts	↑	Initial activation then down-regulation	No change
Transfection	Activated H- <i>ras</i> or v-K- <i>ras</i>	NIH 3T3 or Rat 1	ND	ND	Uncoupling between surface stimulation and PLC
Transfection (<i>ras</i> under inducible promoter)	v-H- <i>ras</i>	PC12	↑	ND	ND
Transfection	Activated H- <i>ras</i> and K- <i>ras</i>	NIH 3T3	ND	ND	ND
Transfection	Activated and wt H- <i>ras</i>	NIH 3T3 and Rat 1	ND	ND	Uncoupling between surface stimulation and PLC

† In microinjection and scrape loading experiments protein was introduced into recipient cells; transfection indicates the introduction of DNA.

‡ ND, not determined.

influence by altering phosphatidylcholine but not phosphoinositide metabolism (Price *et al.*, 1989). Indeed, Macara (1989) has recently shown that there is an increased choline kinase activity in *ras*-transformed NIH 3T3 cells, giving rise to elevated levels of phosphocholine. Similarly, changes in DAG level have been found not to correlate with increased phosphoinositide metabolism (Wolfman & Macara, 1987; Price *et al.*, 1989).

This brief survey of the considerable recent literature on the relationship between *ras* p21 and the second messenger system will give some idea of the general air of confusion which seems to pervade the subject (Table 3). Areas of agreement between different groups of workers seem to be rather limited. Starting from the basic observation that the introduction of activated p21 into cells produces a mitogenic response, it has generally been felt that there ought to be a complementary and marked change in

the activities of the two enzymes central to the second messenger system (PLC and PKC) with a resulting change in metabolite levels. A consideration of the data summarized in Table 3 gives the impression that a greater significance is being attached to the results obtained than they merit and that the variations in intermediary metabolism may be only a by-product of *ras* transformation. More specifically, a number of the experiments performed in this field of research may be criticized from several points of view. Firstly, an examination of the effects of *ras* judged by comparison of intermediary metabolite levels in cells before and after transformation seems to be basically flawed. By the time that transformed cells are established a considerable period of time will have elapsed and a large number of changes within the cell will have occurred. Variations within the second messenger system, which is designed to transmit information relatively

Inositol lipid and inositol polyphosphate metabolism	Phosphocholine metabolism	Other observations	Reference
ND	ND	Oocyte maturation; no change in cyclic AMP level	Birchmeier <i>et al.</i> (1985)
↑ level of PtdInsP_2 , InsP and InsP_3	ND	No change in InsP_3 or InsP_4 , no effect with wt p21	Lacal <i>et al.</i> (1987a)
ND	ND	Oocyte maturation; increase in PDE activity; no effect with wt p21	Sadler & Maller (1989), Sadler <i>et al.</i> (1990)
↑ Small increase in level ↑ PtdIns and PtdInsP turnover	↑ Phosphocholine and choline kinase ↑ Phosphatidylcholine and phosphatidylethanolamine metabolism	Oocyte maturation; no effect with wt p21	Lacal (1990) Pan & Cooper (1990)
ND	ND	Blocks insulin-induced maturation	Deshpande & Kung (1987), Korn <i>et al.</i> (1987)
No change	ND	p21 increased phosphatidylcholine metabolism	Morris <i>et al.</i> (1989)
No change	↑ Phosphocholine and choline level	No effect of wt	Price <i>et al.</i> (1989)
ND	ND		Preiss <i>et al.</i> (1986)
↑ Level of InsP_2 and InsP_3 , ↑ ratio of $\text{DAG}/\text{PtdInsP}_2$	ND		Fleischman <i>et al.</i> (1986)
↑ Level of InsP in response to certain growth factors	ND		Wakelam <i>et al.</i> (1986)*
No change	ND		Wolfman & Macara (1982)
No change	↑ Level of phosphorylcholine and phosphorylethanolamine	No effect of wt or non-transforming p21	Lacal <i>et al.</i> (1987b)
Correlation between <i>ras</i> expression and lack of response to PDGF as measured by InsP_2 and InsP level	ND	Increased bradykinin receptors	Parries <i>et al.</i> (1987)*
↑ Turnover and level of InsP , InsP_2 and InsP_3 in transformed DT line. No change in untransformed cell line (C11)	ND		Kamata <i>et al.</i> (1987b)
No change in InsP turnover (in the presence or absence of growth factors)	ND		Seuwen <i>et al.</i> (1988)
↑ Turnover and level of InsPs	ND	Overexpressed wt p21 had no effect, nor did non-transforming mutants	Hancock <i>et al.</i> (1988)
ND	ND		Weyman <i>et al.</i> (1988)
↑ Level and turnover of InsP_2 and InsP_3	ND	Increase PtdIns turnover in response to growth factors was due to a <i>ras</i> -induced increase in bradykinin receptors	Downward <i>et al.</i> (1988)*
↑ PtdIns , PtdInsP , PtdInsP_2	↑ Phosphatidylcholine metabolism		Huang <i>et al.</i> (1988)
↑ Level of glycerophosphoinositol, ↑ ratio of $\text{GroPIns}/\text{InsPs}$. No change in levels of InsP_2 , InsP_3 , InsP_4 and InsP_5	ND	Increased phospholipase A_2 activity; similar effects were seen with other membrane associated and cytoplasmic oncogenes	Alonso <i>et al.</i> (1988)
↑ InsP_3 turnover	ND	Increased level of cyclic AMP and acetylcholinesterase activity	Sugimoto <i>et al.</i> (1988)
ND	↑ Metabolism	Increased choline kinase activity	Macara (1989)
↑ Level of GroPIns , InsP , InsP_2 and InsP_3 in response to AlF_4^- , but less in normal cells	ND	Increased bradykinin receptors; similar effects were seen with other membrane associated and cytoplasmic oncogenes	Alonso <i>et al.</i> (1990)*

* The results obtained in these papers should be considered in the light of the data presented by Downward *et al.* (1988) and Alonso *et al.* (1990), indicating that cytoplasmic and membrane-associated oncogene mediated transformation causes an increase in bradykinin receptors.

rapidly (over a time scale of seconds and minutes) could well be a very indirect result of the presence of mutant p21. A second major criticism which can be made of virtually all of the experiments where inositol phosphates have been studied is that little or no effort has been made to characterize the heterogeneous water-soluble inositol phosphate population present. This is particularly important since it is now apparent that a large number of different inositol phosphate derivatives are present in the cell and many have different functions (see for example the review by Berridge & Irvine, 1989); thus whilst changes in overall level or rate of metabolism of inositol phosphates may be small, the changes for a particular component may be appreciable. Similarly, changes in certain components of the inositol phosphate pathway may have more far-reaching consequences than others; it is obviously important to distinguish between those

inositol phosphates in which changes in level or metabolism result indirectly from some undefined *ras*-initiated 'transformation event' and those inositol phosphates which trigger other important processes (such as Ca^{2+} release).

It is also clear that 'transformation' *per se* may give rise to changes in the metabolism of inositol lipids and polyphosphates without *ras* p21 having any direct influence. For example, it has been shown that transformation of rodent cells by a number of cytoplasmic and membrane located oncogenes (including *ras*) all cause a similar increase in glycerophosphoinositol/inositol phosphate ratio (Alonso *et al.*, 1988, 1990). Conversely, in cells expressing high levels of v-K-Ras but which are not transformed, the levels of glycerophosphoinositol and inositol phosphates are similar to those seen in the parental NIH 3T3 cells. In another study using these lines, levels of DAG and of inositol mono-, bis

and trisphosphates were elevated in the transformants but not in the high Ras-expressing untransformed line (Kamata *et al.*, 1987b).

Finally, it is important to stress that the introduction of activated *ras* into cells may have effects that are only tenuously connected to the function of the wild type protein in normal cells. This is particularly relevant to a consideration of the relationship between *ras* and second messenger systems, since in most cases unmutated protein has only a limited effect on cellular metabolism.

CONCLUSIONS AND FUTURE PROSPECTS

It is clear from the mass of data presented in this Review that *ras* oncogenes and their protein products have been the subject of intensive study over the past decade. In spite of this, we have little idea of what function these polypeptides perform in either the normal or transformed cell. As a consequence of their ubiquitous distribution and highly conserved structure, it is obvious that *ras* p21 must play a key role in most, if not all, cells. Based on structural and biochemical similarities to the α subunits of the heterotrimeric G proteins, it has been suggested that the Ras proteins are involved in signal transduction at the plasma membrane. With this in mind, a number of workers have endeavoured to demonstrate differences in second messenger systems as a result of the introduction of *ras* genes or proteins (summarized in Table 3). The results obtained from these studies have been equivocal and it still seems likely, although certainly not proven, that there is no direct link between p21 and the control of either phospholipase C or protein kinase C. Changes which have been observed in the activities of these enzymes and in the metabolism of phosphoinositides in *ras*-transformed cells are probably the indirect result of 'the transformation event' and not a central part of that process.

Whilst these conclusions tend to argue against a central position for Ras in the direct regulation of PKC or PLC, recent evidence points towards a close involvement of p21 with a number of growth factor receptors, upstream of, or perhaps laterally displaced from, these enzyme. This link has been established through GAP which is phosphorylated by tyrosine kinases in response to EGF, PDGF and CSF-1. The consequences for Ras of phosphorylation of GAP and its binding to other proteins (e.g. p62 and p190) are not clear, but these data and the apparent involvement of p21 in mediating the cells' response to insulin, place it in close proximity to the receptor-type tyrosine kinases (see Cantley *et al.*, 1991, for a detailed discussion of these points). As a means of examining directly what happens to Ras in stimulated cells the simple protocols recently adopted by Downward *et al.* (1990a) and Satoh *et al.* (1990) appear to have been particularly fruitful. In this way a direct connection seems to have been established between cellular stimulation (by phytohaemagglutinin or PDGF) and an increase in the proportion of cellular p21 in an active GTP-bound conformation. The fact that this is probably mediated through the phosphorylation of a GAP-associated or Ras-associated polypeptide suggests that we must be very close to understanding how Ras is 'turned on' if not what role it then plays. It may be that previous failures to demonstrate clear effects of p21 on second messenger systems may be due to inadequacies in the experimental procedures adopted (discussed above) such that subtleties in the cellular response to the introduction of exogenous p21 may have been swamped by other changes occurring as a result of transformation. Similarly, effects due to the introduction of additional wild type Ras may only be very slight and go undetected.

The complexities of the *ras* system have become apparent with the characterization of a large number of Ras-related proteins.

Presumably each will have a distinct, although perhaps related, mode of action. Rather more progress has been made with these polypeptides than with Ras itself in the attribution of function. For example, members of the Rab family seem to be involved in intracellular transport although their precise activity is still unclear (see above) while the Rho family appear to be involved in cytoskeletal control. Unfortunately, it is the Ras family which is least well understood at present. If these proteins act in a manner analogous to p21, there will exist a large panel of specific GAPs, GNRPs and GDIs within the cell, all of which are involved in the regulation of guanine nucleotide binding and hydrolysis by these small GTP-binding proteins. If this hypothesis is correct it is certain that in the near future these proteins will be purified and characterized; whether this will help to define functions for them, however, is open to question.

Whilst we do not appear to be much closer to a role for Ras now than we were 5 years ago, there have been some noteworthy successes in our attempts to understand the mode of action of the protein. It is unlikely that there is much left to learn about the post-translational modification which Ras undergoes, although the reasons for such a sophisticated set of reactions are only just beginning to be elucidated. It may be that these studies, which are likely to be completed in the near future, will also provide valuable clues to Ras function. A second area in which great progress has been made is in the determination of the three-dimensional structure of p21. This has provided important information relevant to two areas of investigation; firstly it allows us to understand the effects of any mutation on the polypeptide chain and therefore clarifies the physical consequences of 'activating' mutations. Secondly, the use of caged GTP has allowed the mechanism of the GTP hydrolysis reaction to be described in detail. Hopefully, progress in the near future in these physical studies will be expanded so that the effects of GAP on p21 can be shown at the molecular level.

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