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The *ras* oncogenes

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Summary

Oncogenic forms of the p21*ras* genes have been found in a large variety of human malignancies and tumours induced in animals by chemical carcinogens or irradiation. The active form of the p21 *ras* proteins is the GTP bound state and oncogenic mutations result in the protein being constitutively in the GTP bound active state. There is evidence to suggest that activating mutations can occur either as initiating steps in carcinogenesis or as later events in the evolution to frank neoplasia. To transduce a signal for proliferation and transformation the active GTP form of p21*ras* must interact with one or more cellular targets. Genetic experiments suggest that one potential effector molecule is the GTPase activating protein GAP. However, the mechanism by which interaction with GAP results in proliferation and transformation remains to be elucidated.

Introduction

The H, N and K *ras* genes encode closely related $21 M_r \times 10^3$ proteins that are localized on the inner surface of the plasma membrane, bind and hydrolyse guanosine triphosphate (GTP). These genes and proteins have received much attention because in a large fraction of some human malignancies a *ras* proto-oncogene is activated to a transforming gene (oncogene) by a single point mutation. Although it has been possible to analyse the pattern of *ras* gene mutations in great detail, understanding the function of both the normal and transforming proteins has proved more elusive.

Pattern of *ras* gene mutations in human malignancy

Early studies of *ras* oncogenes in malignancies made use of calcium phosphate coprecipitation to introduce DNA into NIH-3T3 cells to search for transforming genes able to cause focus formation on a confluent layer (Shih *et al.* 1979). Subsequently, efforts were made to extend the sensitivity of the transfection assay by cotransfection with a dominant selectable marker followed by injection of the transfected cells into nude mice to assay for tumorigenic cells (Fasano *et al.* 1984). This cotransfection assay appears to allow detection of mutant *ras* genes, when they are present in only a minor fraction of cells, and transforming alleles that are poor at focus production (Bos *et al.* 1985; Toksoz *et al.* 1987). However, while the transfection assays are very sensitive they are very time consuming and have been superseded for most studies by direct DNA analysis. Allele-specific oligonucleotide probes have proved extremely useful in analysing the natural history of *ras* gene

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mutations, first by direct probing of genomic DNA (Bos *et al.* 1984) and then with much more ease and sensitivity on *ras* target sequences amplified by the Polymerase Chain Reaction (PCR) (Saiki *et al.* 1985). Using the PCR and oligonucleotide probing it is possible to analyse large numbers of samples easily.

Analysis of *ras* gene mutations has lead to the conclusion that some tumours such as breast carcinomas show very little evidence of *ras* gene mutation (Bos, 1988) while in others such as colorectal cancer (Bos *et al.* 1987; Forrester *et al.* 1987), acute myeloid leukaemia (Farr *et al.* 1988*a*) and pancreatic carcinoma (Almoguerra *et al.* 1988) the incidence of mutation is 25–50 % or higher. In these situations where there is a high incidence of mutation there is very pronounced bias to one or other particular *ras* gene being mutated. Thus in AML the mutations are invariably in N-*ras* while in colorectal and pancreatic cancer the majority of mutations are in K-*ras*. The reason for this bias is unknown. At present we have no evidence for functional differences between the *ras* proteins, so it is unclear whether the pattern of activation reflects physiological differences between the proteins. Neither is it clear whether the pattern of activation is a consequence of differential expression of the three genes. The restriction to only one of the three genes being mutated in a particular form of malignancy is paralled by the pattern of activation of *ras* genes by chemical carcinogens in animal experiments. In these experiments the vast majority of tumours show the same *ras* gene being activated by the same mutation (Sukumar *et al.* 1983; Quintanilla *et al.* 1986). These results suggest that the specificity of mutations could reflect the nature of the interaction between DNA sequence and a particular carcinogen.

An important question in *ras* gene activation is the timing of the mutational event in relation to tumour initiation and progression. Studies using short lived carcinogens to induce tumours in rodents argue that *ras* gene mutation is an early, possibly initiating event in carcinogenesis (Sukumar *et al.* 1983). Similar conclusions about the timing of *ras* gene mutations have been made from studies of premalignant papillomas in rodent skin carcinogenesis (Balmain *et al.* 1984) and premalignant polyps in human colorectal cancer (Bos *et al.* 1987). Although these observations point to an early involvement of *ras* mutations in neoplastic transformation they do not necessarily imply that it is the initiating event. Strong support for *ras* gene mutations not being the initiating event comes from studies on acute myeloid leukaemia from the same patient in presentation and relapse. Farr *et al.* (1988) and Farr *et al.* (1988*b*) have found in five cases that the presentation AML may carry a *ras* gene mutation but relapses either do not contain any detectable mutations or bear a different mutation. Since relapses appear to be derived from the same leukaemic clone as the presentation (Fearon *et al.* 1986), these results argue that *ras* gene mutation is occurring after some other initiating leukaemic event. Therefore in these leukaemias *ras* gene mutation is more likely to play a role after the initial event(s) in neoplastic transformation.

Similar conclusions can also be drawn from studies on mutation in colorectal polyps where the smallest, most benign polyps do not contain *ras* gene mutations (Farr *et al.* 1988*b*). It is possible however, that while *ras* gene mutation is not

the initiating genetic event it does occur early rather than very late in neoplastic progression because *ras* gene mutation is frequent in the preleukaemic myelodysplastic syndromes (Hirai *et al.* 1987; Padua *et al.* 1988).

Biochemical properties of p21*ras* molecules

Membrane localization

The p21*ras* molecules are synthesized in the cytosol and are then found associated with the plasma membrane. The membrane bound form migrates on SDS–PAGE genes with an apparent molecular weight approximately $1–2\times10^3$ less than the cytosolic form of the protein. The mechanism and significance of this mobility shift remains obscure. It does not appear to be caused by the addition of the fatty acid palmitoyl residue to Cys186 since removal of the palmitoyl group by hydrazine does not shift the mobility of the membrane bound form to that of the cytosolic (McGhee *et al.* 1987).

Addition of a palmitoyl group to Cys186 appears to be a central step in the localization of p21*ras* molecules to the membrane. Mutation of the Cys186 to Ser186 which cannot form the thioester bond to palmititic acid results in a protein which cannot go to the plasma membrane and is incapable of transforming cells (Willumsen *et al.* 1984). Such data strongly argue that the site of action of the active p21*ras* molecule is at the membrane and implies that whatever is the target of p21*ras* must be localized in the membrane. Interestingly the palmitoyl group on Cys186 turns over rapidly with a half life of 10–20 min (McGhee *et al.* 1987) compared to the 20 h half life of the protein (Ulsh & Shih, 1984). The functional significance of this turnover is unclear and it remains to be resolved whether the cycles of removal and addition of palmititic acid are reflected in shuttling of the proteins between membrane and cytosol.

Guanine nucleotide binding

The purification of p21*ras* proteins from recombinant expression systems has permitted a detailed analysis of guanine nucleotide binding. Most workers report affinities for binding GTP or GDP in the 10^{-8} to 10^{-9} M range (McGrath *et al.* 1984; Trahey *et al.* 1987). However, higher affinities of around 10^{-11} M have been reported for proteins that have been prepared nucleotide free (Feuerstein *et al.* 1987). Comparison of the sequences of other guanine nucleotide binding proteins together with X-ray crystallographic analysis has delineated the structural features necessary for guanine nucleotide binding. At present only the crystal structure of the GDP form of p21*ras* has been solved (DeVos *et al.* 1988). GDP is bound in a pocket by four of the nine loops which interconnect α strands, or β strands to helix. The residues of loop 1 which include amino acids 12 and 13 are near the β phosphate of GDP, amino acid 30 of loop 2 is adjacent to the ribose of GDP, while amino acids 116, 117, 119 and 120 of loop 7 together with amino acids 145 and 147 of loop 9 form part of the pocket for the guanine of GDP. As will be discussed later mutations in loop 1 affect guanine nucleotide hydrolysis and loop 7 binding.

Since the active form of p21*ras* appears to be the GTP bound state (Trahey & McCormick, 1987), solution of the structure of the GTP form will be necessary to determine how binding of GTP to p21*ras* and conversion to the active state modifies the structure of the molecule. One level for regulating the activity of p21*ras* proteins is at the level of bound nucleotide. This could be achieved either by regulating the rate at which bound nucleotide is exchanged or by the rate of hydrolysis of bound GTP. Measurements of exchange rates on purified proteins show that in the presence of Mg^{2+} the exchange rate is slow with a half life of around 40 min. The rate of exchange can be considerably increased *in vitro* by decreasing the Mg^{2+} concentration (Hall & Self, 1986). Although it is an attractive idea that the exchange rate of guanine nucleotide is regulated *in vivo* in an analogous way to receptor-bound G proteins, no evidence has been adduced at present to show a stimulated exchange on p21*ras* proteins *in vivo*.

GTP hydrolysis

GTP bound to purified normal p21*ras* proteins is hydrolysed slowly with a half life of around 50 min (Hall & Self, 1986). Oncogenic transforming mutations at codon 12 or 61 reduce this rate about 10-fold (McGrath *et al.* 1984). Structural determination of the GDP form of *ras* shows that the amino acids of loop 1 (10–15) are located just below the phosphate of GDP and presumably in the GTP form straddle the phosphate linkage (DeVos *et al.* 1988). The mechanics of catalysis of hydrolysis of the β–γ bond are still unresolved. However, the fact that loop 1 has a highly constrained conformation explains why virtually any mutation at aa 12 or 13 leads to an inhibition of intrinsic GTPase. Although amino acid 61 of loop 4 is not in contact with the phosphates of GDP it is in contact with loop 1, presumably mutations at this site produce conformational changes in loop 1 and thereby result in reduced intrinsic GTPase activity.

It has been recently found that the rate of GTP hydrolysis by the purified normal proteins does not reflect the true rate in the cell. The rate of GTP hydrolysis by normal p21*ras* is at least 100-fold higher *in vivo* because the normal *ras* proteins interact with a protein called GTPase activity protein (GAP) to elevate the rate of hydrolysis (Trahey & McCormick, 1987). The rate of hydrolysis by the codon 12 and 61 mutants is unaffected by GAP, thus GAP only appears able to enhance the GTPase activity of proteins which already have a sufficient level of intrinsic activity. The mechanism by which GAP enhances GTPase activity remains to be elucidated. Furthermore, the precise role of GAP in the physiology of the *ras* proteins is not yet fully understood. One possibility is that GAP may act as a regulator of the amount of GTP bound to normal p21*ras* proteins and thereby regulate the activity of p21*ras*. Inhibiting the activity of GAP, perhaps as a result of growth factor stimulation, would then lead to an increase in the amount of bound GTP. However, genetic experiments to be described later argue that GAP may be involved in the effector functions of p21*ras*. The role of GTPase activation would therefore be to terminate the effector function.

Transforming mutations in p21*ras* proteins

Since the active form of p21*ras* is the GTP bound state (Trahey & McCormick, 1987; Field *et al.* 1987) any mutation that increases the level of GTP bound to p21*ras* might be expected to lead to a transforming allele. In addition to mutations that lead to transforming activity, overexpression of normal *ras* proteins at 10–50 times the normal level can lead to transformation in some (e.g. see McKay *et al.* 1986) but not all cell types (Ricketts & Levinson, 1988). However, by far the major route to oncogenesis involving *ras* genes in human malignancy results from point mutations, reducing GTPase activity. Amplification and overexpression of normal *ras* genes appear to be rather infrequent.

Mutations resulting in a reduction of intrinsic GTPase activity

The first transforming mutation that was identified in a *ras* oncogene detected by transfection with DNA from a human tumour was the Val^{12} mutation in the H-*ras* gene of the T24 bladder carcinoma (Tabin *et al.* 1982; Reddy *et al.* 1982). Subsequently, all possible single base changes leading to a transforming mutation at codon 12 have been detected in human malignancies (Bos, 1988). However, the single most common mutation appears to be Asp^{12} resulting from a $G \rightarrow A$ transition at the 2nd base of codon 12 (Farr *et al.* 1988; Bos, 1988). Site-directed mutagenesis experiments have shown that replacement of Gly^{12} with any amino acid other than proline leads to a transforming protein (Seeburg *et al.* 1984). Interestingly, this study also showed that different replacements lead to transforming alleles with different potencies. For all cases so far studied, codon 12 replacement leads to a reduction in intrinsic GTPase activity (Colby *et al.* 1986), therefore the explanation of the different transforming strengths of different alleles remains to be seen.

Mutations at codon 12 appear to be the largest single class of *ras* gene mutations in human malignancy. However, mutations have also been detected at codon 13 and 61. The codon 13 mutations that have been found in human malignancy seem to fall into the class of weak transforming alleles, since they are more readily detected in transfection assays using the cotransfection/tumorigenicity assay (Bos *et al.* 1985; Hirai *et al.* 1987). Eighteen of the nineteen possible substitutions at codon 61 lead to reductions in intrinsic GTPase activity, although some of these proteins seem no more transforming than the normal allele (Der *et al.* 1986). For the few codon 13 mutations that have been examined intrinsic GTPase activity also seems to be reduced (C. Calés, personal communication).

The mechanism by which mutations at codons 12, 13 or 61 reduce the instrinsic GTPase is not fully understood. The observation that virtually any substitution at these sites and even deletions around codon 12 (Chipperfield *et al.* 1985) cause a reduction in GTPase is consistent with the idea that the mutations destroy a function. However, the precise mechanisms of the hydrolysis of the β–γ phosphate link is not clear. This terminal phosphate is probably buried deep in the loop forming the phosphoryl binding region (DeVos *et al.* 1988). Which residues in this loop are involved in the catalysis of the hydrolysis is not known but it has been argued that it is

likely to be the positioning of the peptide backbone rather than the side chains which is critical (DeVos *et al.* 1988). Mutation at codons 12, 13 or 61 is likely to move the peptide backbone away from the β–γ phosphate bond.

None of the proteins with transforming mutations at codons 12 or 61 appears to interact with GAP to enhance their GTPase activity (Trahey & McCormick, 1987; C. Calés, personal communication). This result resolves the apparent paradox that some proteins such as Asp^{12} have an intrinsic GTPase activity which is almost 50 % of normal yet are as fully transforming as proteins with only 10 % of wild type activity (Trahey *et al.* 1987). In the presence of GAP, Asp^{12} has less than 1 % of the GTPase activity of normal p21*ras*.

Guanine nucleotide exchange mutants

Since the rate of nucleotide exchange on p21*ras* is slow in physiological divalent cation concentrations ($t_{\frac{1}{2}} = 50$ min) (Hall & Self, 1986) and because the rate of GTP hydrolysis in the presence of GAP is high, the guanine necleotide bound to normal p21*ras* is probably GDP. Increasing the rate of guanine nucleotide exchange will drive more of the protein into the active GTP state because the concentration of intracellular GTP greatly exceeds that of GDP. There may be physiological mechanisms that speed up this exchange but it is also clear that some mutations enhance the rate of exchange and lead to transforming alleles. Mutations at codons 116 a.s.p. or 119 asparagine which are involved in binding of the guanine ring (DeVos *et al.* 1988) vastly increase the rate of nucleotide exchange and lead to transforming alleles (Walter *et al.* 1986). Similarly the Ala59 to Thr59 substitution which is found together with codon 12 substitution in the *ras* oncogenes of the Harvey and Kirsten murine sarcoma viruses leads to a 5- to 10-fold increase in guanine nucleotide exchange (Lacal & Aaronson, 1986). Curiously, none of these exchange mutants has yet been found in human malignancy (Farr *et al.* 1988; C. Farr, personal communication), although they have been seen in rodent tumours (Wiseman *et al.* 1986). One explanation may be that the exchange mutants are insufficiently potent in transforming activity *in vivo*. In our hands the exchange mutations are even weaker in NIH-3T3 focus assays than Val^{13} mutations, which is the weakest transforming mutations we have found in human malignancies (Bos *et al.* 1985; G. Mbamulu and C.J. Marshall unpublished results).

Effector functions of p21*ras*

The disturbances of growth regulation expressed in transformed cells suggests that proto-oncogenes and oncogenes are involved in growth control. Although some oncogenes which are derived from growth factors or growth factor receptors (Waterfield *et al.* 1983; Downward *et al.* 1984) clearly have an obvious role in growth control, the precise mechanism of *ras* transformation remains unclear. Like most transformed fibroblasts, *ras* transformed fibroblasts have reduced requirements for serum growth factors. In part, this reduced requirement may result from the production of autocrine transforming growth factors (Marshall *et al.* 1985), but may

also reflect altered proliferative signals coming directly from transforming mutations. It is therefore of fundamental significance to understand the role of normal p21*ras* in proliferative signals and how this role is perverted by transforming *ras* mutations.

Role of normal p21*ras*

The only experimental approach that has been successful in attempting to analyse the functions of normal p21*ras* proteins has been the injection of the antibody Y13–259 into a variety of cells. This antibody recognizes an epitope contained in amino acids 63–73 of loop 4 of all three p21*ras* proteins (Sigal *et al.* 1986). Binding of Y13–259 blocks nucleotide exchange on p21*ras* and interferes with the stimulation of GTPase activity by GAP (Adari *et al.* 1988). Microinjection of Y13–259 into *ras*-transformed cells reverts the transformed phenotype (Mulcahy *et al.* 1985). Thus this antibody appears to be able to neutralize the effects of p21 *ras*.

When Y13–259 is injected into quiescent non-transformed 3T3 fibroblasts subsequent serum stimulation of DNA synthesis is blocked (Mulcahy *et al.* 1985). Whatever process mediated by *ras* the antibody is blocking appears to be activated shortly after growth factor stimulation and to continue right up until S phase. Microinjection of Y13–259 blocks the early stimulation of c-*fos* expression which occurs 30 min–1 h after adding growth factors (Stacey *et al.* 1987) and inhibition of DNA synthesis is observed even if the antibody is added as late as 1–2 h before the commencement of S phase (Mulcahy *et al.* 1985). One likely interpretation of these results is that normal p21*ras* is involved in transmitting signals from growth factor receptors activated by ligand binding. It is known that not only does growth factor binding initiate rapid events but also that growth factors need to be present for 6–8 h to cause DNA synthesis. At present there appears to be no evidence for specificity of different polypeptide growth factors in this process. DNA synthesis stimulated by the mixture of growth factors in serum, pure PDGF or EGF is blocked by microinjection of Y13–259. Furthermore, in *Xenopus* oocytes maturation stimulated by insulin is inhibited by microinjection of the neutralizing antibody (Deshpande & Kung, 1987).

Strikingly, DNA synthesis stimulated by activation of protein kinase C by phorbol ester treatment is also inhibited by Y13–259 (Yu *et al.* 1988). Since a variety of different stimuli of DNA synthesis which appear to work through different routes (Rozengurt, 1986) are all blocked by inhibiting p21*ras* the site of action of normal p21*ras* may be a point of convergence for all routes to stimulate DNA synthesis. Alternatively p21*ras* may be involved in the generation of a signal which growth factor-activated pathways do not produce but with which they must interact to stimulate DNA synthesis.

Second messenger systems and p21*ras*

Observations that p21*ras* appears to be involved in growth factor signalling pathways coupled with the analogy with classical G proteins (Gilman, 1984) of a regulatory GTPase activity has prompted the search for the involvement of p21*ras* with known

second-messenger generating systems. Because of the ease of molecular genetics with yeast much of the work on the functions of the *ras* proteins has been carried out in yeast rather than mammalian cells. The difficulties in doing biochemistry on microinjected cells has meant that most work on vertebrate cells has had to rely mainly on using cells containing transforming mutant *ras* proteins or overexpressing normal p21*ras* proteins.

Elegant genetic and biochemical experiments show that in the yeast *Saccaromyces cerevisiae ras* is involved in regulating adenylate cyclase activity by a direct interaction between *ras* and adenylate cyclase (Toda *et al.* 1988). However, no evidence has been found for such a role in higher organisms, since p21*ras* does not appear to activate adenylate cyclase in vertebrate cells (Beckner *et al.* 1985; Birchmieir *et al.* 1985).

Attention has therefore been focused on the other well-characterized second-messenger system resulting from activation of receptors, the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP2). Phospholipase C action on PIP2 results in the formation of inositol trisphosphate (IP3), which releases Ca^{2+} from intracellular stores, and diacylglycerol, which activates protein kinase C. Studies on a cell line (TI5) overexpressing a normal N-*ras* gene from an inducible promoter has shown that these cells are sensitized to bombesin as an agonist for PIP2 breakdown (Wakelam *et al.* 1986). These results were first interpreted as indicating that a normal *ras* protein can function as a coupling protein between an activated receptor and a second-messenger generating system in a way analogous to classical G proteins. However, subsequent studies have shown that the situation in this cell line is unusual and other cell lines overexpressing normal N-*ras* do not show such responses (Lloyd *et al.* 1988). In general, responses to PIP2 breakdown agonists appear to be downregulated in *ras*-transformed cells (Parries *et al.* 1987; A. Lloyd, M. Whittaker and C. J. Marshall, unpublished results). Reports from several, but not all (Seuwen *et al.* 1988), studies have shown small but reproducible increases in the turnover of inositol phospholipids in *ras*-transformed cells (Fleischmann *et al.* 1986; Hancock *et al.* 1988). These effects appear dependent on p21*ras* being in the active GTP state since when cells transformed by overexpressing normal *ras* proteins are studied under conditions in which the bound GTP will have been hydrolysed to GDP, no activation of PI turnover is found (Hancock *et al.* 1988). However, it is not clear from these experiments whether p21*ras* activates phospholipase C directly or whether the activation is more indirect involving 'cross talk' between signalling pathways.

Studies relying on the behaviour of comparisons of *ras*-transformed cells with their normal counterparts are fraught with difficulties because of unselected and unknown divergence between cell lines during prolonged tissue culture. Transformation may also indirectly alter many aspects of cell physiology. We have therefore turned to an alternative approach to assay more immediate effects following the introduction of *ras* proteins into cells. By the use of the scrape loading technique (McNeil *et al.* 1984) purified recombinant p21*ras* proteins can be introduced into large numbers of cells at high efficiency (Morris *et al.* 1988). Within five minutes of

introducing a transforming Val^{12} *ras* protein, activation of protein kinase C can be observed (Morris *et al.* 1988). However, there is no measurable increase in inositol phospholipid breakdown indicating that *ras* may be involved in generating messengers which activate protein kinase C from other sources of diacylglycerol (Lacal *et al.* 1987*b*).

The activation of protein kinase C following the introduction of transforming *ras* proteins suggests that p21*ras* is 'upstream' of protein kinase C. However, this is at variance with the observation that blocking normal p21*ras* by microinjection of neutralizing antibody Y13–259 inhibits phorbol ester-stimulated DNA synthesis, which is presumably mediated *via* protein kinase C (Yu *et al.* 1988). The microinjection experiments indicate therefore that normal p21*ras* functions after protein kinase C activation. One resolution of this paradox is that there may be differences between the pathways activated by normal and transforming p21*ras* proteins. Such differences, which may be quantitative rather than qualitative, are possible because the activity of normal p21*ras* is regulated by hydrolysis of GTP whereas transforming mutants are not subject to such regulation. The argument that at least part of the transforming activity of p21*ras* is channelled *via* protein kinase C is supported by the observation that down regulation of protein kinase C blocks *ras*-stimulated DNA synthesis in either microinjected (Lacal *et al.* 1987*a*) or scrape-loaded cells (Morris *et al.* 1988). Furthermore, both p21*ras* and protein kinase C activation by phorbol esters stimulates inactive enhancers presumably *via* the AP1c-jun site (Wasylyk *et al.* 1987).

Is GAP the p21*ras* effector?

Single amino acid substitutions or deletions in the region of amino acids 30–40 (loop 2) of p21*ras* leads to a protein which is transformation defective but still localizes to the membrane, binds guanine nucleotides and has unaltered intrinsic GTPase (Sigal *et al.* 1986; Willumsen *et al.* 1986; Cales *et al.* 1988). Two groups have asked the question whether this class of mutation, which presumably affects the interaction of p21*ras* with its target molecule, alters the interaction with GAP. Effector site mutations that destroy transforming activity also destroy the ability of GAP to enhance GTPase activity but substitutions in this region which do not affect transformation do not affect GAP activity (Calés *et al.* 1988; Adari *et al.* 1988). Mutations at other sites of the molecule do not affect the GAP interaction. Thus the regions defined genetically as being the site at which p21*ras* interacts with its target also seems to be the site at which p21*ras* interacts with GAP. This result therefore provides strong evidence that GAP is the next step in the pathway mediating signal transduction through p21*ras*. The role of GAP in enhancing the GTPase of p21*ras* would then be to turn off its own activation. This model for p21*ras*–GAP interaction predicts that the oncogenic transforming proteins also will interact with GAP to activate it (Vogel *et al.* 1988).

The nature of GAP has yet to be defined. Its likely role as the target of p21*ras* suggests that it will be a regulatory enzyme, perhaps a phospholipase or a kinase.

Furthermore, it also remains to be demonstrated how GAP is involved in the activation of protein kinase C, which is an essential component of the *ras*-mediated proliferative signal.

References

ADARI H., LOWY, D. R., WILLUMSEN, B. M., DER, C. J. & MCCORMICK, F. (1988). Guanosine triphosphatase activating protein GAP interacts with the p21 *ras* effector binding domain. *Science* **240**, 518–521.

ALMOGUERRA, C., SHIBATA, D., FORRESTER, K., MARTIN, J., ARNHEIM, N. & PERUCHO, M. (1988). Most human carcinomas of the Exocrine Pancreas contain mutant c-k *ras* genes. *Cell* **53**, 549–554.

BALMAIN, A., RAMSDEN, M., BOWDEN, G. T. & SMITH, J. (1984). Activation of the mouse cellular Harvey-*ras* gene in chemically induced benign skin papillomas. *Nature, Lond.* **307**, 658–660.

BARBACID, M. (1987). *ras* genes. *A. Rev. Biochem.* **56**, 779–827.

BECKNER, S. K., HATTORI, S. & SHIH, T. Y. (1985). The *ras* oncogene product is not a regulatory component of adenylate cyclase. *Nature, Lond.* **317**, 71–72.

BIRCHMEIER, C., BROEK, D. & WIGLER, M. (1985). *Ras* proteins can induce meiosis in *Xenopus* oocytes. *Cell* **43**, 615–621.

BOS, J. L. (1988). The *ras*-gene family and human carcinogenesis. *Mutat. Res.* **195** (3) 255–271.

BOS, J. L., FEARON, E. R., HAMILTON, S. R., VERLAAN DE VRIES, M., VAN BOOM, J. H., VAN DER EB, A. J. & VOGELSTEIN, B. (1987). Prevalance of *ras* gene mutations in human colorectal cancer cells. *Nature, Lond.* **327**, 293–297.

BOS, J. L., TOKSOZ, D., MARSHALL, C. J., VERLAAN DE VRIES, M., VEENEMAN, G. H., VAN DER EB, A., VAN BOOM, J. H., JANSSEN, J. W. G. & STEENVOORDEN, A. C. M. (1985). Amino-acid substitution at codon 13 of the N-*ras* oncogene in human acute myeloid leukaemia. *Nature, Lond.* **315**, 726–730.

BOS, J. L., VERLAAN-DEVRIES, M., JANSEN, A. M., VEENEMAN, G. H., VAN BOOM, J. H. & VAN DER EB, A. J. (1984). Three different mutations in codon 61 of the human N-*ras* gene detected by synthetic oligonucleotide hybridization. *Nucl. Acids Res.* **12**. 9155–9163.

CALÉS, C., HANCOCK, J. F., MARSHALL, C. J. & HALL, A. (1988). The cytoplasmic protein GAP is implicated as the target for regulation by the *ras* gene product. *Nature, Lond.* **332**, 548–551.

CHIPPERFIELD, R. G., JONES, S. S., LO, K. M. & WEINBERG, R. A. (1985). Activation of Ha-*ras* p21 by substitution, deletion and insertion mutations. *Molec. Cell Biol.* **5**, 1809–1813.

COLBY, W. W., HAYFLICK, J. S., CLARK, S. G. & LEVINSON, A. D. (1986). Biochemical characterisation of polypeptides encoded by mutated human Ha-*ras*-1 genes. *Molec. cell Biol.* **6**, 730–734.

DER, C., FINKEL, T. & COOPER, G. M. (1986). Biological and biochemical properties of human *ras*- genes mutated at codon 61. *Cell* **44**, 167–176.

DESHPANDE, A. K. & KUNG, H. F. (1987). Insulin induction of *Xenopus laevis* oocyte maturation is inhibited by monoclonal antibody against p21 *ras* proteins. *Molec. Cell Biol.* **7**, 1285–1288.

DEVOS, A. M., TONG, L., MILBURN, M. V., MATIAS, P. M., JANCARIK, J., NOGUCHI, S., NISHIMURA, S., MIURA, K., OHTSUKA, E. & KIM, S. H. (1988). Three dimensional structure of an oncogene protein: catalytic domain of human c-H-*ras* p21. *Science* **239**, 888–893.

DOWNWARD, J., YARDEN, Y., MAYES, E., SCRACE, G., TOTTY, N., STOCKWELL, P., ULLRICH, A., SCHLESSINGER, J. & WATERFIELD, M. D. (1984). Close similarity of epidermal growth factor receptor and v-*erb*-B oncogene protein sequence. *Nature, Lond.* **307**, 521–527.

FARR, C. J., MARSHALL, C. J., EASTY, D. J., WRIGHT, N. A., POWELL, S. C. & PARASKERA, C. (1988*b*). A study of *ras* gene mutations in colonic adenomas from familial polyposis coli patients. *Oncogene* **3**, 673–678.

FARR, C., SAIKI, R., ERLICH, H., MCCORMICK, F. & MARSHALL, C. J. (1988*a*). Analysis of *ras* gene mutations in acute myeloid leukaemia using the polymerase chain reaction and oligonucleotide probes. *Proc. natn. Acad. Sci. U.S.A.* **85**, 1629–1633.

FASANO, O., BIRNBAUM, D., EDLUND, L., FOGH, J., & WIGLER, M (1984). New Human Transforming Genes detected by a tumorigenicity assay. *Molec. Cell Biol* **4**, 1695–1705.

FEARON, E. R., BURKE, P. J., SCHIFFER, C. A., ZEHNBAUER, B. A. & VOGELSTEIN, B. (1986).

Differentiation of leukaemia cells to polymorphonuclear leukocytes in patients with acute nonlymphocytic leukaemia. *N. Engl. J. Med.* **315**, 15–24.

FEUERSTEIN, J., GOODY, R. S. & WITTINGHOFER, A. (1987). Preparation and characterisation of nucleotide free and metal iron free p21 'apoprotein'. *J. biol. Chem.* **262**, 8455–8460.

FIELD, J., BROEK, D., KATAOKA, T. & WIGLER, M. (1987). Guanine nucleotide activation of and competition between *ras* proteins from Saccharomyces cerevisiae. *Molec. cell Biol.* **7**, 2128–2133.

FLEISCHMANN, L. F., CHAWALA, S. B. & CANTLEY, L. (1986). Ras transformed cells: altered levels of phosphatidylinositol – 4,5-bisphosphate and catabolites. *Science* **231**, 407–410.

FORRESTER, K., ALMOGUERA, C., HAN, K. & PERUCHO, M. (1987). Detection of high incidence of K-*ras* oncogenes during human colon tumorigenesis. *Nature, Lond.* **327**, 298–303.

GILMAN, A. G. (1984). G Proteins and dual control of adenylate cyclase. *Cell* **36**, 577–579.

HALL, A. & SELF, A. (1986). The effect of Mg^{2+} on the guanine nucleotide exchange rate of p21 N-*ras*. *J. biol. Chem.* **261**, 10 963–10 965.

HANCOCK, J. F., MARSHALL, C. J., MCKAY, I. A., GARDINER, S., HOUSLAY, M. D., HALL, A. & WAKELAM, M. J. O. (1988). Mutant but not normal p21 *ras* elevates inositol phospholipid breakdown in two different cell systems. *Oncogene* **3**, 187–193.

HIRAI, H., KOBAYASHI, Y., MANO, H., HAGIWARAK, MARA, Y., OMINE, M., MIZOGUCHI, H., NISHIDA, J. & TAKAKU, F. (1987). A point mutation at codon 13 of the N-*ras* oncogene in myelodysplastic syndrome. *Nature, Lond.* **327**, 430–432.

LACAL, J. C. & AARONSON, S. A. (1986). Activation of *ras* p21 transforming properties associated with an increase in the release rate of bound guanine nucleotide. *Molec. cell. Biol.* **6**, 4214–4220.

LACAL, J. C., FLEMING, T. P., WARREN, B. S., BLUMBERG, P. M. & AARONSON, S. A. (1987*a*). Involvement of functional protein kinase C in the mitogenic response to the H-*ras* oncogene product. *Molec. cell. Biol.* **7**, 4146–4149.

LACAL, J. C., MOSCAT, J. & AARONSON, S. A. (1987*b*). Novel source of 1,2 diacylglycerol elevated in cells transformed by Ha-*ras* oncogenes. *Nature, Lond.* **330**, 269–272.

LLOYD, A., DAVIES, S., CROSSLEY, I., WHITTAKER, M. J., WAKELAM, M., HALL, A. & MARSHALL, C. J. (1988). Bombesin stimulation of TI5 cells overexpressing N-*ras* causes an increase in the production of IP_3 and a corresponding increase in the release of intracellular calcium. *Biochem. J.* (in press).

MAGEE, A. I., GUTIERREZ, L., MCKAY, I. A., MARSHALL, C. J. & HALL, A. (1987). Dynamic fatty acylation of p21 N-*ras*. *EMBO J.* **6**, 3353–3357.

MARSHALL, C. J., VOUSDEN, K. & OZANNE, B. (1986). The involvement of activated *ras* genes in determining the transformed phenotype. *Proc. R. Soc. Lond.* B **226**, 99–106.

MCGRATH, J. P., CAPON, D. J., GOEDDEL, D. V. & LEVINSON, A. D. (1984). Comparative biochemical properties of normal and activated human *ras* p21 protein. *Nature, Lond.* **310**, 644–649.

MCKAY, I. A., MARSHALL, C. J., CALÉS, C. & HALL, A. (1986). Transformation and stimulation of DNA synthesis in NIH-3T3 cells are a titratable function of normal p21 N-*ras* expression. *EMBO J.* **5**, 2617–2621.

MCNEIL, P. L., MURPHY, R. F., LANNI, F. & TAYLOR, D. L. (1984). A method for incorporating macromolecules into adherent cells. *J. Cell Biol* **98**, 1556–1564.

MORRIS, J. D. H., PRICE, B., LLOYD, A. C., SELF, A. J., MARSHALL, C. J. & HALL, A. (1989). Scrape loading of Swiss 3T3 cells with *ras* protein induces rapid activation of protein kinase C followed by DNA synthesis. *Oncogene* (in press).

MULCAHY, L. S., SMITH, M. R. & STACEY, D. W. (1985). Requirement for *ras* proto-oncogene function during serum-stimulated growth of NIH-3T3 cells. *Nature, Lond.* **313**, 241–243.

PADUA, R. A., CARTER, G., HUGHES, D., GOW, J., FARR, C., OSCIER, D., MCCORMICK, F. & JACOBS, A. (1988). *Ras* mutations in myelodysplasia detected by amplification and oligonucleotide hybridisation. *Leukaemia Res.* (in press).

PARRIES, G., HOEBEL, R. & RACKER, E. (1987). Opposing effects of a *ras* oncogene on growth factor stimulated phosphoinositide hydrolysis – Desensitisation to platelet–derived growth factor and enhanced sensitivity to bradykinin. *Proc. natn. Acad. Sci. U.S.A.* **84**, 2648–2652.

QUINTANILLA, M., BROWN, K., RAMSDEN, M. & BALMAIN, A. (1986). Carcinogen specific mutation and amplification of Ha-*ras* during mouse skin carcinogenesis. *Nature, Lond.* **322**, 78–80.

REDDY, E. P., REYNOLDS, R. K., SANTOS, E. & BARBACID, M. (1982). A point mutation is

responsible for the acquisition of transforming properties of the T24 human bladder carcinoma oncogene. *Nature, Lond.* **300**, 149–152.

RICKKETS, M. H. & LEVINSON, A. D. (1988). High level expression of c-H-*ras* fails to fully transform Rat-1 cells. *Molec. cell. Biol* **8**, 1460–1468.

ROZENGURT, E. (1986). Early signals in the mitogenic response. *Science* **234**, 161–166.

SAIKI, R., SCHARF, S., FALOONA, F., MULLIS, K. B., HORN, G. T., ERLICH, H. A. & ARNSTEIN, N. (1985). Enzymatic amplification of β globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**, 1350–1354.

SEEBURG, P. H., COLBY, W. W., CAPON, D. J., GOEDDEL, D. V. & LEVINSON, A. D. (1984). Biological properties of human c-Ha-*ras*-1 genes mutated at codon 12. *Nature, Lond.* **312**, 71–75.

SEUWEN, K., LAGARDE, A. & POUYSSEGUR, J. (1988). Deregulation of hamster fibroblast proliferation by mutated *ras* is not mediated by constitutive activation of phospholipase C. *EMBO J.* **7**, 161–168.

SHIH, C., SHILO, B., GOLDFARB, M. P., DANNENBERG, A. & WEINBERG, R. A. (1979). Passage of phenotypes of chemically transformed cells via transfection of DNA and chromatin. *Proc. natn. Acad Sci. U.S.A.* **76**, 5714–5718.

SIGAL, I. S., GIBBS, J. B., D'ALONZO, J. S. & SCOLNICK, E. M. (1986). Identification of effector residues and a neutralizing epitope of Ha-*ras* encoded p21. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4725–4729.

STACEY, D. W., WATSON, T., KUNG, H-F. & CURRAN, T. (1987). Microinjection of transforming *ras* protein induces c-*fos* expression. *Molec. cell. Biol.* **7**, 523–527.

SUKUMAR, S., NOTARIO, D., MARTIN-ZANCA, D. & BARBACID, M. (1983). Induction of mammary carcinomas in rats by nitroso-methylurea involves malignant activation of H-*ras*-1 by single point mutations. *Nature, Lond.* **306**, 658–661.

TABIN, C. J., BRADLEY, S. M., BARGMANN, C. I., WEINBERG, R. A., PAPAGEORGE, A. G., SCOLNICK, E. M., DHAR, R., LOWY, D. R. & CHANG, E. H. (1982). Mechanism of activation of a human oncogene. *Nature, Lond.* **300**, 143–149.

TODA, T., UNO, I., ISHIKAWA, T., POWERS, S., KATAOKA, T., BROEK, D., BROACH, J., MATSUMOTO, K. & WIGLER, M. (1988). In Yeast, RAS proteins are controlling elements of the cyclic AMP pathway. *Cell* **40**, 27–36.

TOKSOZ, D., FARR, C. J. & MARSHALL, C. J. (1987). *ras* gene activation in a minor population of the blast population in acute myeloid leukaemia. *Oncogene* **1**, 409–413.

TRAHEY, M. & MCCORMICK, F. (1987). Cytoplasmic protein stimulates normal N-*ras* p21 GTPase but does not affect oncogenic mutants. *Science* **238**, 542–545.

TRAHEY, M., MILLEY, R. J., COLE, G., INNIS, M., PATERSON, H., MARSHALL, C. J., HALL, A. & MCCORMICK, F. (1987). Biochemical and biological properties of the human N-*ras* protein. *Molec. cell. Biol.* **7**, 541–544.

ULSH, L. S. & SHIH, T. Y. (1984). Metabolic turnover of human c-ras^{H} p21 protein of EJ bladder carcinoma and its normal cellular and viral homologs. *Molec. cell. Biol.* **4**, 1647–1655.

VOGEL, U. S., DIXON, R. A. F., SCHABER, M. D., DIEHL, R. E., MARSHALL, M. S., SCOLNICK, E. M., SIGAL, T. S. & GIBBS, J. B. (1988). Cloning of bovine GAP and its interaction with oncogenic *ras* p21. *Nature, Lond.* **335**, 90–93.

WAKELAM, M. J. O., DAVIES, S. A., HOUSLAY, M. D., MCKAY, I., MARSHALL, C. J. & HALL, A. (1986). Normal p21 N-*ras* couples the combesin and other growth factor receptors to inositol phosphate production. *Nature, Lond.* **323**, 173–176.

WALTER, M., CLARK, S. G. & LEVINSON, A. D. (1986). The oncogenic activation of human p21 *ras* by a novel mechanism. *Science* **233**, 649–652.

WASYLYK, C., IMLER, J. C., PEREZ-MUTUL, J. & WASYLYK, B. (1987). The c-Ha-*ras* oncogene and a tumour promoter activate the polyoma virus enhancer. *Cell* **48**, 525–534.

WATERFIELD, M. D., SCRACE, G. T., WHITTLE, N., STROOBANT, P., JOHNSON, A., WASTESON, A., WESTERMARK, B., HELDIN, C. H., HUANG, J. S. & DUEL, T. F. (1983). Platelet-derived growth factor is structurally related to the putative transforming protein p28^{sis} of simian sarcoma virus. *Nature, Lond.* **304**, 35–39.

WILLUMSEN, B. M., CHRISTENSEN, A., HUBBERT, N. L., PAPAGEORGE, A. G. & LOWY, D. R. (1984). The p21*ras* C-terminus is required for transformation and membrane association. *Nature, Lond.* **310**, 583–586.

WILLUMSEN, B. M., PAPAGEORGE, A. G., KUNG, H. F., BEKESI, E., ROBINS, T., JOHNSEN, M., VASS, W. C. & LOWY, D. R. (1986). Mutational analysis of a *ras* catalytic domain. *Molec. cell. Biol.* **6**, 2646–2654.

WISEMAN, R. W., STOWERS, S. J., MILLER, E. C., ANDERSON, M. W. & MILLER, J. A. (1986). Activating mutations of the c-Ha-*ras* proto-oncogene in chemically induced hepatomas of the male B6C3F1 mouse. *Proc. natn. Acad. Sci. U.S.A.* **83**, 5825–5834.

YU, C. L., TSAI, M. H. & STACEY, D. W. (1988). Cellular *ras* activity and phospholipid metabolism. *Cell* **52**, 63–71.