# Post - translational modifications and p21ras function

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# Abstract

The *ras* gene family consists of three members that encode highly similar proteins of 21Kd (p21$^{ras}$/Ras). This protein is plasma membrane associated, binds guanine nucleotides and has intrinsic GTPase activity. Activating point mutations render Ras insensitive to regulation by GAP (GTPase activating protein) and it remains in the active GTP bound state. Membrane association of Ras has been shown to be essential for its biological activity.

The plasma membrane targeting of Ras is accomplished by a series of post-translational modifications which occur in 2 steps. Step 1 involves the CAAX motif (C = cysteine, A = aliphatic and X = any amino acid) at the C-terminus. The cysteine is alkylated by $C_{15}$ farnesyl, the -AAX amino acids are removed and the new C-terminal cysteine undergoes methylesterification. Step 2 involves palmitoylation of cysteine residues near the CAAX motif in the case of H-, N- and K-*ras* (A). Membrane localisation of K-*ras* (B) appears to involve electrostatic interaction of the polybasic region (K175-180) with the membrane.

Other CAAX containing proteins (*rap* 1A, G25K) are prenylated with a $C_{20}$ geranylgeranyl moiety rather than $C_{15}$ farnesyl. Geranylgeranylation of H- and K-*ras* (B) also leads to membrane association of the protein but specific targeting to the plasma membrane requires the presence of the polybasic domain or the palmitoylation sites.

Another family of proteins (p60$^{src}$, Gag, cytochrome $b_5$ reductase) is membrane associated by the addition of myristic acid to the N-terminus. Myristoylation can also allow Ras proteins to be membrane associated but specific plasma membrane targeting remains dependent on the presence of palmitoylation sites or a polybasic region.

Upstream of the CAAX motif is the hypervariable domain - a region that shows less than 20% homology between the *ras* genes. The function of this domain is not known and it may simply connect the N - and C - termini. However this region could also confer specificity on the interaction of different Ras proteins with different effector and/or regulatory proteins. Deletions within this region destroy transforming ability and reduce MAP kinase activity suggesting that effector interaction is disrupted. N17 deletion mutants rescue proliferation of NIH 3T3 cells indicating that exchange factor interaction is also influenced by the hypervariable region.

This thesis attempts to establish a relationship between the biological activity of Ras, its cellular location and post-translational processing events. A functional role for the hypervariable domain is also examined.

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## 1. General Introduction

A major field of scientific research that has developed this decade involves studying cell growth and differentiation and how uncontrolled cell proliferation can lead tumour formation. Analysis of the genes involved in tumour development was greatly aided by the discovery of retroviruses. These RNA viruses can cause tumours in the host and can also transform cells in cell culture. Retroviruses contain genes that are involved in carcinogenesis and these are called viral oncogenes (v-onc). These genes have the dominant mutated forms of the host's own genes (c-onc) or proto-oncogenes. Proto-oncogenes are involved in the signal pathways of cellular proliferation and encode proteins such as growth factors (*v-sis*), growth factor receptors (*v-erb* B, *v-fms, v-kit*), transducers of growth factor responses (*v-src, v-ras, v-raf*) and transcription factors that mediate growth factor-induced gene expression (*v-jun*, v-f*os*).

The first transforming gene isolated from tumour cells was found to be homologous to a retroviral oncogene. A mutation was identified (Gly12 to Val12) in the c-H-*ras* gene of the EJ human bladder carcinoma cell line (Tabin *et al*, 1982; Reddy *et al*, 1982; Taparowsky *et al*, 1982). Point mutations are not the only genetic changes found in tumours - deletions, chromosomal translocations and gene amplification have all been identified.

The *ras* genes having been the first to be discovered have been the most extensively studied. It has now become evident that the *ras* genes belong to a large family 'the *ras* superfamily of proteins' that can be divided into three main groups (Chardin, 1988). One group contains the *ras, ral* and *rap* genes, another the *rho* genes and the third, the *rab* genes.

Ras proteins bind guanine nucleotides (GDP and GTP) with high affinity and possess intrinsic GTPase activity implying that Ras activity is regulated. Ras migrates as a 21kDa protein (p21) which has been extensively studied. Insight into the structure of Ras has been achieved by mutational and crystallographic analyses. Further interest in Ras has come from the identification of factors that regulate Ras activity and the involvement of these factors in physiological signalling and in tumour development. The post-translational modifications of Ras have also been thoroughly investigated and may provide cancer therapy targets.

## 1.1. The Ras superfamily

### 1.1.1. Introduction

*Ras* genes were first identified as the transforming principle of the Harvey and Kirsten strains of rat sarcoma viruses (Harvey, 1964; Kirsten and Mayer, 1967). They are found in many species and their products show remarkable structural homology.

The discovery of Rho in the marine snail *Aplysia* (Madaule and Axel, 1985) and YPT in yeast (Gallwitz *et al*, 1983), two proteins with 30% homology to Ras, lead to the discovery of a large family of Ras proteins. Other members of the Ras superfamily were discovered using an oligonucleotide strategy (Chardin and Tavitian, 1986; Touchot *et al*, 1987) or by low stringency hybridization using a H-*ras* or YPT probe (Lowe *et al*, 1987; Mozer *et al*, 1985; Schejter and Shilo, 1985; Swanson *et al*, 1986).

The Ras superfamily can be divided into three main branches represented by Ras, Rho and Rab. The Ras family includes H-ras, N-ras and K-ras, RalA and B, R-ras, Rap1A, 1B, 2A and 2B, Dras1, 2 and 3, RAS1 and 2 and TC21. These proteins have 50% homology to one another. The Rho family includes RhoA, B and C, Rac1 and 2, RHO1 and RHO2, CDC42Hs and TC10. The Rho and Rac family show 30% homology to the Ras family. The Rab protein family consists of a whole series of Rab proteins (to date 24 have been identified), SEC4 and YPT1 and 2 (Chardin, 1988).

Two further subgroups have recently been identified, the Arf and Ran proteins. A prokaryotic analogue for *ras* has also been reported (March *et al*, 1988). The *E. coli era* gene has 50% homology to yeast *RAS1* in the N-terminal two-thirds of the protein.

Phylogenetic trees for the Ras superfamily represent functional rather than species relationships (Valencia *et al*, 1991).

### 1.1.2. Human *ras* genes

N-*ras* was first identified as the transforming gene of two human sarcoma cell lines, a promyelocytic cell line and a neuroblastoma cell line (Murray *et al*, 1981; Perucho *et al*, 1981). H-*ras* and K-*ras* were isolated from genomic libraries by probing with the viral genes (Chang *et al*, 1982). H-*ras*-1 and K-*ras*-2 are the normal cellular homologues found. The three genes all contain four coding exons plus a fifth upstream non-coding exon. The identical splice junctions indicate a common evolutionary precursor (Brown *et al*, 1984; Cichutek and Duesberg, 1986; McGrath *et al*, 1983). There are two alternative fourth exons of K-*ras*, 4A and 4B. Exon 4B is the most widely

expressed form in mammalian tissues whereas exon 4A is used in the viral oncogene (Capon *et al*, 1983; Shimizu *et al*, 1983). In humans, N-*ras* is located on the short arm of chromosome 1 (1p22 - p32), H-*ras*-1 is on chromosome 11 (11p15.1 - p15.5) and K-*ras*-2 is on chromosome 12 (12p12.1 - pter) (O'Brien, 1984).

Ras can be found in all adult and fetal tissues. In mouse tissues levels of H-ras were found to be the highest in skin and skeletal muscle and lowest in liver, K-ras expression was highest in the thymus and intestine and lowest in skin and skeletal muscle, whilst N-ras was highest in testis and thymus and lowest in the liver and kidney (Leon *et al*, 1987).

The H-ras, N-ras and K-ras(A) proteins are 189 amino acids long while K-ras(B) is 188 amino acids long because exon 4B has one codon less than the other fourth coding exons. They bind guanine nucleotides (both GDP and GTP), have intrinsic GTPase activity and are plasma membrane associated. The first 85 amino acids of all three proteins are identical. The second domain of 80 amino acids shows 85% homology but this is followed by a highly divergent region with only 10% homology. This hypervariable region (HVR) is highly conserved between species and may account for the different functions of the Ras proteins. The extreme C-terminus of the protein forms the CAAX (C=cysteine, A=aliphatic and X= any amino acid) motif which is semi-conserved.

### 1.1.3. Other members of the *ras* subfamily

Another human *ras* gene, R-*ras*, is 55% identical to H-ras (Bourne *et al*, 1991), has a molecular weight of 23KDa and is 218 amino acids long with the extra 26 amino acids at the N-terminus. It is located on chromosome 19, is palmitoylated and membrane associated (Lowe and Goeddel, 1987). It does not cause transformation when it is mutated in positions analogous to those of activated Ras. It does however appear to have intrinsic GTPase activity and reacts with RasGAP (Lowe and Goeddel, 1987). Recent studies (Fernandez-Sarabia and Bischoff, 1993) have shown an interaction between the C-terminus of R-ras and Bcl2 in the yeast two hybrid system indicating that R-ras may be involved in apoptosis.

The Ral proteins are 50% homologous to Ras and have 85% homology to each other. The regions of similarity are those involved in GTP binding and hydrolysis and membrane localisation whilst the effector region shows divergence. RalA is nontransforming (Chardin, 1988) and consists of 206 amino acids with a molecular mass of 23.5kDa. Its N-terminus is 11 amino acids

longer than that of Ras and there are 6 additional amino acids at the C-terminus. The function of Ral has not been determined (Chardin and Tavitian, 1986).

Rap1A and Rap2A were identified (Pizon *et al*, 1988a) by screening a Raji human Burkitt lymphoma cell library with probes based on the *Dras3* gene of *Drosophila* (Schejter and Shilo, 1985). This strategy was also used to identify Rap1B/smgp21 (Pizon *et al*, 1988b). A human fibroblast cDNA library was used to identify Rap1A/Krev-1 (Kitayama *et al*, 1989). Rap2B was identified through molecular cloning from a platelet cDNA library (Ohmstede *et al*, 1990). Rap1A and Rap1B are 95% identical whilst Rap2A and 2B are 90% identical. The Rap1 and 2 proteins are 70% identical to each other.

Rap1A was identified by its ability to suppress transformation of NIH 3T3 cells by K-ras (Kitayama *et al*, 1989) suggesting that Rap1A competes with Ras for effector molecules. Rap1A (Frech *et al*, 1990) and Rap1B (Hata *et al*, 1990) have both been shown to compete with Ras for binding to Ras-GAP. Rap1A and 1B are substrates for cAMP-dependent protein kinase as well as a number of other kinases (Bokoch and Quilliam, 1990; Hoshijima *et al*, 1988; Kawata *et al*, 1989b; Lerosey *et al*, 1991; Quilliam *et al*, 1991). This phosphorylation can regulate Rap macromolecular interactions *in vitro* and suggests a role for Rap in cell regulation.

Elevation in cAMP levels can lead to tumour development in some cell types (Dumont *et al*, 1989; Valler *et al*, 1987) and Rap may be involved in this process. Rap may mediate some of the effects of cAMP in neutrophils (Quilliam *et al*, 1991) where cAMP inhibits NADPH oxidase activity, and in platelets (Lazarowski *et al*, 1990) where activation of phopholipase C is blocked by cAMP. Rap activity may also be regulated by hormones through action on GAP (Martin and Lapetina, 1992).

Rap1A is widely expressed and is unlikely to only regulate a phagocytic-specific enzyme. It is proposed that Rap1 plays a more general role in cell function possibly involving the regulation of cytoskeletal interactions or protein-protein interactions.

## 1.1.2. Functions of the *ras* genes
### 1.1.2.1.Mammals

Since *ras* oncogenes are the cause of many malignancies, they must play a role in the growth of normal cells. Ras proteins have been found to be essential for the transmission of signals from tyrosine kinases such as *src* and the insulin, EGF and NGF receptors, to serine/threonine kinases such as Raf

and MAP kinases or ERKs (extracellular signal regulated kinases) and are therefore involved in cell regulation. Ras promotes differentiation of PC12 cells (Bar-Sagi and Feramisco, 1985, Satoh *et al*, 1987), maturation of *Xenopus* oocytes (Birchmeier *et al*, 1985) and neoplastic transformation of fibroblasts (Barbacid, 1987).

Activation of Ras also leads to a number of physiological responses including increased activity of phospholipase $A_2$ (Bar-Sagi and Feramisco, 1985; Bar-Sagi *et al*, 1988), S6 kinase (Barrett *et al*, 1990), induction of c-*fos* mRNA (Stacey *et al*, 1987), increased levels of diacylglycerol (Fleischman *et al*, 1986) and membrane ruffling (Bar-Sagi and Feramisco, 1985).

### 1.1.2.2.Yeasts

The *S. cerevisiae ras* genes *RAS1* and *RAS2* are essential for stimulating cAMP production via adenyl cyclase. *RAS1RAS2* double mutations are lethal but either gene can sustain growth. Mammalian *ras* genes can induce phenotypic alterations in yeast cells (Clark *et al*, 1985) and can also complement non viable yeast mutants. Yeast-mammalian *ras* chimeras can also transform NIH 3T3 cells (DeFeo-Jones *et al*, 1985). This ability to interchange between species illustrates the high degree of conservation of *ras* genes during evolution.

*S. pombe* only has one *ras* gene that is required for mating (Fukui *et al*, 1986; Nadin-Davis *et al*, 1986).

### 1.1.2.3.Other organisms

Ras can induce meiosis in *Xenopus* oocytes (Birchmeier *et al*, 1985) by an insulin pathway, increases diacylglycerol levels (Lacal *et al*, 1987), increases germinal breakdown in full grown (stage VI) oocytes and activation responses in growing (stage IV) oocytes (Johnson *et al*, 1990).

Expression of activated Ras in *Drosophila melanogaster* leads to reduced viability and abnormal wing and eye development (Bishop and Cories, 1988). Mammalian cells can also be transformed by *Drosophila ras* genes. The *ras* gene of *C. elegans*, *let60*, is essential to vulva development (Beitel *et al*, 1990; Han and Sternberg, 1990). Expression of activated *ras* genes leads to a multivulva phenotype and overexpression of effector mutants is lethal. Tyrosine kinase receptors are also involved in the *C. elegans* Ras pathways.

In *Dictyostelium* discoidium expression of activated Ras adversely affects chemotaxis. cGMP production is impaired due to a reduction in the cAMP binding by chemotaxis receptors (Luderus *et al*, 1988; Van Haastert *et al*, 1987).

Inositol phosphate levels increase when activated *ras* genes are expressed (Europe-Finner *et al*, 1988).

### 1.1.3. Ras and cancer

#### 1.1.3.1.Ras and human tumours

*Ras* genes are the most frequently identified oncogene in human cancer. Their overall incidence is between 10 and 15% and they have been found in carcinomas, haematopoietic tumours of lymphoid and myeloid lineage and in tumours of mesenchymal origin such as fibrosarcomas and rhabdomyosarcomas.

The incidence of mutated *ras* genes varies among different tumour types. More than 80% of pancreatic tumours contain a mutated K-*ras* gene (Bos, 1989) whereas N-*ras* is the predominantly mutated *ras* gene of myeloid leukaemia. The relationship between certain types of tumour and a particular *ras* gene may indicate that the *ras* genes have different functions.

Malignancy is not only due to activating point mutations but, also in rare cases, by over expression of normal Ras products (Westaway *et al*, 1986). *Ras* gene mutations can also be induced by carcinogenic agents. The majority of mutations found in natural or induced malignancies have mutations at positions 12, 13, 59 and 61 (Barbacid, 1987).

#### 1.1.3.2.Ras and animal tumours

The role of Ras in tumour development has been studied using carcinogen - induced animal tumour systems. A single dose of nitroso-methylurea (NMU) during puberty leads to the activation of H-*ras*-1 in 86% of rat mammary carcinomas (Sukumar *et al*, 1983). Ras mutation is therefore an initiating event equivalent to the first step in tumour development. Skin carcinomas can be induced in mice by 7, 12-dimethylbenz [a] anthracene (DMBA) and phorbol esters. These have been found to have an A to T mutation in codon 61 of H-*ras*-1 (Balmain and Pragnell, 1983). Mouse thymomas induced by X-rays or chronic NMU treatment have been found to contain K-ras and N-ras mutations (Guerrero *et al*, 1984). The type of carcinogen and its time of use influences the *ras* gene that is activated (Barbacid, 1986). *Ras* oncogenes can therefore initiate carcinogenesis in appropriate animal models.

The estimate of spontaneous Ras mutation in animal cells is high yet very few mutations actually lead to tumour formation. A single Ras point mutation is not sufficient to transform cultured primary cells (Land *et al*, 1983;

Newbold and Overell, 1983; Ruley *et al*, 1983). Nuclear oncogenes such as c-*myc*, adenovirus E1A or polyomavirus large T antigen have been shown to co-operate with activated Ras to transform primary cells (Land *et al*, 1983; Ruley *et al*, 1983).

## 1.1.4. The Rho family

Rho was first isolated from *Aplysia* (Madaule and Axel, 1985) and three related genes, CDC42Sc, RHO1 and RHO2 have been cloned from *S. cerevisiae* (Johnson and Pringle, 1990). Rho and Ras are thought to function in different pathways since the lethality of RHO1 deletion cannot be suppressed by overexpression of activated RAS2 (Madaule *et al*, 1987). RhoC is ADP-ribosylated by *Clostridium botulinum* exoenzyme C3 and leads to the disruption of actin fibres suggesting a role for RhoC in cytoskeletal control (Chardin *et al*, 1989).

Rho is an essential component of a signal transduction pathway that links growth factor receptors to the assembly of focal adhesions and the organisation of actin into stress fibres (Ridley and Hall, 1992). A small fraction of Rho is plasma membrane localised whilst the majority of RhoA and C is cytosolic. RhoB is associated with early endosomes and a pre-lysosomal compartment. These results indicate that Rho proteins cycle on and off the plasma membrane (Adamson *et al*, 1992).

Rac is involved in a phenomenon known as membrane ruffling which is a result of actin reorganisation at the plasma membrane induced by growth factors. Activation of Rac in turn activates Rho and leads to the formation of stress fibres (Downward, 1992). Rac proteins are also essential components of the NADPH oxidase system that generates superoxide in phagocytes (Abo *et al*, 1991; Knaus *et al*, 1991).

## 1.1.5. The Rab family

The main differences between the Ras and Rab families are at the C-terminus where the CAAX motif is replaced by CC or CXC. The different Rab proteins appear to be associated with particular intracellular compartment and they regulate exocytic and endocytic processes. For example Rab1A, Rab1B and Rab2 are involved in the exocytic pathway from the endoplasmic reticulum to the Golgi (Plutner *et al*, 1991; Tisdale *et al*, 1992). Endocytic pathways involve Rab4 and Rab5 which are both associated with early endosomes (Chavrier *et al*, 1990; Van Der Sluijs *et al*, 1991). An increase in the level of Rab5 stimulates endocytosis and early endosomes become larger

(Bucci *et al*, 1992). Overexpression of Rab4 leads to an increase in the endocytic markers on the cell surface and induces accumulation of endocytic tubules and tubule clusters (Van Der Sluijs *et al*, 1992). Rab proteins can therefore be localised to the same organelle but have distinctly different functions. The cycling of Rab proteins on and off membranes is regulated by interaction with various factors that increase GDP/GTP exchange and the rate of GTP hydrolysis.

### 1.1.6. Other members of the Ras superfamily

The ARF (ADP-ribosylation factor) family is now thought to contain at least fifteen members (Clark *et al*, 1993). Arf is a 20kDa protein that binds GTP and although it is related to Ras, it is the most divergent member of the Ras superfamily (Price *et al*, 1988; Sewell and Kahn, 1988). They achieve membrane localisation by N-terminal myristoylation rather than C-terminal processing events. Arf proteins are required for the efficient ADP-ribosylation of Gsα by cholera toxin (Serventi *et al*, 1992) and are also thought to be involved in membrane vesicle fusion and transport (Balch *et al*, 1992; Donaldson *et al*, 1992; Lenhard *et al*, 1992; Zeuzem *et al*, 1992). Ran proteins (Ras-related nuclear proteins) are involved in mitosis and are abundant nuclear GTPases (Roberge, 1992).

### 1.2. The structure of Ras
### 1.2.1. General structure

Ras exists in two interconvertible conformational states - one active or GTP bound and one inactive or GDP bound. The three dimensional structure of both wild type and mutant H-ras proteins has been identified using n.m.r. and crystallographic studies and has been found to be extremely similar to that of EF-Tu and G protein α chains (Jurnak, 1985; McCormick *et al*, 1985). The resolution of the structure of Ras is significant because it is the first atomic description of an oncogene product and is therefore of great interest to the field of cancer research.

The three dimensional structure of wild type and mutant H-ras associated with GDP has been resolved, and by using Ras crystallised with [$P^3$-1-(2-nitro)phenylethylguanosine 5'-0-triphosphate] or 'caged' GTP at the active site, the GTP-bound form as well as any structural changes during GTP hydrolysis have also been studied (Schlichting *et al*, 1989; 1990a; 1990b).

The overall structure of Ras-GTP, Ras-GDP and Ras-caged GTP was found to be identical in terms of their α-helical and β-sheet organisation. The

protein appears to consist of five α-helices, one six-stranded β-sheet and ten interconnecting loops. Five of the six β-sheet strands are parallel to one another with the connecting helical strands positioned on both sides of the central sheet. Five of the interconnecting peptide loops are located on the convex surface of the β-sheet whilst the others are on the concave face of the molecule **(Figure 1.1.)**.

All the hydrophobic amino acids are on the inside of the structure with only three being exposed on the surface (Ala121, Ala122 and Leu171) whereas the charged amino acids are on the surface apart from two (Lys16 and Asp57) (Tong *et al*, 1991). These polar residues are involved in salt bridge formation and Lys16 and Asp57 are involved in guanine nucleotide binding.

Loop 1 is involved in GDP and GTP binding with the main chain amide hydrogens of several amino acids together with the ε-amino group of Lys16 forming bonds with the α- and β- phosphates of GTP or GDP (Milburn *et al*, 1990; Pai *et al*, 1989; 1990). This loop connects β1, a hydrophobic β strand, to α1, an amphipathic α helix.

Residues 32 - 40 change conformation on GTP binding partly due to an alteration in the orientation of the threonine residue at position 35. This amino acid appears to be involved in the binding of a $Mg^{2+}$ ion which is essential for GTPase activity. Pai and co-workers (1989; 1990) have suggested that the magnesium is co-ordinated to oxygen atoms from the β and γ phosphates, hydroxyl groups of Ser17 and Thr35 and two water molecules which are hydrogen bonded to Asp57 and Asp33. This is in the GTP state whereas when GDP is bound there is direct co-ordination to the carboxyl group of Asp57 and a water molecule replaces Thr35 (Schlichting *et al*, 1990a). Two groups (Milburn *et al*, 1990; Tong *et al*, 1991) suggest that the structure in GDP-Ras is a $Mg^{2+}$ ion co-ordinated by oxygen atoms from the β phosphate of GDP, Ser17 and four water molecules. In the GTP state, one of the water molecules is replaced by the hydrogen side chain of Thr35 (Pai *et al*, 1990; Tong *et al*, 1991).

Residues 53 - 62 are conserved in all GTPases. Asp57 binds the $Mg^{2+}$ ion via a water molecule and Gly60 forms a hydrogen bond with the γ phosphate of GTP. The GDP and GTP forms show great differences in the conformation of residues 60 and 63 and the α2 helix.

Residues 112 - 119 form a hydrophobic β strand followed by a hydrophilic loop. Hydrogen bonds are formed between carboxy oxygens of Asp119 and the guanine ring. The nucleotide binding site is stabilised by Asn116 and Lys117 forming hydrogen bonds to residues 13 and 14.

**Figure 1.1.**
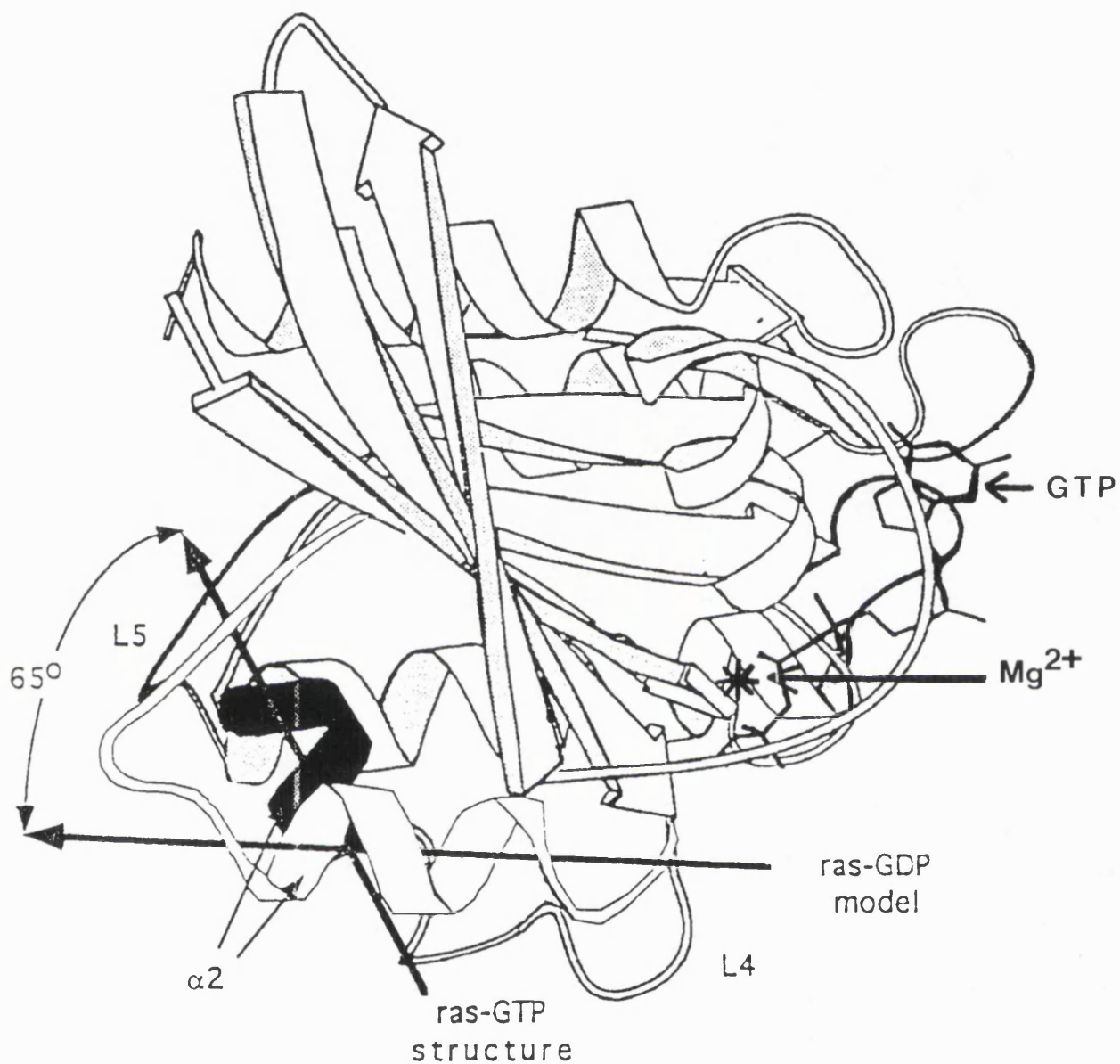


**The RasGTP crystal structure.**

Ras consists of five α helices and a six-stranded β sheet that are connected together by ten loops. The position of αhelix2 alters upon GTP hydrolysis and rotates 65°. The position of GTP (stick representation) and the magnesium ion (asterisk) are shown. αHelix2 is dark in the RasGTP structure and light in the RasGDP model. (This figure is based on that of Stouten *et al*, 1993.)

The loop between the sixth β strand and the α5 helix (residues 144 - 146) interact with the nucleotide through hydrogen bonds stabilising the side chains of Asn116 and Asp119. Ala146 contacts directly with GTP.

A schematic diagram of all the interactions is shown in **Figure 1.2.**

### 1.2.2. Differences between the GDP and GTP bound states

There are three sequence motifs important for nucleotide interaction. Amino acids 16 and 17 are involved in binding to the α and β phosphates. $Mg^{2+}$ is bound by Asp57 whilst Gly60 binds the γ phosphate when GTP is bound. These amino acids are found in the second conserved motif of amino acids 57 - 60. The third motif, 116 - 119, binds the guanine ring. There are marked structural changes when the bound nucleotide is altered from GDP to GTP. These changes are most marked in two regions spanning residues 30 - 38 (loop 2 - the effector region) and residues 60 - 76 (the Y13 259 binding site) which consists of loop 4, loop 5 and α helix 2. These regions are on the outer surface of the molecule and are close to one another (Milburn *et al*, 1990; Schlichting *et al*, 1990a).

The changes in the 'switch I' region (amino acids 30 - 38) that occur between the GTP and the GDP bound forms of Ras involves flips of some peptide units and reorientation of side chains (Milburn *et al*, 1990; Schlichting *et al*, 1990a). The hydrogen bond between the side chains of Tyr32 and Tyr40 broken and the Tyr32 moves out to partially block the entrance to the guanine nucleotide pocket. The Thr35 moves toward the GTP and interacts with the γ phosphate and the $Mg^{2+}$ ion. The orientation of the side chains of residues 36 and 38 also changes.

The 'switch II' region has considerable conformational flexibility especially in the GDP bound state (Milburn *et al*, 1990; Schlichting *et al*, 1990a). Five major differences in the switch II region are observed between the GTP and GDP bound states (Stouten *et al*, 1993). The backbone of Ala59 moves so that the position vacated by the γ phosphate of GTP is occupied by its Cβ atom. Helix α2 is extended by one turn at its N-terminus by the rearrangement of residues 63 - 66 into a helical conformation in the GDP state. A hydrogen bond between the side chain of Glu63 and the backbone NH of Gly60 stabilises this extension. The side chain of Tyr64 fits into a hydrophobic cavity that is also present in the GTP bound form. Loop 5 is extended by the unraveling of the α2 helix (71 - 74). In Ras-GTP, the α2-L5 region contacts with the end of α3 and L7. Helix α2 rotates about its own helical axis and by approximately 65° about an axis perpendicular to this axis.

Figure 1.2.
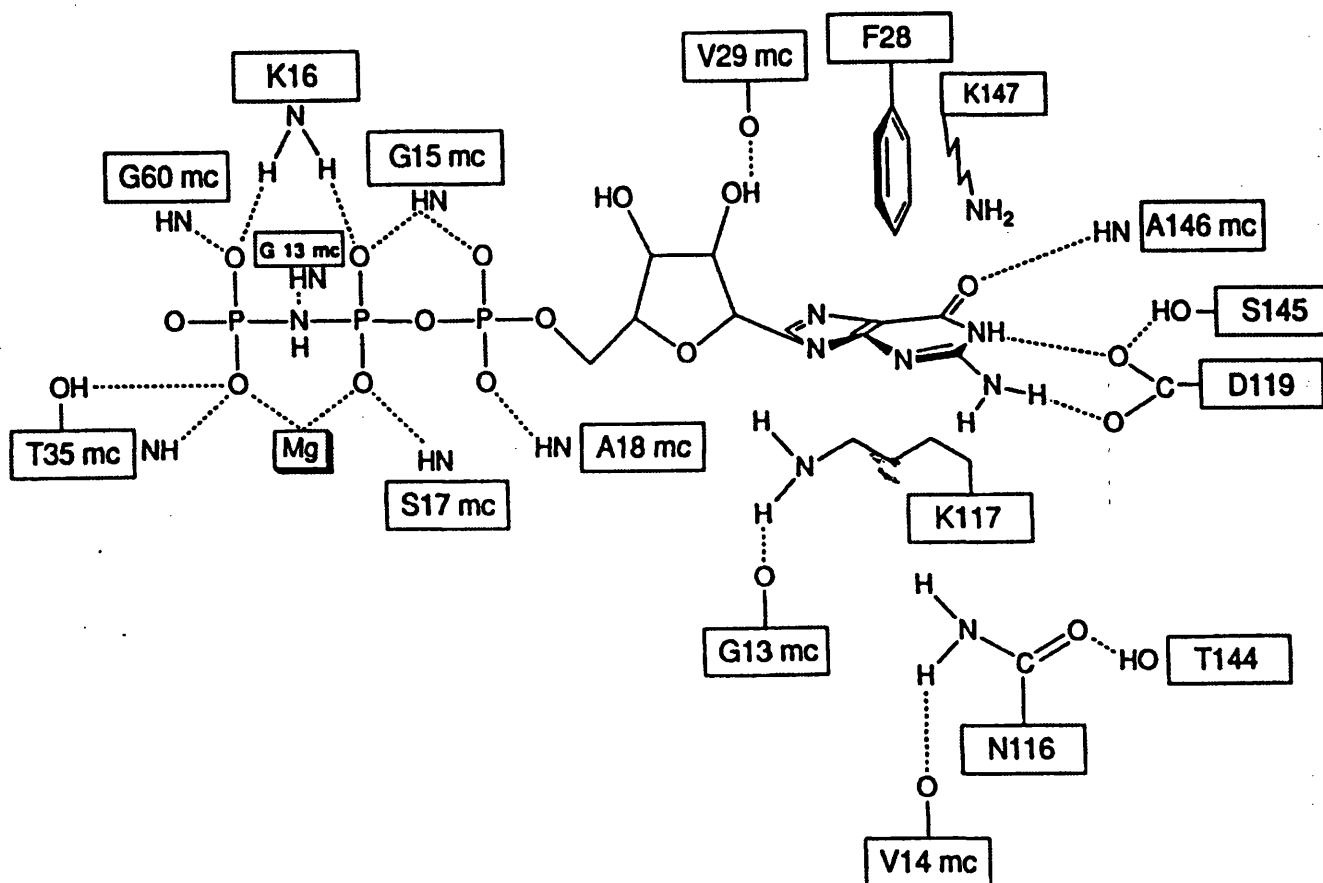


**A Schematic diagram to illustrate the interactions between caged GTP and Ras.**
Hydrogen bonds are represented by dotted lines. mc, main chain. (This figure is based on that of Wittinghofer and Pai, 1991.)

The conformational changes that occur in the different nucleotide bound states are most pronounced in the switch II region. This region is away from the nucleotide binding site and appears to be sensed by other proteins according to the bound nucleotide. The switch sensor is formed by the ends of helices α2 and α3 and loops L5 and L7. Most of the residues that are prominently exposed in both nucleotide states are conserved in a subfamily specific way (Valencia et al, 1991).

p120GAP and NF1 accelerate GTP hydrolysis and are thought to recognise the switch II region since mutations that affect p120GAP/Ras interaction map to this region (Gideon et al, 1992). Ras-GDP is recognised by guanine nucleotide dissociation inhibitors (GDIs) (Araki et al, 1990). Guanine nucleotide exchange factors (GEFs) appear to recognise the state of switch II by binding to helices α2 and α3. Mutations in these regions (63, 71 and 73 - 75 in α2-L5 and 103 - 108 in α3-L7) disrupt GEF stimulated nucleotide exchange (Beitel et al, 1990; Mistou et al, 1992; Verrotti et al, 1992).

## 1.2.3. Ras mutations and their structural effects

Mutations at positions 12, 13 and 59 lead to activated Ras that permanently binds GTP. When the glycine at position 12 is mutated the guanine nucleotide pocket is partially blocked preventing hydrolysis of the GTP. The presence of a hydrophobic side chain in this region would also create an energetically unfavourable situation as it would be in contact with the highly hydrophilic and charged γ phosphate of GDP. Proline is the only non-activating substitution at codon 12 since it causes no distortions in the protein molecule. Mutation of position 13 to valine or aspartate may distort loop 1 and the binding of β phosphate (Tong et al, 1991). Substitution at positions 12 or 13 with proline is non-activating as distortion does not occur.

Mutations at position 59 reduce the intrinsic GTPase activity of Ras since the γ phosphate group can be attacked but at a much slower rate than in wild type Ras (John et al, 1988). A decrease in GTPase has also been found to occur with mutations at position 61 since activation of position 175 cannot take place which would normally lead to hydrolysis of GTP (Kreugel et al, 1990).

Substitutions at positions 16, 28, 35, 36, 38, 116, 117, 119, 144 and 147 also lead to activation. Nucleotide binding is reduced by mutating residues 116, 117, 119 and 144 so activating the protein (Clanton et al, 1986; Der et al, 1986; Feig et al, 1986; Sigal et al, 1986b; Walter et al, 1986). Mutation of Lys16 disrupts the stability of the polypeptide chain, has a steric effect on Ser17

and reduces nucleotide affinity but not specificity since Lys16 does not bind [1 8] the guanine ring (Sigal *et al*, 1986b). Phe28 binds the guanine base through aromatic-aromatic interactions and interacts with Lys147. Mutation of Phe28 or Lys147 leads to a decrease in nucleotide binding and transformation.

A decrease in GTPase activity is commonly seen with oncogenic mutations of Ras and this is true of positions 12, 61 and 35. The ability of these mutants to transform is probably a consequence of their inability to interact with GAP. GAP interaction is abolished by mutations at positions 35, 36 and 38 (Adari *et al*, 1988; Calés *et al*, 1988).

## 1.3.    Proteins that regulate Ras

### 1.3.1.    Introduction

Ras proteins have intrinsic GTPase activity with the protein cycling between an active GTP bound state and an inactive GDP bound form. **(Figure 1.3)**. The rates of GDP dissociation and GTP hydrolysis (~0.008 and $0.02min^{-1}$ respectively) (John *et al*, 1988; Neal *et al*, 1988) of Ras *in vitro* are slow implying that guanine nucleotide exchange factors (GEFs) exist to catalyse guanine nucleotide exchange and other proteins stimulate GTPase. The replacement of GDP with GTP is mediated by proteins known as Guanine nucleotide Dissociation Stimulators (GDS), Guanine nucleotide Releasing factors (GRF) or GEFs (Guanine-nucleotide-exchange factors). Other regulatory proteins known as Guanine nucleotide Dissociation Inhibitors (GDIs) bind the inactive GDP state. The hydrolysis of bound GTP to GDP is due to the action of GTPase Activating Proteins (GAPs).

### 1.3.2.    Guanine nucleotide exchange factors

#### 1.3.2.1.RasGEFs

The CDC25 gene of *S. cerevisiae* was found to be a RAS GEF (Broek *et al*, 1987; Gibbs and Marshall, 1989; Robinson *et al*, 1987). It is required for normal RAS function but it is not essential to the Val19 RAS mutant. It is therefore not required by the 'active' or GTP bound form of RAS (Jones *et al*, 1991). Cdc25 is believed to play a role in determining the nutritional status of the cell and activating RAS when conditions are suitable for growth. Cdc25 contains an SH3-like domain at the amino terminus. Other SH3 (Src-homology-3) containing proteins include cytoskeletal proteins (myosin, spectrin, yeast actin binding protein ABP-1), signal transduction proteins (SHC, GRAB2/sem5, PI3'kinase, phospholipase C-gamma, p120-GAP), non-receptor tyrosine kinases and components of the oxidative burst.

**Figure Legend 1.3.**

**The GDP/GTP exchange cycle for Ras-like GTPases.**

The exchange of GDP for GTP is catalysed by GEFs (guanine nucleotide exchange factors) whilst GAPs (GTPase activating proteins) catalyse the conversion of the GTP bound form back to the GDP state. GDIs (guanine nucleotide dissociation inhibitors) affect GAP action and nucleotide dissociation.

**Figure 1.3**

**The GDP/GTP exchange cycle for Ras-like GTPases.**
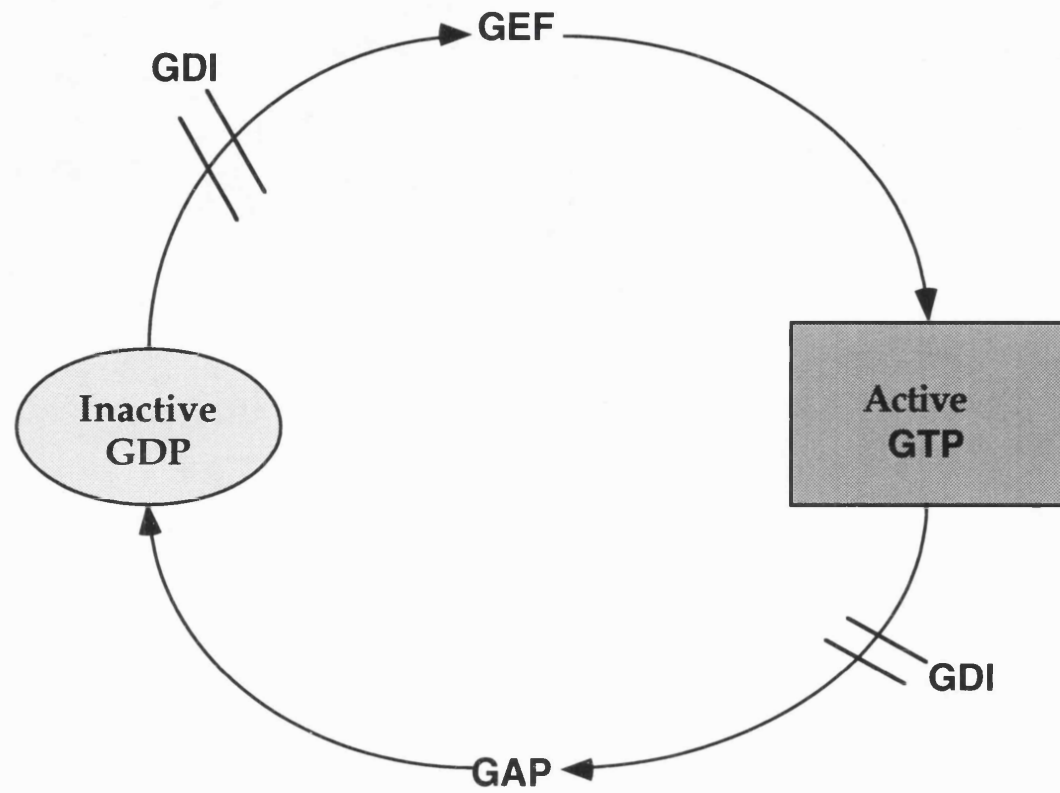
Another Ras GEF from *S. cerevisiae*, Sdc25 (Boy-Marcotte *et al*, 1989; Crechet *et al*, 1990a; 1990b) contains the active region of GEF at its C-terminus. This C-terminal product has been shown to complement a cdc25-5 mutation (Boy-Marcotte *et al*, 1989) whereas the full length product does not (Damak *et al*, 1991). The N-terminal portion of the protein appears to regulate the interaction of the GEF domain with RAS proteins and normally inhibits GEF action. The SDC25 gene is not essential although it may also respond to specific nutrient conditions.

Cdc25 binds to human H-ras (Powers *et al*, 1989) and the GEF domain of sdc25 has been shown to act on H-ras *in vitro* (Crechet *et al*, 1990b). The GEF proteins bind to Ras sequences that are conserved between mammalian and yeast forms. Several of these conserved amino acids are key elements in Ras activation - residues 73 - 75 (Verrotti *et al*, 1992), 102 and 103 (Willumsen *et al*, 1991) residues that bind the purine ring of GDP (Mistou *et al*, 1992) and those that influence co-ordination of $Mg^{2+}$-GDP.

It is thought that the mechanism of GEF action is similar to that of EF-Ts, a GEF that catalyses exchange of GDP and GTP on elongation factor EF-Tu (Bourne *et al*, 1990). The GEF binds to the GDP bound form, the GDP then dissociates from this complex, GTP binds and the GEF dissociates leaving the protein in the active GTP form. Sdc25 has been shown to bind to Ras that is associated with GDP and GTP with comparable affinities (Mistou *et al*, 1992). A stable complex has been demonstrated between Ras and Cdc25 which can be disrupted by GDP or GTP with equal efficiency (Lai *et al*, 1993). Dominant negative mutants of Ras (Asn 17 of H-ras) (Feig and Cooper, 1988) have reduced affinity for GTP and presumably are defective for the displacement of GEF by GTP and therefore remain in an inactive complex (Powers *et al*, 1989).

Another gene in *S. cerevisiae* known as LTE1 contains significant sequence homology to the Ras GEF domain. Loss of Lte1 function appears to result in cold sensitive growth (Wickner *et al*, 1987).

*Ras* 1 of *S. pombe* (Fukui and Kaziro, 1985) is required for mating but not for vegetative growth (Fukui *et al*, 1986; Nadin-Davis *et al*, 1986) and is involved in signal transduction from mating pheromone receptors. Ste6, a sterile mutant of *S . pombe* , encodes a protein with similarities to cdc25 and which presumably activates ras1 function by promoting GTP binding (Hughes *et al*, 1990).

The *Son of sevenless* gene (*Sos*) part of the sevenless pathway affecting eye development in *Drosophila*, activates Dras1 function. The *Sos* gene product contains a GEF domain **(Figure 1.4)** and is presumably a GEF

**Figure Legend 1.4.**

**Homologies between guanine nucleotide exchange factors (GEFs).**

The figure illustrates regions of homology between various GEF proteins. The largest Cdc25 murine homologue is shown (Cdc25Mm). Vav, Bcr and Dbl are mammalian GEFs, Cdc24 and Cdc25 are from the yeast *S. cerevisiae* and Sos is from *D. melanogaster.*

Striped boxes represent CDC25 homology regions whilst black boxes represent regions with homology to CDC24.

**Figure 1.4**

Homologies between guanine nucleotide exchange factors (GEFs)
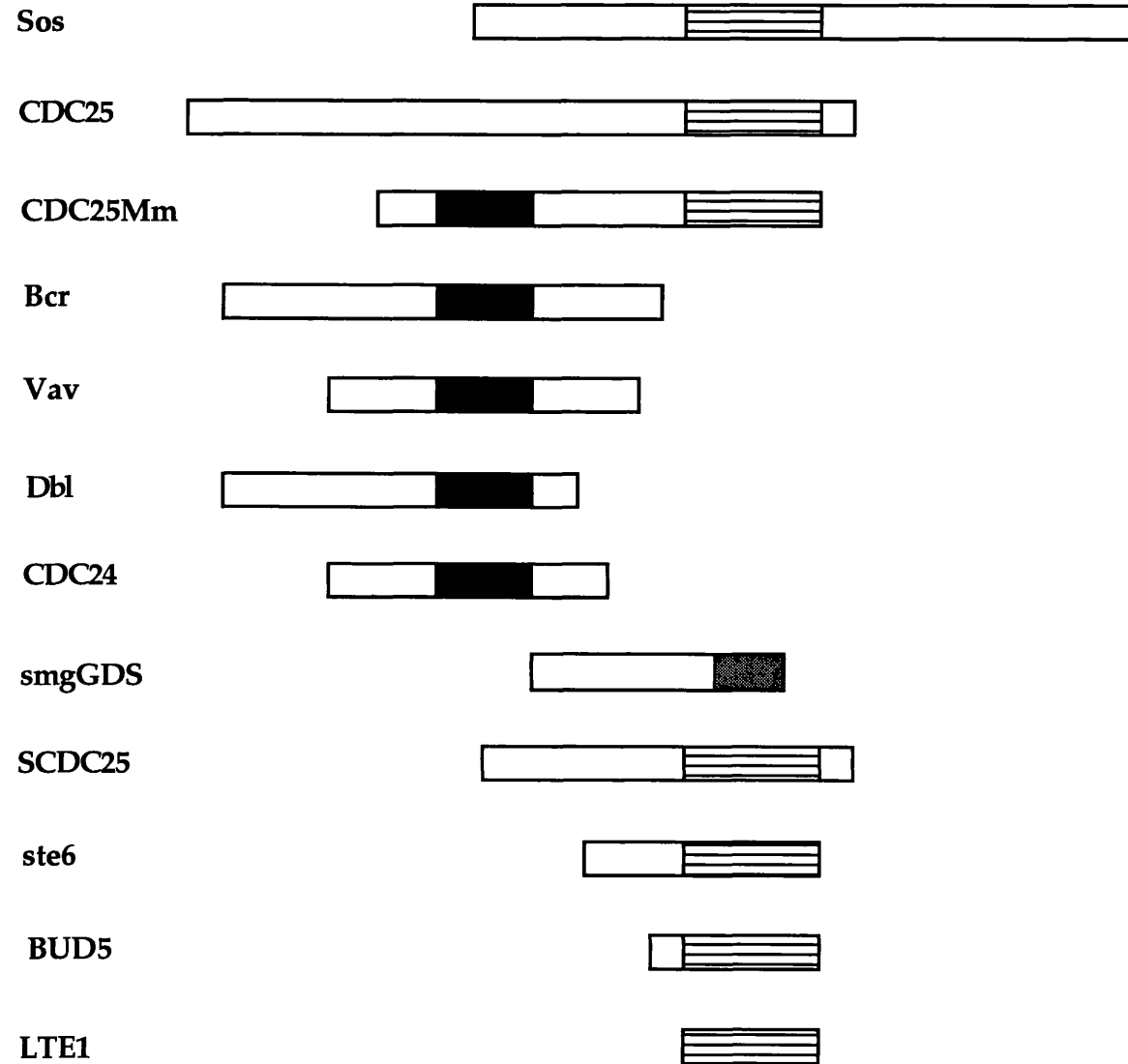
for Dras1 (Simon *et al*, 1991). Two murine homologues, mSos-1 and mSos-2 have been cloned by low stringency hybridisation with the *Drosophila* gene (Bowtell *et al*, 1992). They are 67% identical and are the first examples of mammalian GEF sequences. A rat brain cDNA library was used to isolate another mammalian GEF, p140$^{Ras-GRF}$ (Shou *et al*, 1992) which also contains a RhoGEF domain.

Sos1 is a GEF responsible for Ras activation in response to epidermal growth factor. The EGF receptor dimerises after binding EGF and tyrosine residues in the cytoplasmic domain are autophosphorylated creating SH2 (Src-homology-2) binding sites. Grb2 (homologous to Sem-5 in *C. elegans* and Drk in *Drosophila* ) binds to the EGF receptor via the SH2 domains and also binds to Sos1 through SH3 domains which recognise proline rich regions at the C-terminus of Sos1 (Li, N *et al*, 1993; Olivier *et al*, 1993; Rozakis-Adock *et al*, 1993). **(Figure 1.5).**

These interactions bring the cytoplasmic Sos1 to the plasma membrane where Ras activation occurs. This method of Ras activation could also be used by non-receptor tyrosine kinases through phosphorylation of Shc which binds the Grb2-Sos1 complex in a similar manner to the EGF receptor (Baltensperger *et al*, 1993; Lai *et al*, 1993).

The GEF proteins so far identified show a common conserved domain of approximately 200 residues that consists of three structurally conserved regions (SCRs) separated by less conserved regions. The SCR3 contains a thirteen residue consensus pattern that forms a diagnostic 'signature' that is specific for all known Ras GEF proteins (Boguski and McCormick, 1993) **(Figure 1.4).**

RasGEF contains two pleckstrin homology (PH) domains. Pleckstrin is a 47K protein that is the major substrate for protein kinase C in platelets (Tyers *et al*, 1989). Pleckstrin homology domains have also been identified in Sos, p120GAP and p140$^{Ras-GRF}$.

### 1.3.3. GTPase Activating Proteins (GAPs)
#### 1.3.3.1.RasGAP

p120GAP was the first example of a factor that might regulate Ras activity in vertebrates (Trahey and McCormick, 1987). p120GAP was isolated from *Xenopus* oocytes and from mammalian cells as a cytoplasmic factor that increases the intrinsic GTPase activity of normal Ras. Oncogenic mutants of Ras remain in the active GTP bound state and are resistant to GAP-mediated GTPase stimulation (Adari *et al*, 1988; Trahey and McCormick, 1987) although

**Figure Legend 1.5.**

**The Ras signal transduction pathway.**

EGF binds to its receptor and this leads to phosphorylation. An adaptor protein, Grb2 binds to the EGF receptor via SH2 domains and also binds the GEF (guanine nucleotide exchange factor) Sos through its SH3 domains. Activation of Ras occurs and results in the binding of Raf to Ras. Activated Raf then phosphorylates MEK which in turn phosphorylates MAP kinase. Signals then enter the nucleus by transcription factor phosphorylation and hence regulate gene expression.

**Figure 1.5**
**The Ras signal transduction pathway**
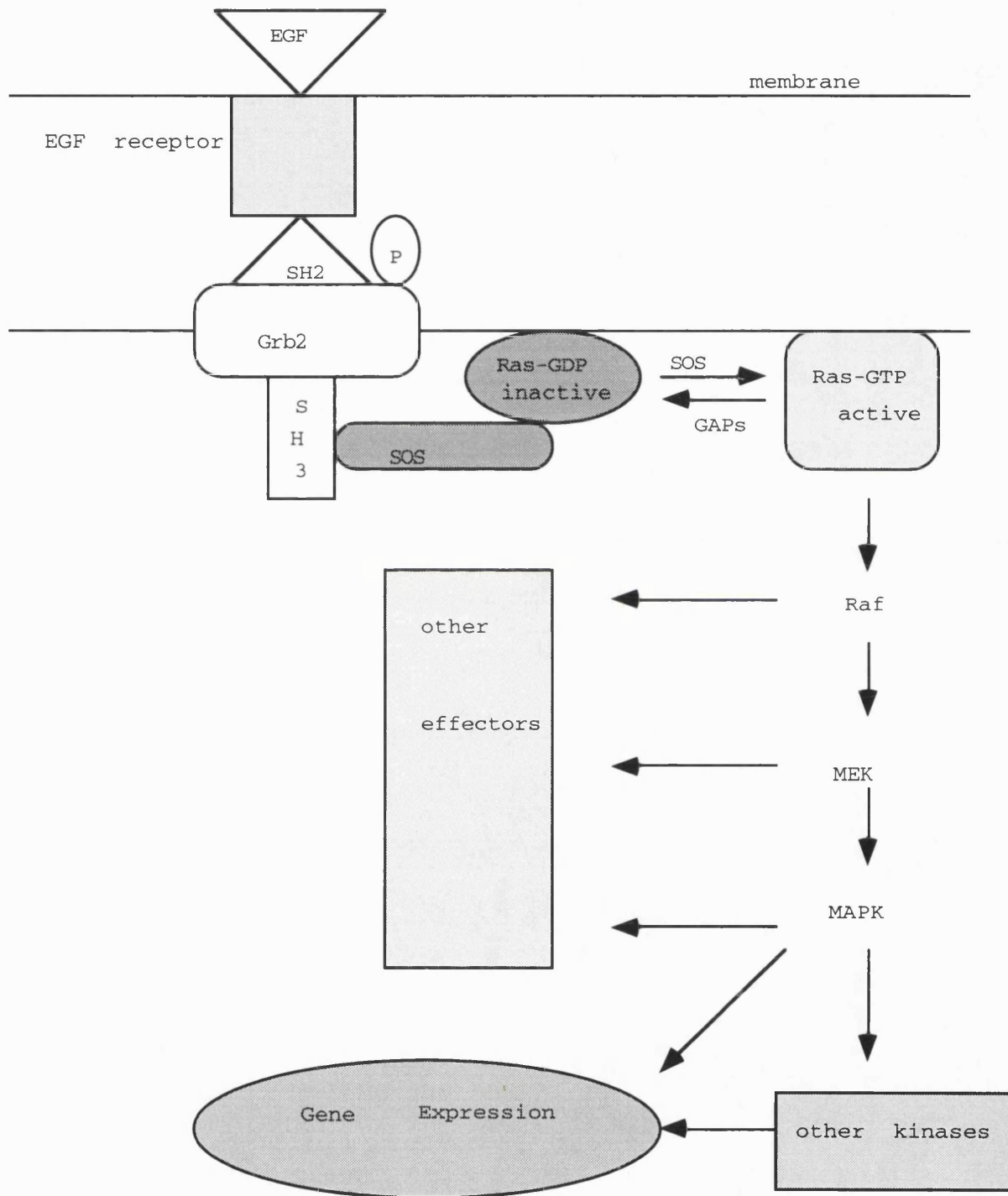
they do interact with p120GAP (Gideon *et al*, 1992; Vogel *et al*, 1988). Full length human placental (Trahey *et al*, 1988) and bovine brain (Vogel *et al*, 1988) GAP cDNA have been cloned. Human GAP was found to be a 120kDa protein - p120GAP. p120GAP increases the intrinsic activity of RasGTPase and this activity has been localised to the C-terminal third of the protein - the catalytic domain. RasGAP preferentially binds RasGTP (Vogel *et al*, 1988), is specific for all normal Ras proteins and R-ras (Garrett *et al*, 1989) but does not effect other members of the Ras superfamily which have their own GAP proteins (Diekmann *et al*, 1991; Rubinfeld *et al*, 1991). p120GAP has a hydrophobic amino terminus, one SH3 region, two SH2 domains, a pleckstrin homology (PH) domain and a region with homology to the CalB region of phospholipase $A_2$ which is believed to be a phospholipid-binding sequence involved in the $Ca^{2+}$-dependent translocation of certain regulatory proteins to membranes (Clark *et al*, 1991) **(Figure 1.6)**. A less widely expressed splice variant has also been identified in human placental tissue that lacks the hydrophobic amino terminus and is known as p100GAP (Halenbeck *et al*, 1990).

Ras is negatively regulated by the GTPase stimulatory activity of p120GAP (Zhang *et al*, 1990; 1991). Overexpression of p120GAP leads to a decrease in the amount of GTP-bound endogenous Ras (Gibbs *et al*, 1990). Transformation by overexpression of normal Ras, Src or the CSF-1 receptor can be prevented or reverted by p120GAP (Bortner *et al*, 1991; DeClue *et al*, 1991; Nori *et al*, 1991; Zhang *et al*, 1990). Mutant Ras transformation is not inhibited by p120GAP since the protein is resistant to p120GAP. Yeast RAS can also be negatively regulated by GAP (Ballester *et al*, 1989; Tanaka *et al*, 1990a).

Mutation of the Ras effector region (amino acids 32 - 42) whilst allowing p120GAP binding (Farnsworth *et al*, 1991b; Gideon *et al*, 1992; Marshall *et al*, 1991; Schaber *et al*, 1989; Vogel *et al*, 1988), renders Ras resistant to GAP activity (Adari *et al*, 1988; Calés *et al*, 1988) suggesting an effector role for p120GAP. The monoclonal antibody Y13 259 inhibits p120GAP and Ras action (Adari *et al*, 1988; Martin *et al*, 1990; Rey *et al*, 1989; Srivastava *et al*, 1989; Trahey and McCormick, 1987) whilst the Y13 238 antibody does not inhibit p120GAP or Ras activity (Adari *et al*, 1988; Kung *et al*, 1986; Rey *et al*, 1989).

The Krev1/Rap1A protein reverts K-ras transformed cells (Kitayama *et al*, 1989). p120GAP binds to Rap1A but does not stimulate GTPase activity (Frech *et al*, 1990; Hata *et al*, 1990). Activating mutations of Rap1A potentiate its suppressor ability (Kitayama *et al*, 1990) and lead to Rap1A-GTP accumulation.

**Figure Legend 1.6.**

**Sequence homologies among GTPase activating proteins.**

The domains of the various GTPase activating proteins are shown. The catalytic domain (black box) is conserved between all GTPase activating proteins. Other regions of extended homology are also found (shaded boxes) in NF1, IRA1 and IRA2. GAP contains SH2 and SH3 gap1 homology domains, a pleckstrin homology domain (PH) and a CaLB motif.

Figure 1.6

Sequence homologies between GTPase activating proteins
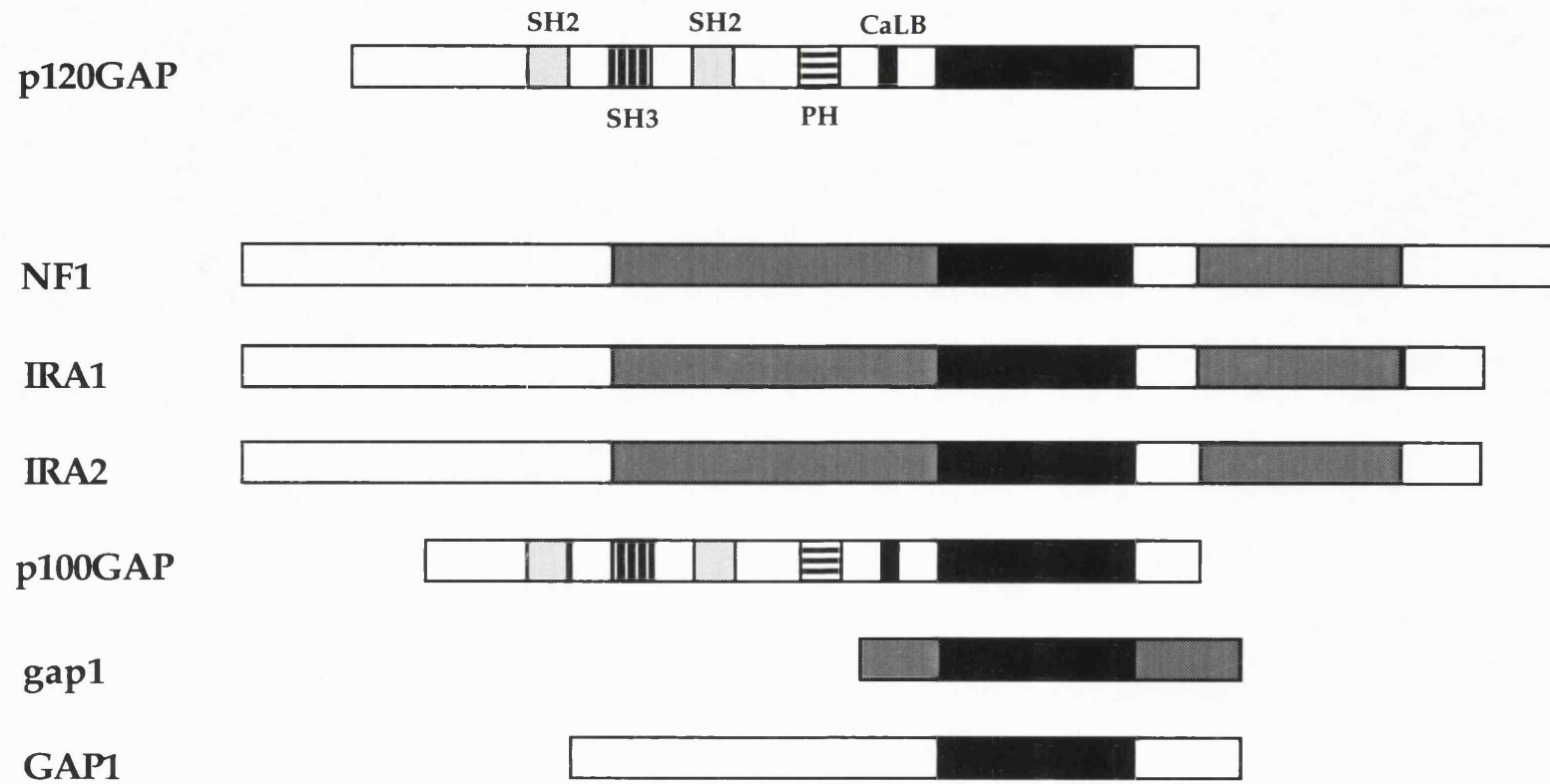
This increases its affinity for the Ras effector which could be p120GAP since the binding affinity between p120GAP/Rap1A-GTP is high (Frech *et al*, 1990).

Other evidence for an effector role of p120GAP has involved the study of muscarinic receptors and K$^+$ channel currents (Yatani *et al*, 1990). Anti-GAP antibodies prevent the ability of Ras to uncouple a heterotrimeric G protein (GK) from muscarinic receptors and K$^+$ channel activation. The requirement for Ras can be overcome by a SH2/SH3 GAP mutant without the catalytic domain (Martin *et al*, 1992). Binding of Ras would appear to expose the SH2 and SH3 domains activating the target function of p120GAP. However an alternative explanation of these data is that the PH domains of p120GAP compete with $\alpha$K for the binding to $\beta\gamma$, since it has recently been reported that PH domains bind to the $\beta\gamma$ subunits of heterotrimeric G-proteins (Lefkowitz, unpublished data).

The effector role of p120GAP has also been supported by evidence that phospholipase C$\gamma$, the effector of Gq, stimulates GqGTPase activity twenty fold (Berstein *et al*, 1992) and the $\gamma$ subunit of phosphodiesterase the effector of transducin G-alpha, also has GAP activity (Arhavsky and Bownds, 1992).

Mutation of Ras residues 61 - 65, the second loop of the protein, reduces the affinity of GAP for Ras (Gideon *et al*, 1992; Martin *et al*, 1991; Zhang *et al*, 1991). The catalytic domain of GAP may interact with both of the Ras loops which alter their conformation in a GTP-dependent manner. These studies all suggest that p120GAP may function as a downstream effector of Ras.

IRA1 and IRA2 are *S. cerevisiae Ras* GAP genes that contain C-terminal domains homologous to p120GAP (Tanaka *et al*, 1989; 1990a; 1990b) **(Figure 1.6)**. Without either of the IRA gene products, RAS accumulates in the GTP bound form and over production of cAMP results (Wigler *et al*, 1990). IRA is therefore a negative regulator and not an effector of RAS in yeast. The catalytic C-terminal domain of p120GAP can complement *S. cerevisiae* mutants lacking IRA1 or IRA2 function (Tanaka *et al*, 1990a). Therefore p120GAP must recognise RAS sequences that are highly conserved between mammals and yeast. However IRA1 and 2 have only limited ability to function on mammalian H-ras (Gibbs *et al*, 1987).

The RasGAP of *S. pombe, sar* 1 or *gap* 1, can complement IRA1 or IRA2 function in *S. cerevisiae* (Imai *et al*, 1991; Wang *et al*, 1991) and is

not required for the effector function of Ras1. In *S. cerevisiae*, overexpression of the RPI1 gene inhibits the function of normal but not activated Ras. It requires IRA1 and IRA2 to function and it is therefore possible that it activates these negative regulators. It may also inhibit Cdc25 (Kim and Powers, 1991).

GAP1 of *Drosophila* is a component of the sevenless (sos) tyrosine kinase/Ras1 signalling pathway (Gaul *et al*, 1992) that is not an effector of Ras1 action since loss of GAP1 stimulates Ras1 function. GAP1 does not appear to be related to neurofibromin outside the Ras-GAP domain and does not contain SH2 or SH3 domains.

### 1.3.3.2. NF1

The NF1 gene responsible for von Recklinghausen's neurofibromatosis has been cloned (Cawthon *et al*, 1990; Viskochil *et al*, 1990; Wallace *et al*, 1990; Zhang *et al*, 1990) and encodes the protein neurofibromin (Marchuk *et al*, 1991). The protein contains a central 350 amino acid region (the GAP-related domain) with homology to p120GAP, IRA1 and IRA2 (Xu *et al*, 1990b) **(Figure 1.6)**. The GAP-related domain of NF1 binds Ras (Bollag and McCormick, 1991) and stimulates RasGTPase activity (Ballester *et al*, 1990; Martin *et al*, 1990; Xu *et al*, 1990a). p120GAP and NF1 are both detected in most cells and tissues (Bollag and McCormick, 1991) suggesting that Ras activity is regulated by one or both proteins.

In T-cells either p120GAP or NF1 clearly act as the negative regulator of Ras since T-cell activation leads to an increase in RasGTP by an inhibition of total GAP activity (Downward *et al*, 1990). Oncogenic Ras binds to NF1 more avidly than to p120GAP (Bollag and McCormick, 1991) suggesting that NF1 is the primary target of Ras.

Neurofibromatosis is characterised by abnormal proliferation of cells of neural crest origin and leads to 'cafe-au-lait' spots formed by abnormal growth of melanocytes or neurofibromas containing predominantly Schwann cells. Schwannoma cell lines from NF1 patients (Basu *et al*, 1992; DeClue *et al*, 1992) have no neurofibromin or very low levels with no GTPase activity, have normal levels of GAP and high levels of RasGTP that is not a consequence of a Ras activating mutation. This supports the idea that NF1 acts as a tumour suppressor gene with NF1 mutations leading to defective negative regulation of Ras. By contrast, the negative regulator of RasGTP in fibroblasts is GAP (Zhang *et al*, 1991).

Activated Ras proteins (RasGTP) cause growth arrest in Schwann cells whereas loss of NF1 leads to RasGTP accumulation and cell proliferation (Basu *et al*, 1992; DeClue *et al*, 1992). This again suggests that NF1 plays a negative regulator role or that RasGTP/NF1 interaction generates a negative growth signal.

Mutant NF-1 alleles are also associated with sporadic cancers unrelated to neurofibromatosis and away from neural crest tissues (Li *et al*, 1992). The same codon is mutated in all of these cases and does not lead to loss of function. Ras mutations are rare in sporadic tumours indicating that increasing active Ras levels by loss of neurofibromin function has a different effect to raising levels by Ras mutation. Juvenile leukaemias are associated with Ras mutations in sporadic cases but with loss of both normal NF-1 alleles in neurofibromatosis patients. In this case neurofibromin loss is biologically equivalent to oncogenic Ras activation (Shannon *et al*, 1994).

### 1.3.4. Regulators of Ras-related proteins
### 1.3.4.1.Regulators of Rab

GDP release from Rab3A is stimulated by Mss4, a mammalian suppressor of sec4 (Burton *et al*, 1993). RabGDI (Matsui *et al*, 1990) inhibits GDP/GTP exchange and is involved in removing Rab3A from membranes (Araki *et al*, 1991). RabGDI has some sequence homology with hCHM, the human choroideremia gene which is a subunit of RabGGTase with no GDI activity (Fodor *et al*, 1991). Homologues of RabGDI have also been found in several species. GAPs for Rab proteins have also been identified (Strom *et al*, 1993; Tan *et al*, 1991b; Walworth *et al*, 1992).

### 1.3.4.2.Regulators of Rap

Rap1GAP (Rubinfeld *et al*, 1991) acts on Rap1A (Kitayama *et al*, 1989) a protein which reverts transformation by activated K-ras. Rap1A binds p120GAP but its GTPase activity is not enhanced by this interaction suggesting that it competes with Ras for p120GAP (Frech *et al*, 1990). Activation of GTPase by Rap1GAP and RasGAP is thought to be brought about by different mechanisms possibly involving residue 61 of the target protein. Rap1A has a threonine at this position whilst most of the small GTPases including Ras, have a glutamine here. Substitution of Thr61 for Gln in Rap1A leads to p120GAP stimulation of Rap1GTPase whilst Rap1AGAP activity is unaffected (Frech *et al*, 1990).

# 1.3.4.3.Regulators of Rho and Rac proteins

Overexpression of CDC42 can overcome defects in the CDC24 gene suggesting that CDC24 is an activator of yeast Rho/Rac-like proteins. Dbl is a mammalian GEF for CDC42Hs/G25K, Rho and Rac1 (Hart *et al*, 1991; Miki *et al*, 1993). The GEF activity of Dbl does not seem to account for cell transformation (S. Munemitsu, unpublished). CDC42Hs could cycle between the GDP and GTP bound states but Dbl acts on other Rho-like proteins to produce transformation.

Bcr, Vav and RasGRF have sequence homology to the GEF domain of Dbl and CDC24 (Adams *et al*, 1992; Boguski *et al*, 1992a; Ron *et al*, 1991) **(Figure 1.6)**. Vav antibodies have shown GEF activity in activated T-cells and *in vitro* translation of Vav generates RasGEF activity (Gluons *et al*, 1993). Vav could have be a RasGEF or it could associate with a RasGEF in a similar manner to that proposed for Dbl.

RhoGDI is involved in the movement of Rho proteins between different membrane compartments. GAPs for Rho and Rac have also been identified (Garrett *et al*, 1989) that have homology to Bcr (Diekmann *et al*, 1991) and n-chimaerin (Hall *et al*, 1990), both of which have GAP activity on Rac1 and Rac2. The p190 protein is a potent GAP for Rho/Rac proteins (Settleman *et al*, 1992a; 1992b). A Rho/Rac GAP in *Drosophilia* involving the *rotund* locus has also been identified (Agnel *et al*, 1992).

# 1.3.4.5.Other protein regulators

GEFs have also been identified for Ran (Ras-related nuclear protein), a nuclear protein of the TC4 subfamily (Bischoff and Ponstingl, 1991), and Ral (Albright *et al*, 1993). A GAP for Ral has also been identified (Emkey *et al*, 1991).

# 1.4. Signal Transduction
# 1.4.1. Introduction

The mechanisms by which cell division and differentiation are controlled have intrigued scientists for years. Signalling pathways start with cell surface receptors and finish in the nucleus with proteins that regulate gene expression. Chains of interconnecting proteins form signalling pathways within cells. Upstream activating signals are integrated by each protein component and passed on to various downstream targets or effector proteins. Signal transduction pathways

integrate signals received at the cell surface and regulate cell division
and cell differentiation.

The importance of Ras in cell proliferation was shown by the fact that serum or growth factor induced mitosis could be inhibited by Y13 259, a monoclonal antibody directed against Ras (Mulcahy *et al*, 1985). Cell transformation by protein tyrosine kinases such as Src and Fms was shown to require endogenous Ras activity whilst other oncogenes for example Mos and Raf, transformed independently of Ras (Smith *et al*, 1986). These results all suggested a role for Ras in a growth signalling pathway.

## 1.4.2. Ras effectors

RasGTP is the active form of Ras and interacts with one or more cellular effector molecules. The effector domain of Ras, residues 35 -40, was detected by mutation analysis (Sigal *et al*, 1986a; Willumsen *et al*, 1986) that disrupted biological activity but not GTP binding. p120GAP and NF1 are both candidate effector proteins for mammalian Ras whilst in yeast it has been shown that Ras activates an adenyl cyclase complex (Toda *et al*, 1985). However Ras does not activate adenyl cyclase in higher eukaryotes (Beckner *et al*, 1985; Birchmeier *et al*, 1985) and a signal transduction pathway for mammalian Ras remained elusive.

Ras signalling pathways are present in eukaryotes ranging from yeast to man. In *S. cerevisiae*, the Ras pathway is involved in detecting nutrient conditions and controlling mitosis (Broach, 1991; Powers, 1992). In *S. pombe* Ras controls meiosis, sporulation, cell shape and the response to mating pheromone (Egel *et al*, 1990; Powers, 1992). Ras in this case is upstream of a series of protein kinases (Errede and Levin, 1993).

In *Drosophila*, eye and embryo development proteins form a pathway that begins with a cell-surface tyrosine kinase (Greenwald and Rubin, 1993; Perrimon, 1993). A similar Ras centered pathway exists in *C. elegans* that is involved in vulva development (Horvitz and Sternberg, 1991).

These pathways involve threonine and tyrosine phosphorylation of MAP (mitogen activated protein) kinases and form MAP kinase cascades **(Figure 1.7)**. They also position the serine/threonine kinase Raf downstream of Ras.

**Figure Legend 1.7.**

**A comparison of MAP kinase cascades.**

Signal transduction pathways involving MAP kinase cascades have been identified in several different species. There are several different cascades in *S. cerevisiae* one of which, the pheromone response acts through a G-protein mediated pathway which includes a downstream MAP kinase cascade. The *S. cerevisiae* HOG genes encode components of a MAP kinase cascade that is involved in the response to increased osmolarity. The cascade in *S. pombe* also involves the pheromone response and acts through Ras and a heterotrimeric G-protein. The *C. elegans* cascade is responsible for vulval induction.

36

# Figure 1.7

## A comparison of MAP kinase cascades



| vertebrate | S. pombe | C. elegans | S. cerevisiae |
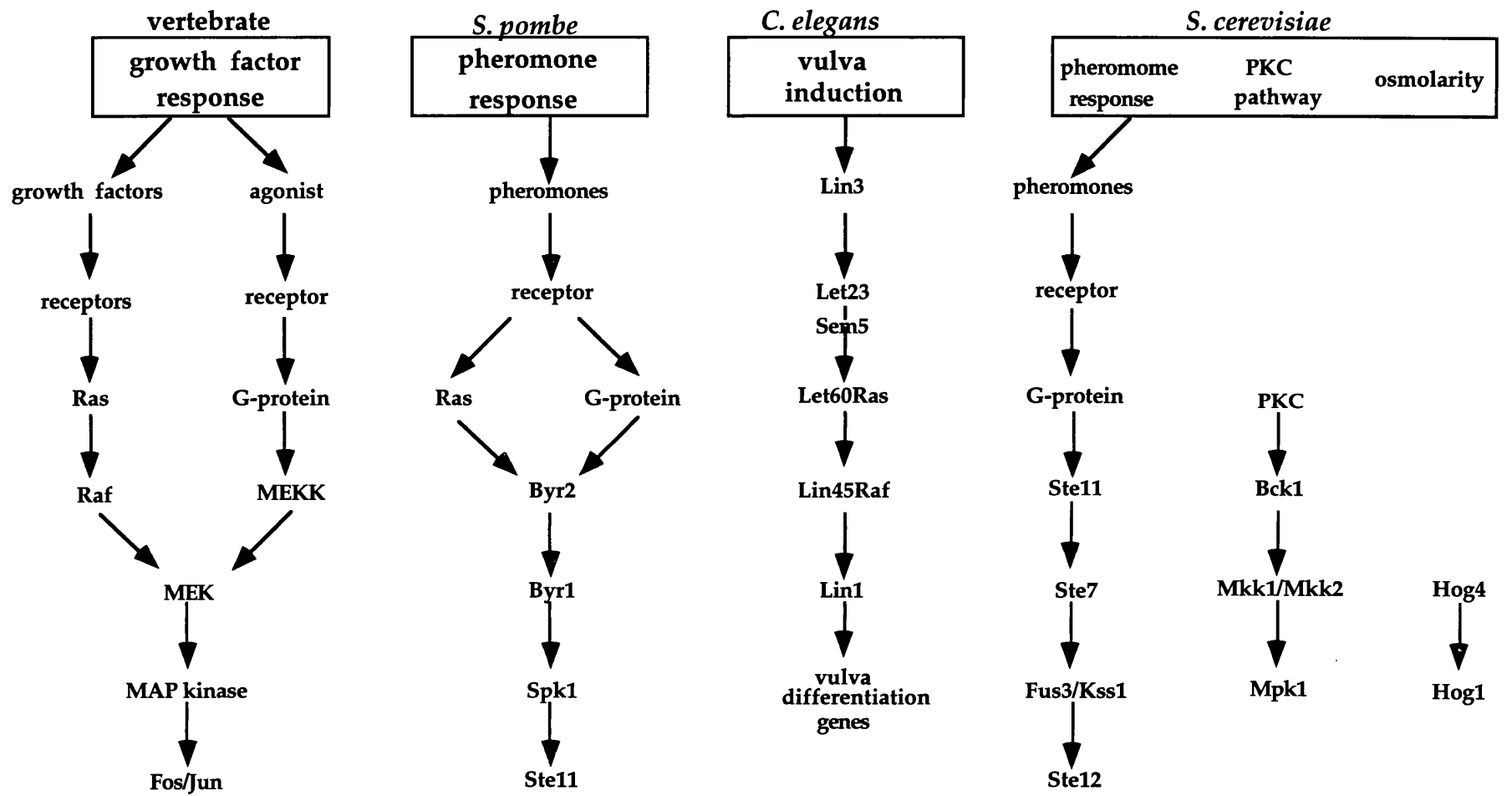
**1.4.2.1.Raf**

The Raf family of serine/threonine kinases consists of 3 members : C-Raf (Raf1), A-Raf and B-Raf (Heidecker *et al*, 1991; Morrison, 1990). The Raf proteins have three conserved structural domains, CR1 (conserved region 1), CR2 and CR3 (Heidecker *et al*, 1989; Morrison, 1990). The N-terminal CR1 region is cysteine rich and contains a putative zinc binding region (Berg, 1986; Heidecker *et al*, 1989). CR2 is also N-terminal and is rich in serine and threonine residues whilst the C-terminal CR3 domain contains the protein kinase domain (Heidecker *et al*, 1989). The Raf proteins have 75% homology in the kinase domain (Heidecker *et al*, 1991) but show different expression patterns. Raf1 is found in all tissues with the highest levels in cerebellum, fetal brain and skeletal muscle (Storm *et al*, 1990). A-Raf is expressed in ovaries, kidney and epididymis and at low levels in other tissues. B-Raf is present in high levels in fetal brain and cerebrum being localised to hippocampal neurons and dendritic spines (Milhaly *et al*, 1991; Olah *et al*, 1991). B-Raf is cytosolic and is autophosphorylated at Thr372 (Stephens *et al*, 1992).

*Drosophila* Raf function is required for both the receptor- and Ras-stimulated phenotypes of the eye and torso (Ambrosio *et al*, 1989; Dickson *et al*, 1992). In *C. elegans,* Ras and Raf are both required for signalling through Let23, an epidermal growth factor-like receptor tyrosine kinase (Han *et al*, 1993) **(Figure 1.7)**. Raf was also positioned downstream of Ras in proliferation and transformation of NIH 3T3 cells (Bruder *et al*, 1992; Kolch *et al*, 1991).

The discovery that Raf complexes to Ras (Moodie *et al*, 1993; Van Aelst *et al*, 1993; Vojtek *et al*, 1993; Warne *et al*, 1993; Zhang *et al*, 1993) and that GRFs activate Ras by causing the release of GDP led to the deduction of a signal transduction pathway for Ras in mammals.

As discussed earlier, an adaptor protein, Grb2 binds to activated EGF receptors through its SH2 domains. Grb2 complexes to Sos (a Ras GRF) via the SH3 domain binding to proline rich regions of Sos. **(Figure 1.5)**. EGF has been shown to stimulate the formation of a ternary complex containing Sos-Sem5/Grb2-EGF tyrosine kinase receptor in fibroblasts (Buday and Downward, 1993; Egan *et al*, 1993). The binding of Sos to Grb2 moves Sos from the cytoplasm to the plasma membrane where Ras activation is thought to occur.

Activation of Ras by the GEF action of Sos is then thought to lead to activation of a kinase cascade. **(Figure 1.5)**. GTP-bound (activated) Ras

binds to the N-terminal CR1 domain of Raf (Vojtek *et al*, 1993; Warne *et al*, 1993). This interaction between Ras and Raf leads to the translocation of Raf from its normal cytosolic location to the plasma membrane where it then activates the MAP kinase cascade. Activated Raf phosphorylates MAP kinase kinase (MAPKK) (Howe *et al*, 1992; Hughes *et al*, 1993; Kyriakis *et al*, 1992) or MEK (Crews *et al*, 1992). Raf therefore has a role as a MAPKK kinase or MEK kinase. The association of Raf with MEK has been shown *in vivo* and *in vitro* by using baculovirus expressed proteins (Huang *et al*, 1993b; Macdonald *et al*, 1993). This system showed direct activation of MEK by Raf in the presence of Ras or Src. A MEK kinase distinct from Raf has also been cloned (Lange-Carter *et al*, 1993) and suggests that there are multiple pathways that lead to MEK activation.

MEK then activates another kinase, MAP kinase (MAPK) or ERK (extracellular signal related kinase) (Errede and Levin, 1993; Pelech and Sanghara, 1992). MEK has dual specificity mediating activation of ERK1 (p44MAPK) and ERK2 (p42MAPK) (Ahn *et al*, 1991; Gomez and Cohen, 1991). MAP kinase itself and other kinases it may activate, send signals into the nucleus by phosphorylating transcription factors that include oncogene products such as Fos and Jun (Alvarez *et al*, 1991; Chen *et al*, 1992; Gutman and Wasylyk, 1991; Hunter and Karin, 1992; Pulverer *et al*, 1991). MAP kinase is found in the nucleus and cytoplasm. The cytoplasmic to nuclear translocation would allow the rapid transmission of growth-regulating information from the membrane, through the cytoplasm to the nucleus (Chen *et al*, 1992).

The discovery that Ras and Raf interact formed the link between Ras and tyrosine kinases and established a vertebrate signal transduction pathway. This area is currently the most exciting field of Ras research and other Ras effectors and pathways may be discovered.

Raf may now be the effector for Ras rather than GAP since it interacts directly with Ras (Vojtek *et al*, 1993; Warne *et al*, 1993), is activated by Ras and in the active state can sometimes replace Ras (Duchesne *et al*, 1993).

## 1.5. Membrane localisation of Ras

## 1.5.1. Introduction

In order to be biologically active Ras proteins need to be associated with the inner surface of the plasma membrane (Willingham *et al*, 1980; Willumsen *et al*, 1984a). Ras is not detected in the nucleus or outside the

cell and very little is found in the cytosol implying that the site of Ras action is the plasma membrane (Der and Cox, 1991). Ras proteins are sensitive to proteolytic degradation suggesting that they are not significantly embedded in the lipid bilayer (Grand *et al*, 1987) and they do not contain hydrophobic signals or membrane spanning sequences. The proteins are synthesised on free cytosolic polysomes and within 5 - 20 minutes they are converted to membrane bound forms with greater electrophoretic mobilities (Shih *et al*, 1982), hydrophobicity and isoelectric points (Gutierrez *et al*, 1989). The translocation of Ras from its site of synthesis in the cytosol to its site of action in the membrane is achieved by a complex series of C-terminal post-translational modifications (Hancock *et al*, 1989).

## 1.5.1.1.Identification of Ras post-translational modifications

Two forms of p21 were identified which migrated at different rates when separated by SDS-PAGE (Shih *et al*, 1979). The slower form, pro-p21, was found to be converted into a faster migrating form, p21. Pro-p21 fractionated into the cytosol whereas the p21 product was associated with the membrane fraction (Shih *et al*, 1982). It was speculated that peptide cleavage or other post-translational modifications accounted for the mobility change and membrane localisation of the mature protein. The intramolecular pro-p21 processing site was determined by selective cleavage of p21 and was found to be located in the C-terminus of the protein (Shih *et al*, 1982).

p21 Ras was shown to be acylated (Sefton *et al*, 1982) by the addition of palmitic acid (Chen *et al*, 1985) and this modification was thought to be responsible for the membrane association of the mature protein. The C-terminus of Ras was shown to be essential for transformation and membrane association by the use of a series of deletion mutants (Willumsen *et al*, 1984a). Further work by this group (Willumsen *et al*, 1984b) showed that the cysteine at position 186 was vital for both transformation and processing of the protein to its membrane associated form. The cysteine 186 is present in all known *ras* genes (Powers *et al*, 1984) suggesting that this amino acid has a vital role.

Mutation of Cys186 to Ser186 abolished palmitoylation, membrane association and resulted in loss of biological activity leading to the conclusion that Cys186 was the site of Ras palmitoylation. The recognition sequence for the palmitoylation of Ras was found to be a

tetrapeptide with the sequence - Cys - A - A - X (Chen *et al*, 1985) where C=cysteine, A=aliphatic and X=any amino acid. This CAAX motif is also found in other proteins such as the nuclear lamins, Ras - related proteins and certain of the $\alpha$ and $\gamma$ subunits of heterotrimeric G proteins.

The importance of the CAAX motif and C-terminal palmitoylation was also shown in yeast. Inactivation of the enzyme responsible for fatty acid acylation (Powers *et al*, 1986) or the equivalent of the Cys186 to Ser186 mutation in the yeast RAS2 protein (Cys319 to Ser) abolished palmitoylation and membrane localisation of this protein. However overexpression of this mutant protein produced an activity in complementation assays (Deschenes and Broach, 1987) leading to the conclusion that acylation of RAS2 was important for its membrane localisation but was not essential for RAS2 function.

Mutational analysis of YPT1, another yeast Ras protein with two consecutive cysteines at the C-terminus, showed that mutation of both cysteines led to loss of membrane association and biological activity (Molenaar *et al*, 1988). Mutation of only one of the cysteine residues allowed palmitoylation and membrane association.

Several workers (Feuerstein and Ali, 1985; Fuhrer *et al*, 1986; Polonis *et al*, 1987; Shih *et al*, 1982) have shown that there are several species of Ras proteins differing in isoelectric point and SDS-PAGE mobility. These mobility differences were accounted for by phosphorylation of the proteins. Not all Ras proteins are phosphorylated however, and other processes must be responsible for these differences.

Yeast RAS was shown to be processed via two precursors (Tamanoi *et al*, 1988) and other acylated proteins, for example the fungal mating factors (Akada *et al*, 1987; Betz *et al*, 1987; Brake *et al*, 1985; Kamiya *et al*, 1979), undergo proteolysis which removes the last three amino acids of their CAAX motifs.

The addition of a methyl ester to the $\alpha$-carboxyl group of a carboxy-terminal cysteine residue was also detected in fungal mating factors (Ishibashi *et al*, 1984; Sakagami *et al*, 1981) and lead to the identification of H-ras C-terminal methylation (Clarke *et al*, 1988). The conversion of a carboxylate anion at the carboxyl terminus to a methyl ester increases the hydrophobicity of the protein and accounts for the changes observed in isoelectric point.

When further researched, it was found that the post-translational processing of Ras is a two-step process (Gutierrez *et al*, 1989). The starting

product pro-p21 is converted (step 1) into an intermediate form, c-p21, which has different SDS-PAGE mobility, hydrophobicity and pI and is cytosolic. The increase in SDS-PAGE mobility of c-p21 results from the removal of the three C-terminal amino acids from pro-p21 (Clarke *et al*, 1988; Gutierrez *et al*, 1989). This proteolysis event would not account for the changes in isoelectric point that occur and this was found to be due to α carboxyl - methylation (Gutierrez *et al*, 1989). Both c-p21 and m-p21 are methylesterified in a similar manner to the fungal mating factors (Ishibashi *et al*, 1984; Sakagami *et al*, 1981). The conversion of c-p21 to the membrane associated form, m-p21, is due to the addition of palmitic acid (step 2). Carboxymethylation would increase the pI of the protein as it progresses from pro-p21 to c-p21 but the further increase seen on the conversion of c-p21 to m-p21 would not be accounted for by the addition of palmitic acid. The Ser186 mutation as well as blocking acylation, membrane binding and transformation by Ras (Willumsen *et al*, 1984a) also blocks the processing of pro-p21 to c-p21. This conversion event therefore precedes acylation and the CAAX motif may therefore be important for signaling modifications preceding acylation.

The fungal mating factors have a C-terminal methylated cysteine residue with a polyisoprenoid side chain attached to the thiol group via an ether linkage (Akada *et al*, 1987; Anderegg *et al*, 1988; Ishibashi *et al*, 1984; Sakagami *et al*, 1979). The nuclear lamins were also shown to be isoprenylated (Wolda and Glomset, 1988).

Research to further investigate the sequence requirements of Ras palmitoylation lead to the discovery that Cys186 was not the palmitoylation site of H-ras but is in fact an isoprenylation site (Hancock *et al*, 1989). All Ras proteins are isoprenylated at position 186 by the addition of a farnesyl or $C_{15}$ moiety (Casey *et al*, 1989). The farnesyl ($C_{15}$) is derived from the mevalonate pathway which produces cholesterol (Goldstein and Brown, 1990) **(Figure 1.8)**. Inhibition of mevalonate biosynthesis by the use of drugs (compactin and lovastatin/Mevinolin) completely abolishes Ras prenylation (Goldstein and Brown, 1990).

All other Ras modifications are blocked by inhibition of isoprenylation suggesting that farnesylation is required for all subsequent CAAX processing steps (Hancock *et al*, 1989; Jackson *et al*, 1990; Kim *et al*, 1990; Schafer *et al*, 1989). Palmitoylation of H-ras then occurs at cysteines

**Figure Legend 1.8.**

**The Mevalonate pathway.**

Mevalonate is the key intermediate in the biosynthesis of cholesterol and other isoprenoids. The rate limiting enzyme in the pathway is 3-hydroxy-3-methylglutaryl-CoA (HMG CoA) which can be inhibited by compactin and lovastatin. Inhibition of this enzyme therefore prevents all isoprenoid biosynthesis, blocks protein prenylation and cholesterol biosynthesis.

Mevalonate is converted to isopentenyl pyrophosphate (IPP). Sequential condensation with other IPP molecules leads to the formation of isoprenoids or prenoids. Geranyl, farnesyl and geranylgeranyl are isoprenoids commonly found attached to proteins, a process referred as prenylation or isoprenylation.

**Figure 1.8**

**The Mevalonate pathway**



HMG CoA

HMG CoA
reductase ——— Lovastatin, Compactin

**MEVALONATE**

**Mevalonate-PP**

**Isopentenyl-PP ($C_5$)** ⇌ **Dimethylallyl-PP**

**CHOLESTEROL**

**Geranyl-PP ($C_{10}$)**

**SQUALENE**

IPP

**Farnesyl-PP ($C_{15}$)**

**Farnesylated Proteins**

IPP

**Geranylgeranyl-PP ($C_{20}$)**

**Geranylgeranylated Proteins**
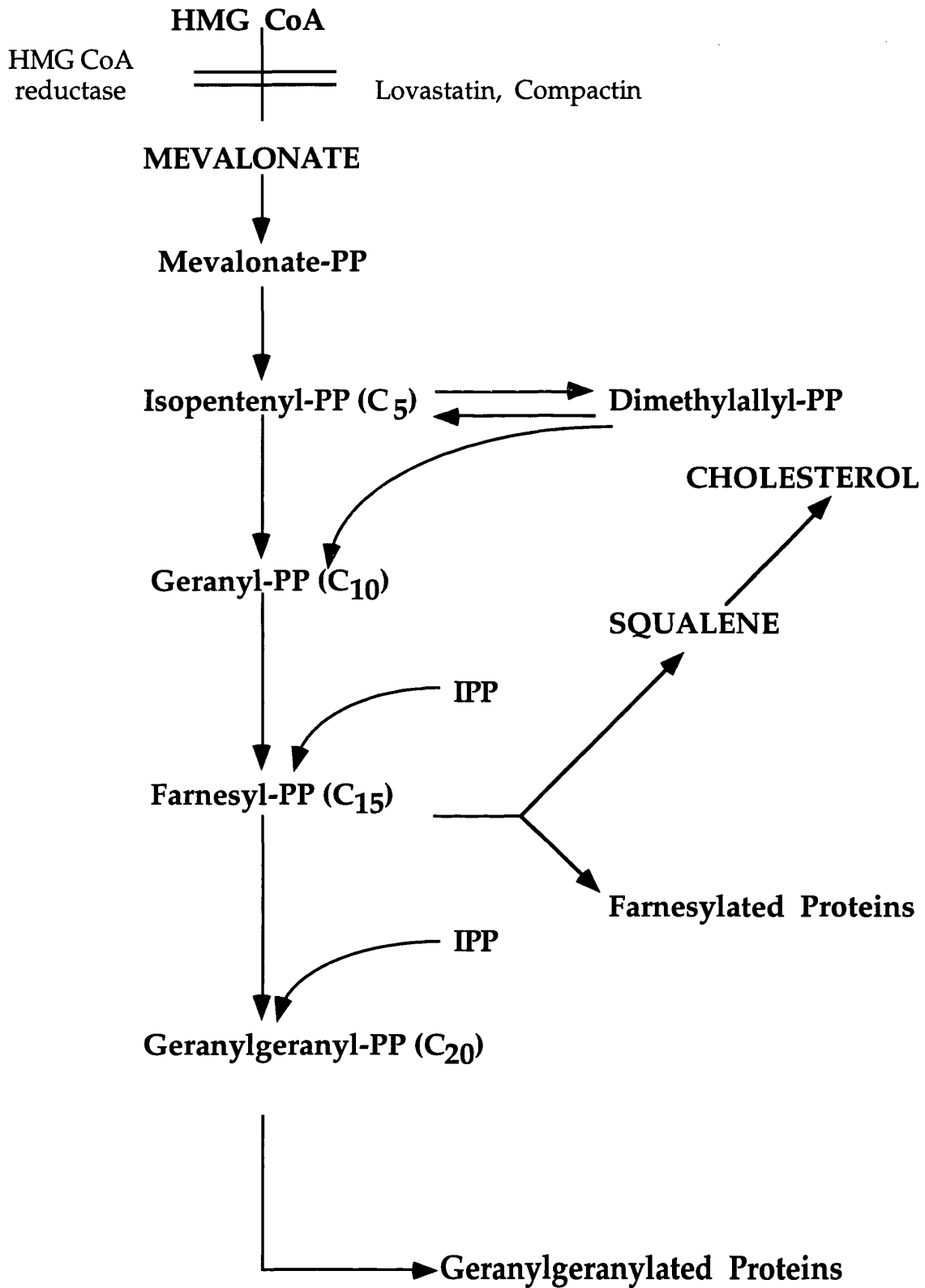
181 and 184. K-ras(B) has no palmitoylation sites at its C-terminus but contains a polybasic domain instead (Hancock *et al*, 1989).

The earlier observations of Willumsen and co-workers (1984a; 1984b) were vital discoveries to the field of Ras research since they recognised the importance of the CAAX motif and especially Cys186. Mutation of Cys186 to Ser blocks membrane association by preventing prenylation, leaving the protein cytosolic and inactive. Palmitoylation occurs after acylation leading to a membrane associated, biologically active Ras protein.

**1.5.1.2.The CAAX modifications all contribute to membrane localisation.**

The triplet of Ras post-translational modifications involving the CAAX motif have now been fully identified and characterised. First, a polyisoprenoid chain is added via a thioether bond to the cysteine of the CAAX motif (Casey *et al*, 1989; Hancock *et al*, 1989), second, proteolysis removes the -AAX amino acids (Gutierrez *et al*, 1989) and finally, the now C-terminal cysteine residue is carboxylmethylated (Clarke *et al*, 1988; Gutierrez *et al*, 1989). **(Figure 1.9).**

These CAAX modifications increase the hydrophobicity of the processed Ras protein so facilitating cell membrane association. *In vitro* studies with CAAX containing peptides show that farnesylation can take place in soluble cell extracts (Reiss *et al*, 1990; Schafer *et al*, 1990) and in rabbit reticulocyte lysates (Vorbuger *et al*, 1989).

The individual contributions of the CAAX modifications to membrane association have been examined *in vitro* using a rabbit reticulocyte lysate to translate and process K-ras(B) (Hancock *et al*, 1991a). In reticulocyte lysates, it was found that the only processing event that took place was prenylation. Prenylation was confirmed by supplementing translation reactions with [$^{14}$C] mevalonolactone. Incorporation of label was seen in all proteins with an intact CAAX motif but was absent in the C186S mutant protein. The addition of pancreatic microsomes was required to produce a fully processed K-ras protein which underwent proteolysis and methylation as well as prenylation. Methylation was inhibited by the use of methylthioadenosine and it was possible to examine the effect of the individual CAAX processing events on membrane association.

**Figure Legend 1.9.**

**Post-translational modifications of Ras.**

CAAX processing comprises farnesylation of the cysteine residue, removal of the -AAX amino acids and methylation of the $\alpha$ carboxyl of the now C-terminal cysteine. These modifications increase the hydrophobicity of the protein but not sufficiently to cause membrane association. This cytosolic intermediate c-p21 requires a second membrane targeting signal in order to become plasma membrane associated.

For H-ras, the addition of palmitic acid at cysteines 181 and 184 provides the second membrane targeting signal.

The second signal for the plasma membrane targeting of K-ras(B) is provided by a polybasic domain that consists of six consecutive lysine residues.

These second signals are contained within the hypervariable region of the protein.

**Figure 1.9**

**Post-translational processing of Ras**



PLASMA MEMBRANE

Palmitoyltransferase

c-p21
cytosolic
hydrophobic

pro-p21
cytosolic
hydrophilic

H-ras

K-ras(B)

OMe = methyl

P = palmitate

++++++ = polybasic domain
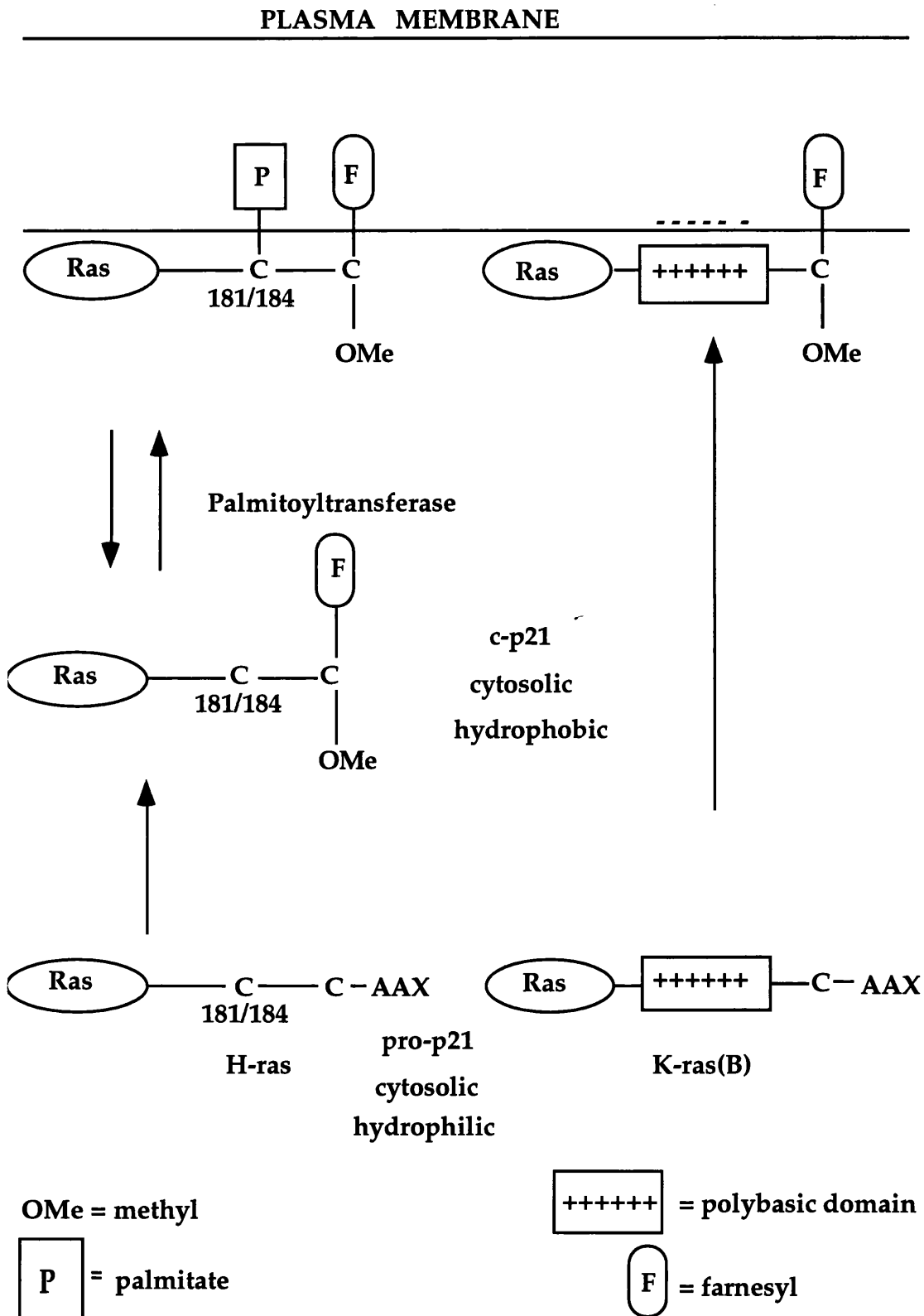
F = farnesyl

Farnesylation increases the hydrophobicity of the protein sufficiently to cause partitioning into the detergent phase of Triton X-114 but is insufficient for efficient membrane binding. Removal of the -AAX amino acids produced a 2-fold increment in the extent of membrane binding *in vitro* and subsequent methylation resulted in a further 2-fold increase in membrane association. All three CAAX modifications are therefore required for the efficient membrane binding of K-ras(B).

The -AAX amino acids are presumably removed to prevent steric hindrance with the farnesyl/membrane interaction. Methylation of the carboxyl group of the cysteine residue probably occurs to neutralise the negative charge on the ionised carboxyl group which could cause repulsion from the negatively charged head groups of membrane phospholipids (Hancock *et al*, 1991a).

This work (Hancock *et al*, 1991a) also gave an insight into the location of the Ras processing enzymes. The enzyme responsible for Ras prenylation is cytosolic since prenylation is observed in lysates without the addition of membranes. The enzymes responsible for the proteolysis and methylation of Ras are membrane associated since the addition of pancreatic microsomes was required for these processes to occur.

### 1.5.1.3. The CAAX modifications do not lead to plasma membrane association.

The post-translational modifications of the CAAX motif whilst increasing the hydrophobicity of Ras are insufficient to target the protein to the plasma membrane *in vivo*. Palmitoylation of Ras was identified several years before the CAAX processing events were known (Chen *et al*, 1985; Sefton *et al*, 1982). C-terminal deletions upstream of the CAAX motif were found to reduce or abolish palmitoylation without affecting step 1 processing (Hancock *et al*, 1989). This suggested that cysteine residues other than Cys186 were the palmitoylation sites of H-ras. Further experiments identified that H-ras was palmitoylated at two sites - cysteine 181 and cysteine 184 (Hancock *et al*, 1989) **(Figure 1.9)**. N-ras and K-ras(A) have also been found to undergo palmitoylation at cysteines N-terminal to the CAAX motif. The site of palmitoylation of N-Ras is cysteine 181 and in K-Ras(A), cysteine 180. Palmitic acid is a saturated $C_{16}$ fatty acid which is attached as a thioesterto cysteine residues. The addition of palmitate can only occur after the CAAX modifications have taken place.

Further examination of H-ras palmitoylation mutants (Hancock *et al*, 1990) led to the discovery that nonpalmitoylated H-ras was predominantly cytosolic with reduced avidity of membrane binding. The modifications of the CAAX motif are insufficient to direct plasma membrane localisation and a combination of the post-translational modifications at Cys186 and palmitoylation at Cys181 and Cys184 is required.

K-ras(B) undergoes step 1 processing of the CAAX motif but has no cysteine residues in the hypervariable region (HVR) which could be palmitoylated (Hancock *et al*, 1989). The HVR of K-ras(B) contains a polybasic domain of six consecutive lysine residues (amino acids 175-180) which were investigated for a role in membrane association. Mutations made to replace the lysines with uncharged glutamine residues identified the polybasic domain as the second membrane targeting signal of K-ras(B) (Hancock *et al*, 1990) **(Figure 1.9)**. Progressive replacement of the lysines with glutamines led to an increase in the amount of cytosolic protein.

The polybasic domain of K-ras(B) and palmitoylation of H-ras act in combination with the CAAX motif modifications to target Ras to the plasma membrane **(Figure 1.9)**. Disruption of either signal results in the protein remaining cytosolic.

H-ras and Ras-related proteins are not the only proteins to be palmitoylated. Numerous other proteins including viral glycoproteins, cytoskeletal proteins, cell surface receptors and rhodopsin are all post-translationally modified by palmitate to become membrane associated. It has recently been shown that several myristoylated proteins are also N-terminally palmitoylated. The myristoylated α subunits of heterotrimeric G proteins are palmitoylated at Cys3 (Linder *et al*, 1993; Mumby *et al*, 1994; Parenti *et al*, 1993; Wedegaertner *et al*, 1993) so the consensus sequence for palmitoylation is myr-Gly-Cys-. This sequence is found in 7 of the 9 Src family members (Resh, 1994). The palmitoylation of these proteins as well as increasing the efficiency of membrane binding (Mumby *et al*, 1994; Wedegaertner *et al*, 1993), may influence cellular processes such as endocytosis and receptor coupling. Regulation of acylation is suggested by results that show increased palmitoylation of G α subunits upon agonist stimulation (Mumby *et al*, 1994).

Polybasic domains are also found in other Ras-related proteins such as Rap1A, Rap1B, RhoA, RhoC and Ral. The polybasic domain functions as a nuclear localisation signal in SV40 large T (Kalderon *et al*, 1984), the

nuclear lamins (Loewinger and McKeon, 1988) and c-*abl* (Van Etten *et al*, 1989).

**1.5.1.4.Importance of the second signal for Ras biological activity**

When the palmitoylation sites of H-ras (Cys181 and 184) are mutated to serine residues, the protein is cytosolic but is still transforming although with only a third of the activity of fully processed H-ras (Hancock *et al*, 1990). The Cys186--->Ser mutation on the other hand produces a cytosolic protein but one that is completely inactive in transformation assays. This indicates that the CAAX motif modifications are required for transformation rather than membrane association (Casey *et al*, 1989; Hancock *et al*, 1989; 1990; Jackson *et al*, 1990). Since palmitoylation is not required for transformation, the function of palmitoylation is to increase the avidity of membrane association and through this, enhance transformation.

Similar results were found with K-ras (Hancock *et al*, 1990) in that mutation of Cys186 destroys biological activity even in the presence of an intact polybasic domain. The transforming activity of the protein is reduced when the polybasic domain is mutated and Cys186 is intact. The polybasic domain is therefore not required for transforming activity by oncogenically activated K-ras.

The palmitoylation sites or the polybasic domain are not required for transformation of oncogenically activated proteins, whilst Cys186 is absolutely required. The post-translational modifications of the CAAX motif are essential for transforming activity but the second signals, which lead to plasma membrane association, are not.

The importance of the other CAAX processing events to biological activity was shown by the loss of function exhibited by a chemically demethylated form of the yeast mating factor-a (Anderegg *et al*, 1989). Yeast mutants, ste14⁻, defective for carboxyl methyltransferase activity (Hrycyna and Clarke, 1990) secrete a less hydrophobic, farnesylated, nonmethylated, biologically inactive a-factor (Marr *et al*, 1990). In contrast, yeast RAS function does not require methylation of RAS1 or RAS2 (Hrycyna *et al*, 1991). Farnesylated K-ras that is not proteolysed or methylated also shows transforming potency (Kato *et al*, 1992).

The results of earlier work (Willumsen *et al*, 1984a; 1984b) that proposed that membrane association of Ras was essential for

transformation are now challenged. The original conclusions were formed before the complete story concerning Ras processing events was established and are no longer strictly accurate. The absolute requirement for Cys186 does hold true but not because this residue is palmitoylated but due to its prenylation.

## 1.5.1.5. Reversible processing events and protein cycling

Palmitoylation of H-ras is a dynamic process with the half life of the palmitate being 20 minutes compared with the protein half life of about 24 hours (Magee et al, 1987). Fatty acid moieties have also been shown to turnover on the transferrin receptor (Omary and Trowbridge, 1981) and ankyrin (Staufenbiel, 1987). More recently several myristoylated proteins have also been shown to be reversibly palmitoylated. This group of proteins includes the protein tyrosine kinase p56[lck] (Paige et al, 1993) and G-protein α subunits (Linder et al, 1993; Parenti et al, 1993; Wedegaertner et al, 1993). H-ras, N-ras and K-ras(A) are therefore potentially in dynamic equilibrium between the cytoplasm and the plasma membrane since palmitoylated Ras is membrane localised whilst non-palmitoylated H-ras is predominantly cytosolic.

The turnover of palmitate on the myristoylated Src proteins may account for the ability of these proteins to interact with multiple membrane-bound targets and to participate in several signal transduction pathways. The importance of dynamic palmitoylation was shown with GAP43 where palmitoylation regulates interaction with $G_O$ and therefore nucleotide-exchange activity (Sudo et al, 1992).

By contrast, the K-ras(B) protein might be in permanent contact with the plasma membrane since it is not palmitoylated and relies on electrostatic interaction between the polybasic domain and the negatively charged phospholipid head groups of the membrane to achieve plasma membrane localisation. However the avidity of K-ras membrane binding is much lower than that of H-ras (Hancock et al, 1989) and this may enable the protein to dissociate from the plasma membrane.

Other members of the Ras superfamily also contain polybasic domains. It has been shown that phosphorylation of Rap1B results in its dissociation from membranes to become cytosolic (Lapetina et al, 1989). The phosphorylation site, Ser179, is within the Rap1B polybasic domain. K-ras is phosphorylated at Ser181 again within the polybasic domain (Hancock and Marshall, unpublished data). However only a small proportion of K-

ras is phosphorylated (Ballester *et al*, 1987). The positive charges of the polybasic domain could be neutralised by phosphorylation and lead to reversible membrane association of K-ras.

It is therefore possible that the different Ras proteins have different target sites and functions within the cell as they have different targeting signals (palmitoylation or a polybasic domain) that lead to a temporary or permanent association with the plasma membrane.

### 1.5.1.6.Geranylgeranylation

The first post-translational event to take place at the C-terminus of Ras is the addition of a farnesyl (or $C_{15}$ group) to the cysteine of the CAAX motif via a thioester bond. Other CAAX containing Ras proteins are prenylated by a geranylgeranyl ($C_{20}$) moiety. Prenylation of proteins with $C_{20}$ geranylgeranyl is 10 times more common than farnesylation (Epstein *et al*, 1990). Geranylgeranyl pyrophosphate ($C_{20}$) is formed by the condensation of farnesylpyrophosphate with isopentenyl pyrophosphate (Goldstein and Brown, 1990). **(Figure 1.8).**

This group of $C_{20}$ modified proteins includes Krev 1/Rap1A (Buss *et al*, 1991; Kawata *et al*, 1990), G25K/CDC42 (Maltese and Sheridan, 1990), RhoA (Hori *et al*, 1984) and the $\gamma$ subunit of brain G-proteins (Mumby *et al*, 1990; Yamane *et al*, 1990). These proteins terminate in a CAAX motif where the X amino acid is generally a leucine or phenylalanine residue (Finegold *et al*, 1991; Seabra *et al*, 1991) whereas for farnesylated proteins it is alanine, serine or methionine. Once geranylgeranylation has occurred, the three C-terminal amino acids are removed and the C-terminal cysteine is then $\alpha$ carboxylmethyl esterified. The Rho proteins are membrane associated by co-operation between the geranylgeranyl group and a polybasic region.

The Rab proteins do not have a conventional CAAX motif and terminate in closely spaced cysteine residues (Peter *et al*, 1992). Rab1, Rab2, Rab9 and Rab10 have a cysteine doublet at the C-terminus (-CC), whereas Rab3, Rab4, Rab6 and Rab7 have a CXC C-terminal motif. Rab5 contains a CCXX motif and Rab11 a CCXXX motif. Rab3A, with a CXC motif, is geranylgeranylated on both cysteines and is also carboxylmethylated (Farnsworth *et al*, 1991a; Newman *et al*, 1992). The CC motif of YPT5 (*S.pombe*) has both cysteines geranylgeranylated and methylated but the C-terminal cysteine of YPT1 and YPT3 does not undergo carboxylmethylation and presumably is not geranylgeranylated (Newman

*et al*, 1992; Wei *et al*, 1992). There have also been reports that CC motifs can be palmitoylated. This has been shown for YPT1 of *S. cerevisiae* (Molenaar *et al*, 1988) and for *S. pombe* YPT3 but not for *S. pombe* YPT1 (Newman *et al*, 1992).

The prenylation of a protein by geranylgeranyl or farnesyl may account for different functional roles of the Ras superfamily proteins. Geranylgeranyl and farnesyl are both branched charge acyl groups and may not readily insert into the lipid bilayer unlike palmitate. Geranylgeranylated proteins have been found to be more avidly bound to membranes (Hancock *et al*, 1991b) possibly due to the more hydrophobic nature of geranylgeranyl compared to farnesyl or palmitate (Black, 1992). The addition of farnesyl or geranylgeranyl together with or without palmitoylation may allow different functions by virtue of the differences in strength of membrane association. There may also be specific membrane receptors that recognise farnesyl or geranylgeranyl groups.

Prenylation only accounts for part of the membrane localisation signal and does not account for the ability of proteins with the same isoprenoid group to localise to different membranes. The Rab proteins are localised to discrete intracellular compartments within the secretory and endocytotic pathways. For example Rab1 is localised to the endoplasmic reticulum (Plutner *et al*, 1991) and Rab3A is localised to clear synaptic vesicles (Fischer *et al*, 1990), both components of the exocytic pathway.

### 1.5.1.7.Prenylation is vital for Ras protein interactions

Several geranylgeranylated proteins have been shown to be found in membrane associated and soluble forms. The soluble forms form stable complexes with specific binding proteins. Prenylated, GDP bound Rab3A forms a soluble complex with a GDI (guanine nucleotide dissociation inhibitor). This RabGDI was isolated from bovine brain (Matsui *et al*, 1990) and inhibits the GDP/GTP exchange reaction and the binding of Rab3A-GDP to membranes (Araki *et al*, 1991). RabGDI has some sequence similarity to the product of the human choroideremia gene (hCGM) (Fodor *et al*, 1991). The choroidereamia gene product, p95CHM, is a subunit of the Rab geranylgeranyltransferase (RabGGTase) and has not been shown to have GDI activity. Homologues of RabGDI have been found in *Drosophila* (Zahner *et al*, 1992), *C. elegans* (Waterston *et al*, 1992) and *S. cerevisiae* (Waldherr *et al*, 1992).

The association between Rab3A and RabGDI removes the protein from membranes (Fischer von Molland *et al*, 1991) and without interaction with proteins such as GDIs, it is hard to imagine how proteins with two geranylgeranyl groups can cycle on and off membranes.

A GDI for Rho and Rac proteins has been identified in bovine brain (Fukumoto *et al*, 1990), guinea pig (Abo *et al*, 1991), nematode (Waterston *et al*, 1992) and in humans (Mulheron *et al*, 1992). RhoGDI dissociates GDP from post-translationally modified Rho proteins (Isomura *et al*, 1991; Ohga *et al*, 1989). It also removes Rho from membranes in cell-free systems and could therefore regulate the amount of membrane associated Rho or facilitate the movement of Rho between membrane compartments. The translocation of p47 and p67, components of the neutrophil superoxide generating system, from the cytosol to the membrane involves release of Rac from the Rac/RhoGDI complex (Abo *et al*, 1991).

The transfer of Rap1B/smgp21B to the cytosol involves interaction with smgGDS (guanine nucleotide dissociation stimulator). Smg(small GTP-binding proteins)GDS promotes GDP/GTP exchange on Rap, K-ras, Rho and Rac proteins (Hiraoka *et al*, 1992; Kaibuchi *et al*, 1991; Yamamoto *et al*, 1990) and only functions on prenylated proteins (Mizuno *et al*, 1991).

A GDS for Rho and Rac has also been identified - Rho/RacGEF (guanine nucleotide exchange factor). In yeast defects in the CDC42 gene can be overcome by overexpression of the CDC24 gene (Drubin, 1991) suggesting that CDC24 activates CDC42. The mammalian homologue, Cdc42Hs and Rho interact with Dbl (Hart *et al*, 1991) which also binds several Rac/Rho-like proteins (Rho, RhoC, Rac1, CDC42Mm) *in vitro* (Miki *et al*, 1993).

GDSs and GDIs also affect the nucleotide state of their target proteins. GDIs were originally thought to bind to GDP associated proteins and prevent GDP release keeping the protein in an inactive state however it has recently been shown that RhoGDI also binds to Ras in the GTP form (Hancock and Hall, 1993). RhoGDI inhibits GAP stimulated GTP hydrolysis and the intrinsic GTPase rate. GDSs bind both GDP and GTP forms and stimulate nucleotide release leading to increased levels of the GTP/active form of the protein. A model has been proposed whereby the cycling of proteins on and off membranes involves prenylated proteins being recognised by a GDS, the protein binds GTP and becomes membrane targeted **(Figure 1.10)**. GTPase action removes the GTP and the

**Figure Legend 1.10.**

**A model for membrane cycling of prenylated proteins.**

The post-translationally modified GDP bound protein becomes GTP bound and membrane associated after interaction with GDS. Hydrolysis of the GTP is stimulated by GAP, the protein binds to GDI and returns to the cytosol.

**Figure 1.10**

**A model for membrane cycling of prenylated proteins**
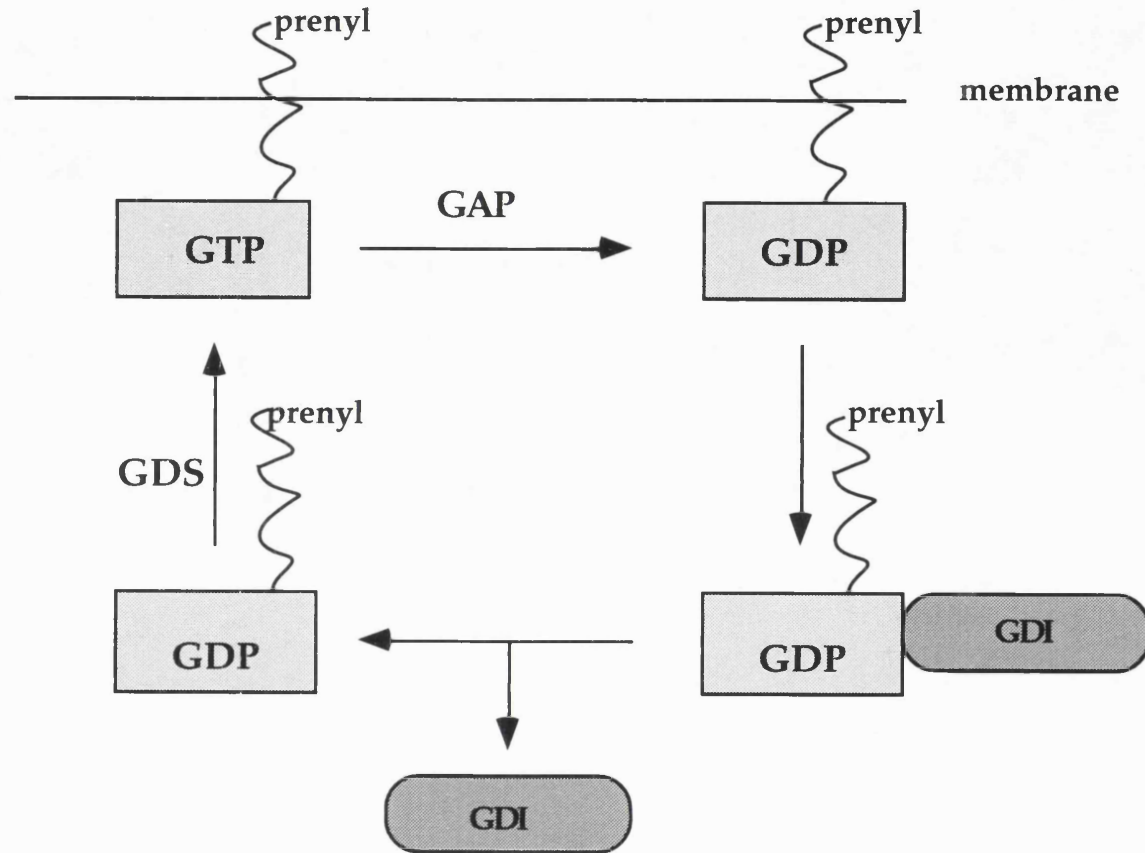
now GDP bound protein complexes with a GDI to become cytosolic.

One controlling point for these protein interactions would be the GTPase step and another may involve phosphorylation. The ability of smgp21B/Rap1B to interact with smgGDS is increased by phosphorylation and results in release of smgp21B/Rap1B from membranes and increased nucleotide exchange (Hata *et al*, 1991; Miura *et al*, 1992). Rab1A and Rab4 have also been shown to be phosphorylated and may be regulated in a similar manner (Bailly *et al*, 1991).

To date a GDI specific for Ras has not been identified however since GDIs have been identified for members of the Ras superfamily it is extremely likely that one or more exist and will be discovered in the near future.

### 1.5.2. Ras processing enzymes

The enzymes responsible for Ras processing **(Figure 1.11)** are currently being identified, characterised and purified by several research groups. The inhibition of Ras processing by enzyme inhibition may be of great therapeutic interest especially as blocking the CAAX motif modifications renders the protein inactive.

### 1.5.2.1.Prenyltransferases

The prenylation of Ras and other members of the Ras superfamily is essential for their cellular localisation and function. Prenylation is due to the attachment of a farnesyl ($C_{15}$) or geranylgeranyl ($C_{20}$) group to the C-terminus of the protein. These reactions are catalysed by prenyltransferases that differ in both their protein target specificity and their isoprenoid substrate.

### 1.5.2.1.1.Farnesyl protein transferase (FTase)

The yeast RAM1 gene was identified by its involvement in **a**-factor production (Michaelis and Herskowitz, 1988; Powers et al, 1986; Wilson and Herskowitz, 1987) and as a suppressor of G protein function (Nakayama et al, 1988). ram1⁻ extracts are defective in **a**-factor and farnesyltransferase activity (Goodman et al, 1990; Kohl et al, 1991; Moores et al, 1991; Schafer et al, 1990). Farnesyltransferase activity can be restored to a ram1⁻ extract by E. coli produced RAM1 (Schafer et al, 1990) even though it lacks enzyme activity on its own. This showed that the RAM1 gene product was a component of FTase but other components were required. Studies with

**Figure Legend 1.11.**

**The Ras processing enzymes.**

The CAAX motif of Ras undergoes a series of post-translational modifications. Ras is prenylated at the cysteine of the CAAX motif by farnesyltransferase. An endopeptidase removes the -AAX amino acids whilst a methyltransferase methylates the $\alpha$ carboxyl group of the now C-terminal cysteine residue. All of the CAAX modifications are irreversible.

H-ras, N-ras and K-ras(A) are palmitoylated at upstream cysteine residues. This reaction is catalysed by a palmitoyltransferase and is a reversible process.

Figure 1.11
The Ras processing enzymes
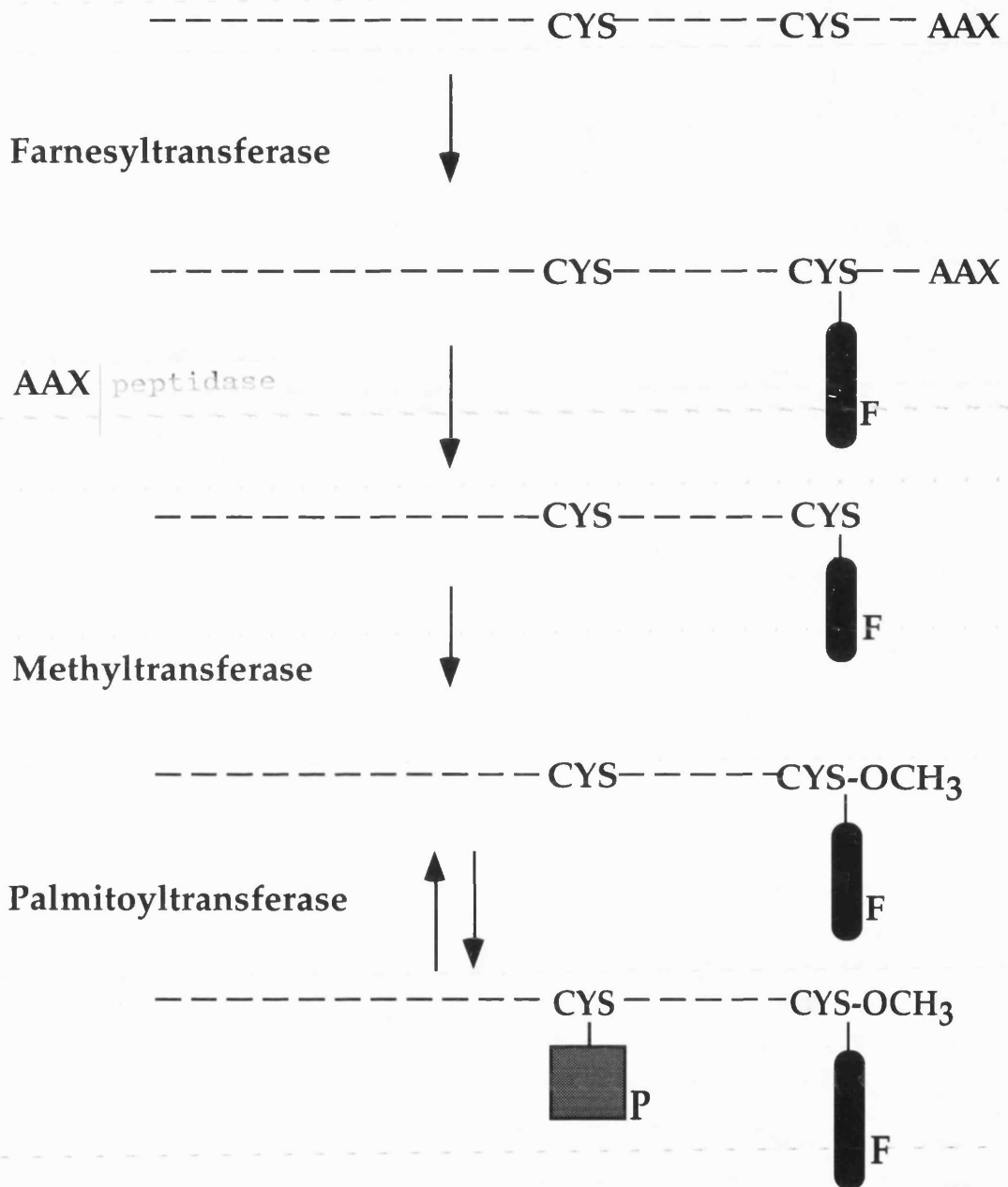


F = Farnesyl

P = Palmitate

lamin B2 showed that prenylation could occur in rabbit reticulocyte lysates (Vorburger *et al*, 1989) and led to the identification of a FTase in the cytosol of bovine brain cells (Schaber *et al*, 1990).

FTase is widely distributed and has been found in all eukaryotic cells investigated (Manne *et al*, 1990). It has also been isolated from human placental tissue (Ray and Lopez-Belmonte, 1992). Farnesyl-protein transferase (FTase) is cytosolic, has a molecular mass of approximately 110kDa and exists as a dimer *in vivo*. The protein consists of an $\alpha$ and $\beta_1$ subunit. **(Figure 1.12).** The $\alpha$ subunit has a molecular weight of 49kDa (Reiss *et al*, 1991a) and the $\beta_1$ subunit has a mass of 46kDa (Chen *et al*, 1991; Reiss *et al*, 1991a). The enzyme requires the presence of divalent cations such as $Mg^{2+}$, $Mn^{2+}$ and $Zn^{2+}$ (Manne *et al*, 1990; Reiss *et al*, 1990; Schaber *et al*, 1990). The $\alpha$ and $\beta$ subunits are tightly associated and can only be separated by denaturation (Reiss *et al*, 1991a). The enzyme utilises farnesyl pyrophosphate (FPP) as the prenyl donor to farnesylate the cysteine residue of the CAAX motif. FPP is an intermediate in the biosynthesis of squalene and isoprenic acid (Goldstein and Brown, 1990). **(Figure 1.8).** The enzyme binds and farnesylates peptides as short as 4 amino acids in length provided that they terminate in a CAAX motif (Moores *et al*, 1991; Reiss *et al*, 1990; Reiss *et al*, 1991b).

The sequence of the $\beta$ subunit of rat brain farnesyltransferase deduced from the cloned cDNA sequence (Chen *et al*, 1991) showed 37% homology to RAM1 (Chen *et al*, 1991; Goodman *et al*, 1988). Transfection of cells with either the $\alpha$ or $\beta$ subunit DNA did not produce FTase activity. Both subunits were required for FTase activity (Chen *et al*, 1991).

Mutation of RAM2, a suppressor of RAS2Val19, leads to defective FTase activity (Goodman *et al*, 1990; Moores *et al*, 1991). RAM2 encodes a protein of 38kDa which when expressed together with RAM1 efficiently farnesylates a-factor peptide and Ras protein substrates (He *et al*, 1991). The yeast FTase is therefore composed of Ram1 and Ram2 polypeptides. ram1⁻ mutations are not lethal whereas ram2⁻ mutations are, suggesting that Ram2 has an essential function in addition to protein farnesylation. It has been shown that CAAX peptides that are efficiently farnesylated, can also act as less efficient substrates for the geranylgeranyl transferase (GGTase I). Ram2 is homologous to the $\alpha$ subunit of mammalian FTase and also forms the $\alpha$ subunit of GGTase I (Kohl *et al*, 1991; Seabra *et al*, 1991). ram2⁻ mutants are nonviable as a result of being unable to farnesylate or geranylgeranylate proteins. Other evidence that the $\alpha$ subunits of FTase and

**Figure Legend 1.12.**

**A comparison of the mammalian and yeast prenyltransferase subunits and their preferred substrates.**

Farnesyltransferase (FTase) consists of an α and β subunit and recognises a Cys-A-A-Ser/Met C-terminal motif. Geranylgeranyltransferase 1 (GGTase 1) recognises a Cys-A-A-Leu C-terminal motif and consists of an α subunit identical to that of FTase and a different β subunit. The RabGGTase is specific for Rab proteins and consists of unique α and β subunits together with the Rab Escort Protein (REP), a homologue of the choroideremia gene (CHM) product.

**Figure 1.12**

A comparison of the mamalian and yeast prenyltransferase subunits and their preferred substrates



|  | **FTase** | | **GGTase1** | | **REP** | **RabGGTase**<br>**GGTase** | |
|---|---|---|---|---|---|---|---|
| Yeast<br>RAM2 | α1 | Yeast<br>RAM2 | α1 | human<br>CHM | α2 | Yeast<br>MAD2 |
| Yeast<br>RAM1 | β1 | CDC43 | β2 | | β3 | BET2 |

|  | | | |
|---|---|---|---|
| C-terminal<br>motif<br>recognised | Cys-A-A-Ser / Met | Cys-A-A-Leu | Rab-Cys-X-Cys  or  Rab-Cys-Cys |
| Preferred<br>substrates | Ras<br>nuclear lamins<br>transducin γ<br>rhodopsin kinase | Rho<br>Rac<br>G proteins  γ | Rab  proteins |

GGTase I are identical is the ability of this subunit to bind both FPP and geranylgeranyl pyrophosphate (GGPP). The enzymes must contain different β subunits since FTase cannot transfer GGPP to its substrate which is recognised by the β subunit (Reiss *et al*, 1992).

The function of the β subunit, peptide recognition, was determined by chemical cross-linking studies (Reiss *et al*, 1991a). The α subunit may form complexes with FPP since geranylgeranyl pyrophosphate competes with FPP for binding to the FTase but is not transferred to the peptide receptor (Reiss *et al*, 1992).

The subunits both contain 5 repeats of a weakly conserved sequence that is unique to each subunit but is conserved in the yeast and animal homologues (Boguski *et al*, 1992b). In the α subunit, the repeat contains glutamine, asparagine, tryptophan and arginine residues in a highly conserved pattern known as ENWR repeats (Feng and Kung, 1993). Mutation of Lys164, which is conserved in all 4 of the known species (rat, human, bovine and yeast), permitted normal binding of FPP and H-ras and formed a stable complex with the β subunit. The rate of transfer of FPP to H-ras was reduced suggesting that the α subunit plays a catalytic role as well as stabilising the β subunit (Andres *et al*, 1993).

The binding of Ras to the β subunit requires $Zn^{2+}$ whilst binding of the prenyl pyrophosphate appears to be independent of divalent cations but $Mg^{2+}$ is required for the transfer of the prenyl group to the peptide receptor (Reiss *et al*, 1992).

## 1.5.2.1.2. Geranylgeranyl transferase (GGTase), type I

Genetic and biochemical work in yeast showed that ram2⁻ mutants were deficient in FTase and GGTase I activity suggesting that these enzymes share a common subunit (Moores *et al*, 1991). GGTase I was isolated and separated from FTase by ion exchange chromatography (Seabra *et al*, 1991; Yoshida *et al*, 1991). Immunological data showed that the FTase and the GGTase I contain identical α subunits since both enzymes cross-reacted with antibodies directed against the α subunit of FTase. The GGTase I did not react with an antibody directed against the β subunit of FTase. Since both enzymes show the same molecular weight by gel filtration (approximately 100,000), it was proposed that GGTase I was a heterodimer with an α subunit identical to that of FTase but with a different β subunit (Seabra *et al*, 1991).

The yeast α subunit of GGTase is encoded by the RAM2 gene (Moores *et al*, 1991). Mutation of the CDC43 gene leads to loss of GGTase I activity and cell cycle defects (Schafer and Rine, 1992). This gene shows homology to RAM1 and codes for the β subunit of yeast GGTase I (Finegold *et al*, 1991; Kohl *et al*, 1991). The cDNA inferred molecular mass of the yeast β subunit is 41.7kDa (Ohya *et al*, 1991) which is similar to the size of the mammalian β subunit ( Yokoyama and Gelb, 1993).

The GGTase I recognises substrates that terminate in a CAAX motif where the X amino acid is leucine or phenylalanine. The GGTase I does not appear to require metal cations for function (Yokoyama and Gelb, 1993).

### 1.5.2.1.3.Geranylgeranyl transferase, (GGTase), type II, RabGGTase.

Other geranylgeranylated proteins do not possess the normal CAAX motif at their C-terminus but have CXC (Rab3A) or CC (Rab1A) sequences (Farnsworth *et al*, 1991a; Khosravi-Far *et al*, 1991; Kinsella and Maltese, 1992). A RabGGTase was isolated from rat brain (Seabra *et al*, 1992a; 1992b) and cloned (Armstrong *et al*, 1993).

RabGGTase consists of a catalytic component, component B, which is a dimer of tightly associated α and β subunits with molecular masses of 60 and 38kDa respectively. Component B requires an accessory protein, component A, which has a molecular mass of 95kDa (Seabra *et al*, 1992a; 1992b) **(Figure 1.12)**.

The function of component A, binding to the Rab product, was identified by sequence analysis which revealed homology to the human CHM protein. This protein is defective in choroideremia, an X-linked retinal degenerative disease (Cremers *et al*, 1992). The sequence of CHM is related to that of smgGDI/Rab3AGDI (Fodor *et al*, 1991). Rab3AGDI forms a soluble complex with prenylated Rab3A and inhibits nucleotide exchange (Araki *et al*, 1990; Matsui *et al*, 1990; Sasaki *et al*, 1990). Since component A shows homology to a protein that binds Rab3A, its function is presumably to bind the Rab substrate and present to the catalytic component B. Component A is now known as REP - Rab Escort Protein whilst component B is known as RabGGTase. The RabGGTase does not recognise COOH-terminal peptides corresponding to its target proteins (Khosravi-Far *et al*, 1992; Seabra *et al*, 1992a). It appears that recognition involves sequences upstream of the C-terminus which correlates with the existence of a REP protein.

Mutations in the CHM gene leading to deficient RabGGTase activity can be rescued by addition of rat REP-1 (Seabra *et al*, 1993). This provides more evidence that the mammalian REP is CHM. A CHML (choroideremia-like) gene on chromosome 1 (Cremers *et al*, 1992) can geranylgeranylate most Rab proteins through its product REP-2 (Cremers *et al*, 1994). REP-2 can substitute for REP-1 in all tissues apart from the retina which may explain why REP-1 deletions only lead to retinal degradation despite Rab proteins being involved in numerous secretion and exocytotic pathways. REP-1 and REP-2 may also recognise different Rab proteins.

REP remains bound to Rab after prenylation and this may be important for proteins that are modified by two geranylgeranyl moieties (Andres *et al*, 1993).

Yeast genetics and sequence homology led to the identification of the components of RabGGTase. MAD2 is involved in feedback control of mitosis in budding yeast (Li and Murray, 1991). This gene has significant homology to the α subunits of other prenyltransferases (Boguski *et al*, 1992b) and forms the α subunit of yeast RabGGTase (Jiang *et al*, 1993). Mutation of MAD2 leads to two different phenotypes. The mad2-1 mutation does not affect geranylgeranylation (Li, R. *et al*, 1993) but fails to arrest mitosis when microtubular polymerisation is slowed by inhibitors (Li and Murray, 1991). The mad2-2$^{ts}$ mutation disrupts geranylgeranylation of Ypt1 and Sec4 at the nonpermissive temperature. The mutants are also defective for secretion but mitosis is arrested normally (Li, R. *et al*, 1993).

The mad2-1 mutation may disrupt the α subunit so that it can geranylgeranylate Ypt1 or Sec4 but cannot geranylgeranylate another Rab protein that regulates mitotic arrest. The role of MAD2 in mitotic arrest may also be unrelated to prenylation. MAD2 may also form the α subunit of more than one RabGGTase in a similar manner to RAM2 which can interact with RAM1 of FTase and CDC43 of GGTase (Kohl *et al*, 1991; Seabra *et al*, 1991). This would account for the differences observed in the geranylgeranylation abilities of the two MAD2 mutations (Li, R. *et al*, 1993). Alternatively the MAD2 mutation affects interaction with only one of the other REP proteins accounting for the effects on different subunits of small G-proteins. The differences also suggest that MAD2 has distinct roles in protein transport and mitotic feedback control.

Another yeast gene BET2 shows homology to RAM1 and CDC43, the β subunits of FTase and GGTase I respectively, suggesting that it is the β subunit of RabGGTase (Rossi *et al*, 1991). BET2 and the β subunit

of mammalian RabGGTase are 52% identical (Armstrong *et al*, 1993). BET2 geranylgeranylates Ypt1, a protein involved in transport from the endoplasmic reticulum to the Golgi (Bacon *et al*, 1989; Baker *et al*, 1990; Segev *et al*, 1988). Bet2 mutants are unable to geranylgeranylate Ypt1 or Sec4. BET2 and MAD2 form a heterodimeric complex similar to RabGGTase (Jiang *et al*, 1993) **(Figure 1.12).**

### 1.5.2.2.The Ras-AAX protease

Farnesylated and geranylgeranylated proteins with CAAX motifs are further modified by the removal of the three carboxyl-terminal amino acids. A protease for Ras has been identified in rat liver cell membranes (Ashby *et al*, 1992), calf liver microsomes (Ma and Rando, 1992) and canine microsomes (Hancock *et al*, 1991). Proteolyis therefore occurs at a membrane organelle not the plasma membrane. The enzyme is an endopeptidase (Ashby *et al*, 1992) that recognises prenylated substrates. Specific cleavage of a farnesylated tetrapeptide between the isoprenylated cysteine residue and the adjacent residue has been shown (Ma *et al*, 1992). Mutation of the $A_2$ amino acid of the $CA_1A_2X$ motif prevents proteolysis suggesting this is the recognition site for the Ras protease (Kato *et al*, 1992). A membrane associated carboxyl-terminal protease activity has also been identified in yeast (Ashby *et al*, 1992; Hrycyna and Clarke, 1992). This is an endopeptidase which again only recognises prenylated proteins. Geranylgeranylated peptides are proteolysed by a membrane associated protease which may be different to the farnesyl-dependent membrane protease (Ashby, *et al*, 1992).

Two other proteases have been identified in yeast that are soluble. One is a progressive exopeptidase that has a molecular weight of about 110,000 and catalyses the removal of free amino acids from farnesylated a-factor (Ashby *et al*, 1992; Hrycyna and Clarke, 1992). This soluble activity has been purified and represents a novel metalloendopeptidase that preferentially cleaves substrates on the C-terminal side of hydrophobic amino acid residues (Hrycyna and Clarke, 1993). The enzyme appears to be a single polypeptide that is encoded by the YCL57*w* gene which was previously identified as an open reading frame on chromosome III in *S. cerevisiae* (Oliver *et al*, 1992). It can also remove the farnesylated cysteine if it is not carboxylmethylated and may therefore be irrelevant to the *in vivo* processing of prenylated proteins. The other soluble activity was shown to be the vacuolar protease carboxypeptidase Y (Hrycyna and Clarke, 1992).

Another membrane associated protease catalyses the removal of a prenylated oligopeptide from farnesylated substrates such as lamin A, a-factor and M-mating pheromone of *S. pombe* (Beck *et al*, 1990; Schafer *et al*, 1989). It is speculated that this protease may be encoded by STE19 (Schafer and Rine, 1992).

### 1.5.2.3.Carboxyl-methyltransferase

In yeast a methyltransferase was identified by mutation of the STE14 gene which leads to production of defective a-factor (Hrycyna and Clarke, 1990; Hrycyna *et al*, 1991). It has also been demonstrated that the STE14 methyltransferase is responsible for methylation of RAS1 and RAS2 (Hrycyna *et al*, 1991). Methylation appears to take place in membranes since the enzyme is membrane associated (Hrycyna and Clarke, 1990) and the sequence reveals multiple potential membrane spanning domains indicative of integral membrane proteins (Sapperstein *et al*, 1994).

Methylation of a-factor is essential for it to bind to its MATα cell receptor or for subsequent steps that lead to $G_1$ arrest and mating. The ste14 mutation has no effect on cell viability showing that methylation is not essential for Ras function at least in yeast. The mutation does however slow down RAS processing (prenylation and proteolysis) suggesting that the CAAX modifications are coupled *in vivo* (Hrycyna *et al*, 1991).

Methyltransferase activity has also been identified in crude membrane fractions from mammalian cells (Stephenson and Clarke, 1990), in membranes from retinal rod outer segments (Perez-Sala *et al*, 1991) and in rat liver microsomes (Stephenson and Clarke, 1992). Farnesylated and geranylgeranylated peptides are both excellent substrates for methyltransferase (Stephenson and Clarke, 1992) whilst a geranylated peptide is not. This suggests that the enzyme recognition site is the prenylated cysteine residue (Hrycyna and Clarke, 1990; Hrycyna *et al*, 1991). Methylation of N-acetyl farnesyl cysteine occurs *in vitro* indicating that additional upstream sequences are not required (Hrycyna *et al*, 1991). Methylation was also observed with farnesylthiopropionic acid indicating that the amino side chain was not required for recognition (Tan *et al*, 1991a). The STE14 gene product is the only methyltransferase in yeast and methylates both farnesylated and geranylgeranylated proteins (Hrycyna and Clarke, 1990; Hrycyna *et al*, 1991). It is therefore possible that the same mammalian methyltransferase is responsible for methylation of farnesylated and both

types of geranylgeranylated proteins, that is, those with a CAAX motif and those with ∴ CXC C-termini.

In some proteins, such as lamin B, methylation appears to be reversible (Chelsky *et al*, 1987). This would require the existence of a methyltransferase and a methylesterase enzyme. Rod outer segment membranes have been found to contain active methyltransferase and methylesterase activities (Perez-Sala *et al*, 1991). Methylation is important for the interaction of transducin with activated rhodopsin and may be way of regulating the visual signal transduction pathway.

Carboxylmethylation of Rac proteins has been shown in neutrophils following stimulation with the chemoattractant *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) (Philips *et al*, 1993). Carboxylmethylation was also shown to be linked to GTP-GDP exchange. It was postulated that release from GDI allows the methyltransferase access to the COOH terminus of the Rac protein and methylation occurs. The human neutrophil prenylcysteine-directed carboxyl methyltransferase has been shown to be plasma membrane associated, phospholipid dependent and present speculation is that it may preferentially modify GTP bound substrates (Pillinger *et al*, 1994).

Methylation of Ras is a stable modification (Gutierrez *et al*, 1989) whilst for lamin B it appears to be a reversible process (Chelsky *et al*, 1987). This implies that both methyltransferases and methylesterases exist. Methylation could therefore be a way of regulating signal transduction pathways.

### 1.5.2.4.Palmitoyltransferase

The identification of the palmitoyltransferase specific for Ras has proved difficult although palmitoylation is a common modification of viral and eukaryotic proteins. However an activity has been found that is associated with the Golgi membranes (Grand *et al*, 1987; Gutierrez and Magee, 1991) and palmitoylates cytosolic N-ras precursors using palmitoyl-CoA as a substrate. The Golgi localisation is confusing since Ras is plasma membrane localised. Yeast YPT1 is palmitoylated and Golgi localised (Molenaar *et al*, 1988; Segev *et al*, 1988) and it is feasible that this palmitoyltransferase is in fact the enzyme responsible for YPT/Rab protein palmitoylation.

It has also been suggested that since palmitoylation of Ras is a dynamic process (Magee *et al*, 1987), the acylation and deacylation may be

enzymatically regulated with the deacylating enzyme being plasma membrane associated. Recently a cytosolic 37kDa palmitoyl-Ras thioesterase was purified from bovine brain (Camp and Hoffman, 1993) that recognises H-ras as a substrate only when it is bound to $Mg^{2+}$ and guanine nucleotides. Removal of $Mg^{2+}$ destabilises the protein and could cause a conformational change that renders the palmitate inaccessible to cleavage. Alternatively $Mg^{2+}$ and/or guanine nucleotides form part of the thioester recognition site or stabilises a three-dimensional determinant on the H-ras protein that is necessary for enzyme recognition.

Palmitoylation of rhodopsin is also a dynamic process and appears to be nonenzymatic (O'Brien et al, 1987) since the palmitoylation activity cannot be destroyed by boiling. The maturation of rhodopsin involves the nonenzymatic covalent attachment of 11- cis-retinal, a carotenoid molecule, to a specific amino acid of the opsin polypeptide (Zhukovsky et al, 1991) which sets a precedent for nonenzymatic protein modifications. It is possible that the palmitoylation and depalmitoylation of Ras involves a similar nonenzymatic process.

## 1.6. Prospects for anti-Ras chemotherapy
### 1.6.1. Introduction

Ras genes are frequently mutated in human malignancy and the incidence of ras mutation is particularly high in pancreatic (90%) and colon (50%) carcinomas (Bos, 1989). A therapeutic agent against Ras would therefore have a significant impact on the war against cancer.

Since Ras function appears to be dependent on the post-translational modifications of the CAAX motif (Hancock et al, 1989; 1990) prevention of the CAAX processing events would seem to be the simplest method of blocking the oncogenic effect of Ras.

It has been shown that HMG CoA reductase inhibitors block Ras processing and membrane association in vitro (Hancock et al, 1989; Kim et al, 1990). In vivo the use of lovastatin and compactin to treat tumours would be of little therapeutic use as mevalonate is the essential precursor for all isoprenoid biosynthesis including cholesterol (Goldstein and Brown, 1990). (Figure 1.8). These inhibitors also have to be used at relative high concentrations before Ras prenylation is abolished whilst cholesterol biosynthesis is blocked at much lower concentrations. These drugs would also be nonspecific in their effect, blocking processing of all prenylated cellular proteins. Not surprisingly use of these HMG CoA reductase

inhibitors leads to cell toxicity (Sinensky *et al*, 1990) and inhibitors of the enzymes that catalyse the post-translational modifications of the CAAX motif are more likely to be of therapeutic interest.

An anti-Ras drug would ideally be specific not only for Ras but for oncogenic Ras leaving endogenous Ras unaffected. A Ras mutant with Gln61--->Leu (an activating mutation) together with the Cys186--->Ser mutation is transformation deficient and growth inhibitory (Gibbs *et al*, 1989; Stacey *et al*, 1991). It is proposed that this mutant avidly binds the Ras effector so blocking Ras signalling. Gibbs (1991) suggests that a farnesyltransferase inhibitor would lead to the accumulation of non-farnesylated oncogenic Ras in the cytosol which would then act like the dominant negative Leu61, Ser186 protein. This effect would therefore be specific for oncogenic Ras since normal cytosolic Ras does not exert a dominant negative effect.

## 1.6.2. Enzyme inhibitors

The enzyme farnesyltransferase (FTase) would appear to make an ideal anti-Ras drug target since prenylation is required for Ras activity and it is also the first step in Ras processing. An inhibitor of FTase may also affect other prenylated proteins within the cell that are modified by the addition of geranylgeranyl since the FTase and GGTaseI enzymes have identical $\alpha$ subunits. Farnesylation of Ras has been shown to be blocked by CAAX tetrapeptides and larger peptides containing the Ras C-terminal sequence (Moores *et al*, 1991; Reiss *et al*, 1990). FTase inhibiting peptides have been made with serine or methionine as the X amino acid. These have minimal effects on the geranylgeranyltransferases (GGTase1 and RabGGTase) and are highly specific for FTase.

The uptake of these peptides into cells is extremely slow and degradation occurs rapidly so their therapeutic use is be limited (Kim *et al*, 1990). These problems can be overcome by microinjection of high concentrations of CAAX peptide into *Xenopus* oocytes; germinal vesicle breakdown induced by co-injected recombinant Ras is blocked, validating the principle that CAAX peptides can function as anti-Ras agents *in vivo*. One potent inhibitor of FTase activity was the tetrapeptide CVKM (Goldstein *et al*, 1991), this peptide is itself farnesylated. The addition of a bulky aromatic residue onto the $A_1$ of the $CA_1A_2X$ tetrapeptide abolished farnesylation of the peptide without affecting its ability to bind and therefore inhibit the $\beta$ subunit of FTase.

Recently two CAAX analogues have been tested with some success as FTase inhibitors. L731,735, based on the CAAX motif of K-ras (Kohl *et al*, 1993) showed no significant activity against GGTase I or RabGGTase. The compound blocked FTase activation *in vitro* but had no effect on H-ras processing *in vivo*. A related analogue, L731,734, blocked H-ras processing *in vivo*, and reverted Ras transformed NIH 3T3 cells but did not effect Raf or Mos transformed cells. Other analogues, BZA-2B and BZA-5B members of the benzodiazepine family (3-amino-1-carboxylmethyl-5-phenyl-benzodiazepin-2-one, BZA), showed low GGTase I and RabGGTase inhibition and were also shown to be growth inhibitory to and to revert Ras transformed Rat-1 cells (James *et al*, 1993).

Three proteins involved in light perception are farnesylated. The membrane association of G protein $\beta\gamma$ dimers and the GTP loading of $G_{t\alpha}$ by activated rhodopsin depends on farnesylation of $\gamma$ transducin. Rhodopsin kinase initiates deactivation of light-bleached rhodopsin and depends on farnesylation to become membrane associated (Lefkowitz, 1993). Farnesylation is also critical to the membrane association of cGMP phosphodiesterase, the enzyme that modulates cGMP levels in response to photon activation (Anan *et al*, 1992). Since the choroid layer of the retina has an extremely good blood supply, FTase inhibitors may reach the rod cells although the blood/brain barrier may prevent this. It is therefore possible that use of a FTase inhibitor may result in impaired vision.

The effect of farnesyl diphosphate analogues on farnesylation has also been studied. ($\alpha$-hydroxyfarnesyl) phosphonic acid was found to specifically inhibit FTase (Gibbs *et al*, 1993) and to inhibit Ras processing in H-ras transformed NIH3T3 fibroblasts at concentrations as low as $1\mu M$.

Limonene is one of the end products of the mevalonate pathway in plants (Crowell and Gould, In Press). It has been shown to inhibit the prenylation of 21-26kDa proteins in NIH3T3 and human mammary epithelial cells (Crowell *et al*, 1991).

The methylesterification of the C-terminal cysteine whilst increasing the extent of membrane binding of K-ras is not absolutely required (Hancock *et al*, 1991a) and yeast ste14 mutants lacking methyltransferase activity are viable. These data would indicate that blocking Ras methylation may not sufficiently abolish Ras function to have an antiproliferative effect. Inhibitors of the Ras palmitoyltransferase are unlikely to be of great therapeutic use since palmitoylation is not essential for Ras activity (Hancock *et al*, 1989) and would be ineffective against K-ras(B).

## 2.  Perspectives

The main research interest of this thesis is the post-translational processing of Ras. Although the membrane targeting signals of Ras have been fully defined, the post-translational modifications of Ras are still of interest since any anti-Ras agent developed will most likely be aimed against these processes, specifically prenylation.

The effect of geranylgeranylation rather than farnesylation of Ras was examined because this is the more common prenylation event that takes place on the Ras superfamily of proteins. Since the geranylgeranylated Rap proteins are Golgi localised, it was wondered whether geranylgeranylated Ras would localise to a membrane compartment other than the plasma membrane. The ability of geranylgeranyl to substitute for farnesyl to produce biologically active Ras proteins was also examined.

The Src protein family achieve membrane localisation by N-terminal myristoylation. The localisation and membrane targeting requirements of myristoylated Ras were investigated along with the effect of myristoylation on the biological activity of Ras.

The hypervariable region is a domain that has the greatest variability in length and sequence amongst the Ras family. The region has previously been shown to have no biological function but its conservation between Ras family members implies a functional role. The effect of increasing or decreasing the size of the hypervariable domain was examined.

# Chapter 2

## Prenylation of Ras : Farnesylation versus Geranylgeranylation

### 2.1. Introduction

As discussed in the introduction chapter, Ras proteins are prenylated by the addition of a farnesyl or $C_{15}$ group. However the majority of Ras-related proteins are prenylated by a geranylgeranyl, $C_{20}$ moiety.

This chapter aims to address whether the addition of a geranylgeranyl group onto K-ras affects the localisation and biological activity of the protein. It also investigates whether having been prenylated by $C_{20}$ there is still a requirement for a second signal, in the case of K-ras the polybasic domain, to direct plasma membrane targeting. The Rap proteins, Rap1A and Rap1B, have C-terminal polybasic domains similar to K-ras but they are geranylgeranylated and are Golgi localised (Beranger *et al*, 1991a). It was therefore of interest to investigate if K-ras would become Golgi localised if it was prenylated with $C_{20}$.

The effect of geranylgeranylation on the cellular localisation of H-ras will also be examined to see if it would be targeted to other intracellular compartments when it was geranylgeranylated. The requirement for palmitoylation is also investigated when H-ras is geranylgeranylated.

Another aspect of the work described in this chapter is an investigation into the nature of the polybasic domain and to determine whether the interaction between the plasma membrane and the polybasic domain of K-ras is a charge/charge interaction or whether a specific membrane docking protein for K-ras exists.

Using protein A, a heterologous protein, the specific signals required for plasma membrane targeting of K-Ras and H-ras were examined. This work would address whether the only domains within Ras required for plasma membrane targeting are an intact CAAX motif and the upstream hypervariable region containing the polybasic domain or palmitoylation sites.

### 2.2. Results

#### 2.2.1. Construction of the $C_{20}$ modified K-ras mutants

Mutants of K-ras were made which contained different CAAX motifs to the wild type protein and which were expected to be geranylgeranylated by GGTase I rather than farnesylated. Two different mutants were made, one had the wild type CVIM CAAX motif sequence changed to CAIL, the CAAX

motif of a brain G-protein $\gamma$ subunit, and the other had a CCIL motif the same as Ral.

The CCIL mutant was made using the site directed mutagenesis protocol described in the Materials and Methods chapter. The mutant was fully sequenced and cloned into the EXV-3 mammalian expression vector (Miller and Germain, 1986). The CAIL mutant was made using the PCR mutagenesis strategy described in the Materials and Methods section. The C-termini of these $C_{20}$ modified K-ras proteins are shown in **Figure 2.1**.

The $C_{20}$ modified K-ras proteins were also made with a mutated polybasic domain (the K6Q versions) as this would determine whether geranylgeranylated proteins still required the polylysine sequence for specific localisation within the cell or if the $C_{20}$ modification alone was sufficient to direct protein targeting.

## 2.2.2. Prenylation of K-ras CAIL and CCIL mutants

The K-ras cDNAs encoding the CAIL and CCIL proteins were cloned into pGem plasmids. The genes were transcribed *in vitro* and the mRNA produced was used to direct *in vitro* translation in a nuclease treated (message dependent) rabbit reticulocyte lysate. This method was used as it had previously been shown that the nuclear lamins A and B could be prenylated using a rabbit reticulocyte lysate (Vorburger *et al*, 1989).

This system was used to show that the CAIL and CCIL proteins were geranylgeranylated by translating *in vitro* in a rabbit reticulocyte lysate that was labelled with [$^3$H] mevalonic acid (Hancock *et al*, 1991a). Following SDS-PAGE and fluorographic detection, the Ras proteins were excised from the polyacrylamide gel, digested out of the gel slices and subjected to methyliodide cleavage (Casey *et al*, 1989). The products of the cleavage were analysed by HPLC. For both proteins the counts retained on the column co-eluted with the $C_{20}$ geranylgeranyl standard and no label was detected in the position of farnesyl. These experiments were kindly performed by C. J. Marshall and J.F. Hancock.

The rabbit reticulocyte lysate system was also used to show that the $C_{20}$ modified K-ras proteins were able to be methylesterified. For this analysis the translations were performed in the presence of S-adenosyl-[$^3$H] methionine (SAM) which would act as the methyl donor. Since the Ras methyltransferase has been localised to intracellular membranes (Hrycyna and Clarke, 1990; Stephenson and Clarke, 1990), canine pancreatic microsomes were added to ensure that methylation could take place. Both the CAIL and CCIL mutants

**Figure 2.1.**

**C-termini of C20 modified K-ras and H-ras mutants.**

|  |  |  | **C** | **A** | **A** | **X** |
|---|---|---|---|---|---|---|
| wild type K-ras | Thr | Lys | Cys | Val | Ile | Met |
| CCIL | Thr | Lys | Cys | Cys | Ile | Leu |
| CAIL | Thr | Lys | Cys | Ala | Ile | Leu |
| wild type H-ras | Cys | Lys | Cys | Val | Leu | Ser |
| HCCIL | Cys | Lys | Cys | Cys | Ile | Leu |

The CAAX motifs of K-ras and H-ras are shown along with the CAAX motifs of the C20 modified mutants.

were found to incorporate label from the S-adenosyl-[$^3$H] methionine and were therefore assumed to be methylesterified. Translations were also performed in the presence of 3mM methylthioadenosine (MTA), an inhibitor of in vivo nuclear lamin methylesterification (Chelsky et al, 1989) and incorporation of SAM was significantly reduced under these conditions, again indicating that the mutant proteins were methylesterified.

The CCIL motif has also been found to be geranylgeranylated in a study of the post-translational processing of full length Ral (Kinsella et al, 1991). Thus these CAA(X=L) motifs direct geranylgeranylation rather than farnesylation, are methylated and therefore -AA(X=L) proteolysed.

## 2.2.3. Subcellular localisation of K-ras CAIL and CCIL mutants

To investigate whether the presence of a $C_{20}$ group affected the subcellular distribution of the K-ras proteins, transfected COS cells expressing the $C_{20}$ modified proteins were fractionated into membrane (p100) and cytosolic (s100) fractions, immunoprecipitated and separated by SDS-PAGE. **Figure 2.2** shows that the CAIL and CCIL proteins were both associated with the p100 fraction. An interesting result was that the $C_{20}$ modified proteins containing the K6Q mutation were also p100/membrane associated. This is in direct contrast to the farnesylated K6Q protein which is 90% cytosolic. Therefore the presence of a $C_{20}$ rather than a $C_{15}$ alkyl group has no effect on the localisation of K-ras proteins that contain an intact polybasic domain. However when the polybasic domain is changed to six uncharged glutamine residues the $C_{20}$ group has a dramatic effect localising the protein to the p100 fraction whereas the $C_{15}$ polybasic mutant is s100 associated.

The subcellular localisation of these proteins was also investigated by immunofluorescence of NIH 3T3 cells. **Figure 2.3** shows that the CAIL and CCIL proteins were found to be plasma membrane associated, whereas the K6QCAIL and K6QCCIL proteins exhibited cytosolic staining which did not appear to be associated with any particular intracellular compartment. The presence of a geranylgeranyl group together with a polybasic domain has no effect on the normal localisation pattern of K-ras. However in the absence of a polybasic domain although the $C_{20}$ modified K-ras(B) proteins are p100/membrane associated, they are not localised to the plasma membrane appearing to bind nonspecifically to intracellular membranes.

This confirms previous results (Hancock et al, 1990) that both the polybasic domain and the CAAX motif are required for the plasma membrane localisation of K-ras.

**Figure 2.2.**
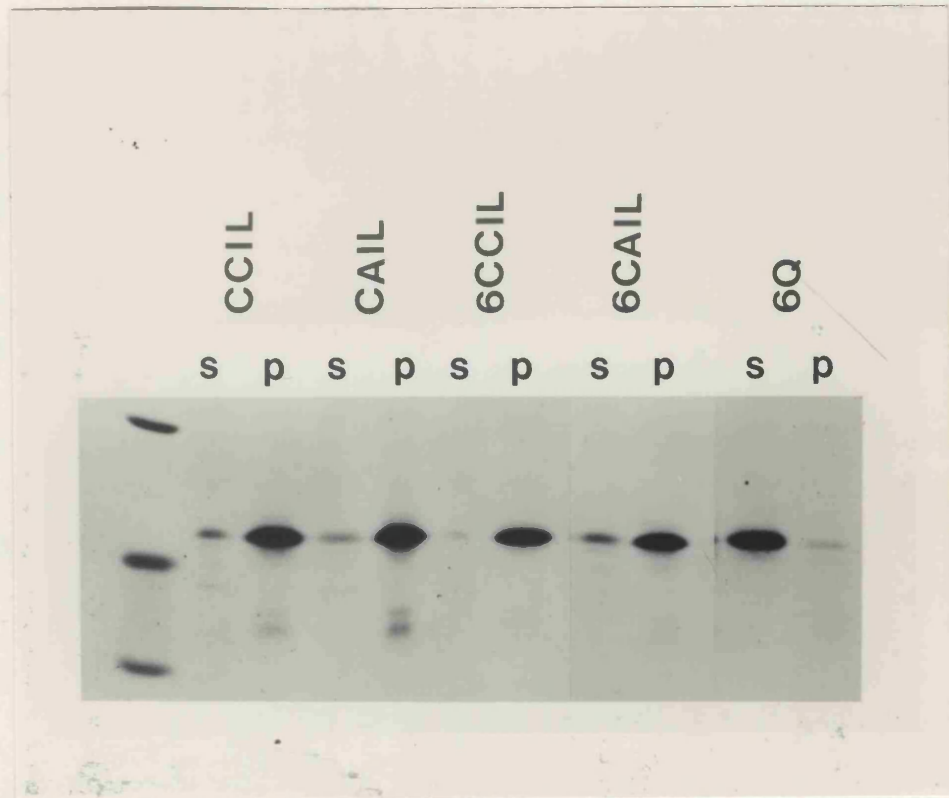


**Fractionation of geranylgeranylated K-ras proteins.**

COS cells expressing K-ras proteins were metabolically labelled for 18hr with [$^{35}$S] methionine and fractionated into p100 (p) and s100 (s) fractions. Equal proportions of each fraction were immunoprecipitated, resolved by SDS-PAGE and autoradiographed.

CCIL     = K-ras with a CCIL C-terminal motif

CAIL     = K-ras with a CAIL C-terminal motif

6CCIL    = K-ras with a CCIL C-terminal motif and the
                mutation K[175 - 180]Q

6CAIL    = K-ras with a CAIL C-terminal motif and the
                mutation K[175 - 180]Q

6Q        = K-ras with the mutation K[175 - 180]Q

**Figure Legend 2.3.**

**Immunofluorescence of geranylgeranylated K-ras proteins.**

NIH 3T3 cell lines derived from the focus assays described in **Table 2.2** were examined for immunofluorescence after incubation with the monoclonal antibody Y13 - 238 followed by an anti - rat FITC conjugate. This methodology allows specific staining of transfected K-ras proteins. (a) K-ras with a CCIL C-terminal motif : plasma membrane staining. (b) K-ras with a CCIL C-terminal motif and the polybasic mutation K[175 - 180]Q : no plasma membrane staining, the P100 associated protein is diffusely localised throughout the cell. (c) K-ras with a CAIL C-terminal motif : plasma membrane staining. (d) K-ras with a CAIL C-terminal motif and the polybasic basic mutation K[175 - 180]Q : no plasma membrane staining, the p100 associated protein is diffusely localised throughout the cell.

# Figure 2.3.
## Immunofluorescence of geranylgeranylated K-ras proteins.
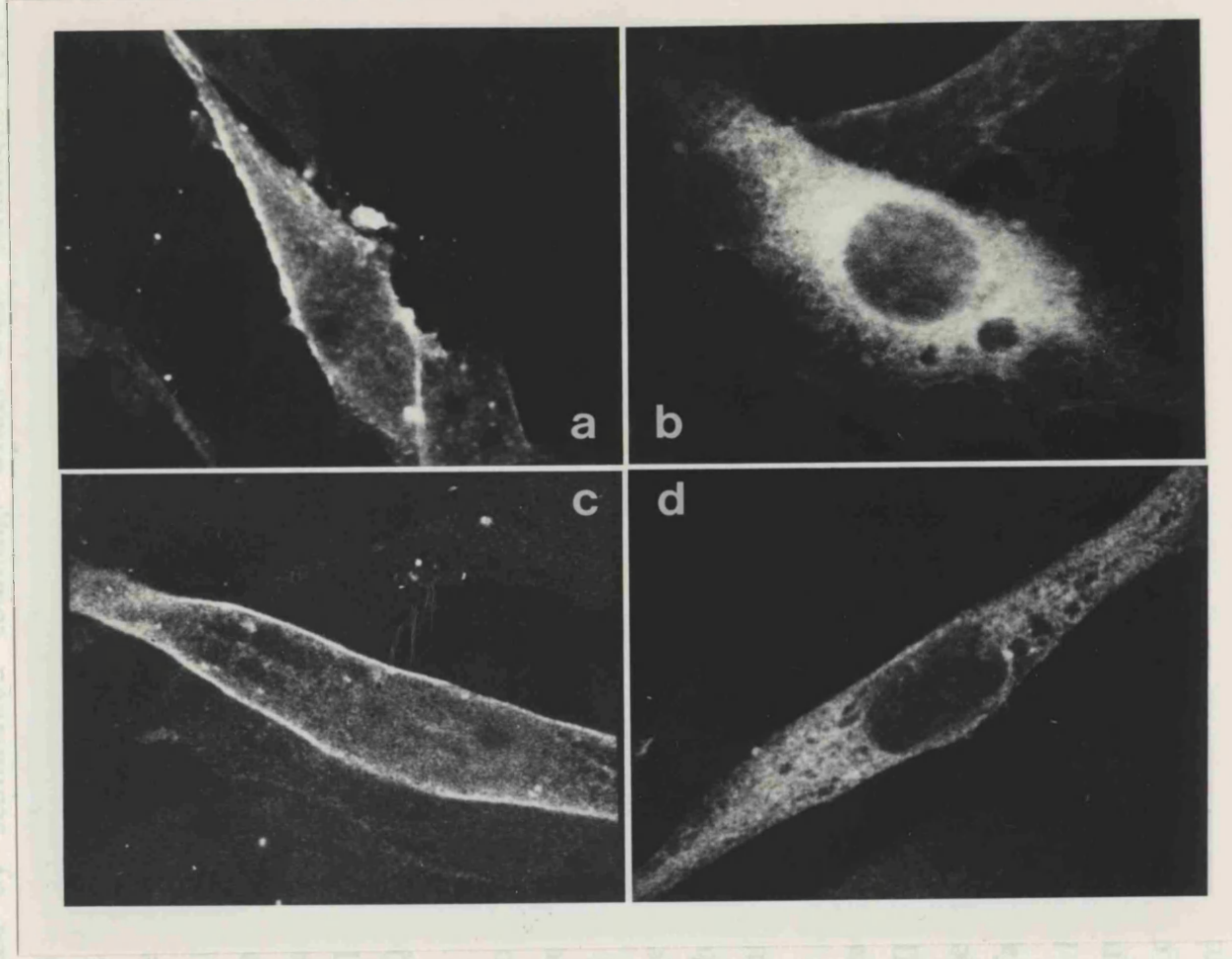
**2.2.4. Avidity of membrane association of C20 modified K-ras**

In order to determine the strength of membrane association of the $C_{20}$ modified K-ras proteins, aliquots of p100 fractions from electroporated COS cells were washed with 1M salt (NaCl) for 30minutes at $20°C$. The samples were then recentrifuged to reisolate s100 and p100 fractions. The fractions were then immunoprecipitated and analysed by SDS-PAGE. The gel bands were excised and subjected to pronase digestion before being quantitated by scintillation counting. Under these conditions 78% of the farnesylated protein was found to be removed from the p100 fraction **(Table 2.1).** By contrast less than 15% of the $C_{20}$ modified proteins was lost from the p100 fraction. This indicates that the geranylgeranylated proteins have a much more avid association with the membrane pellet than the farnesylated protein. This tighter membrane association of the geranylgeranylated proteins is independent of the presence of the polybasic domain because the geranylgeranylated polybasic domain mutants (K6QCCIL and K6QCAIL) were found to bound as avidly as the $C_{20}$ modified K-ras proteins with a polybasic domain (CAIL and CCIL).

However washing membrane/p100 fractions of the farnesylated and geranylgeranylated proteins with 0.5% Triton X-100 solubilises more than 90% of the protein **(Table 2.1).** This suggests that the K6Q geranylgeranylated proteins are associated with an intracellular membrane rather than a high molecular weight cytoplasmic protein complex since this would be resistant to Triton X-100 washing.

**2.2.5. Transforming efficiency of C20 modified K-ras**

NIH 3T3 focus assays were performed to see if the presence of a geranylgeranyl moiety affected the biological activity of oncogenic (Val 12) K-ras. **Table 2.2** shows that the transforming efficiency of the oncogenic (Val 12) geranylgeranylated proteins in NIH 3T3 focus assays was only slightly reduced compared to that of the farnesylated proteins. Similarly the activity of the geranylgeranylated K6Q mutants was not found to be compromised compared to that of the farnesylated K6Q mutant. Prenylation by a $C_{20}$ alkyl chain does therefore not significantly affect the transforming ability of K-ras.

**Table 2.1.**

**Salt and Triton wash of COS cell membrane K-ras protein.**

| | Percentage P100 washout | |
| --- | --- | --- |
| | 1M NaCl | 0.5%Triton |
| K-ras | 78 | 92 |
| K-ras CCIL | 11 | 90 |
| K-ras CAIL | 13 | 94 |
| K6Q CCIL | 12 | 92 |
| K6Q CAIL | 15 | 95 |

[35S] methionine - labelled p100 fractions were prepared from COS cells expressing K-ras proteins. Aliquots of these fractions were washed with 1M NaCl for 30min at 20°C or 0.5% Triton X-100 for 10min on ice and the membranes repelleted at 120,000g. K-ras protein was immunoprecipitated from the 'new' s100 and p100 fractions, resolved by SDS-PAGE and cut out of the gel following autoradiography. The amount of K-ras remaining in the membrane pellet and the amount washed out into the s100 fraction was then quantified by scintillation counting of pronase digests of the gel slices. Results (mean of two experiments) are presented as percentage p100 washout, calculated as (c.p.m. in Ras in the s100 fraction x 100% /(total c.p.m. in Ras). The mutant K-ras proteins are described in the legend to **Figure 2.2.**

**Table 2.2.**

**NIH 3T3 focus assays of $C_{20}$ modified K-ras proteins.**

| ras construct | Relative activity |
|---|---|
| K-ras | 1.0 |
| K6R | 1.0 |
| K-ras CCIL | 0.88 |
| K-ras CAIL | 0.64 |
| K6Q | 0.38 |
| K6Q CCIL | 0.64 |
| K6Q CAIL | 0.2 |

Relative transforming efficiencies of K-ras (Val 12) cDNAs with altered C-termini. 20ng of each EXV plasmid was transfected with 20ug of normal human DNA as carrier onto $1.3 \times 10^5$ NIH 3T3 cells. Foci were scored 14 - 16 days following transfection. K-ras (Val 12) with a wild type C-terminus gave 1 - 1.9 foci / ng and results were expressed relative to this value.

## 2.2.6. The effect of geranylgeranylation on the subcellular localisation of H-ras

It was also decided to examine the effect of geranylgeranylation on the subcellular localisation of H-ras. Since plasma membrane targeting of H-ras has been shown to require the CAAX post-translational modifications as well as palmitoylation (Hancock *et al*, 1990), it was of interest to examine whether geranylgeranylation of H-ras was sufficient to target the protein to the plasma membrane or whether there was still a requirement for palmitoylation.

## 2.2.7. Construction of the $C_{20}$ modified H-ras mutants

A $C_{20}$ modified H-ras protein was made using the PCR reaction to mutate the CAAX motif of H-ras to one representing the C-terminus of Ral. The mutation was made in H-ras containing cysteine residues available for palmitoylation at positions 181 and 184 to give HCCIL and also in a H-ras mutant where these cysteines had been replaced by serine residues to give H1/4SCCIL **(Figure 2.1)**.

## 2.2.8. Subcellular localisation of $C_{20}$ modified H-ras mutants

H-ras is modified by the addition of palmitic acid to cysteines at positions 181 and 184. Palmitoylation only occurs after farnesylation of the protein has taken place. To confirm that prenylation by a $C_{20}$ group did not effect palmitoylation of the protein, electroporated COS cells were labelled with [$^3$H]palmitic acid before being lysed in 1% Triton X-114, immunoprecipitated and resolved by SDS-PAGE. **Figure 2.4** shows that both farnesylated (H) and geranylgeranylated (HCCIL) H-ras undergo palmitoylation whereas the two proteins containing serine mutations at positions 181 and 184 (H1/4S and H1/4SCCIL) did not incorporate palmitic acid. This result indicates that geranylgeranylation of H-ras can substitute for farnesylation in providing a substrate for palmitoyltranferase.

The step 1 processing (or CAAX modifications) of the $C_{20}$ modified H-ras proteins was examined by using electroporated COS cells and labelling the cells for 10 minutes with [$^{35}$S] methionine before lysing the cells in 1% Triton X-114 immediately after labelling (pulse) or after a 4 hour incubation with cold methionine (chase). The lysates were incubated at 37°C to separate the detergent and aqueous fractions which were then immunoprecipitated and resolved by SDS-PAGE.

# Figure 2.4.
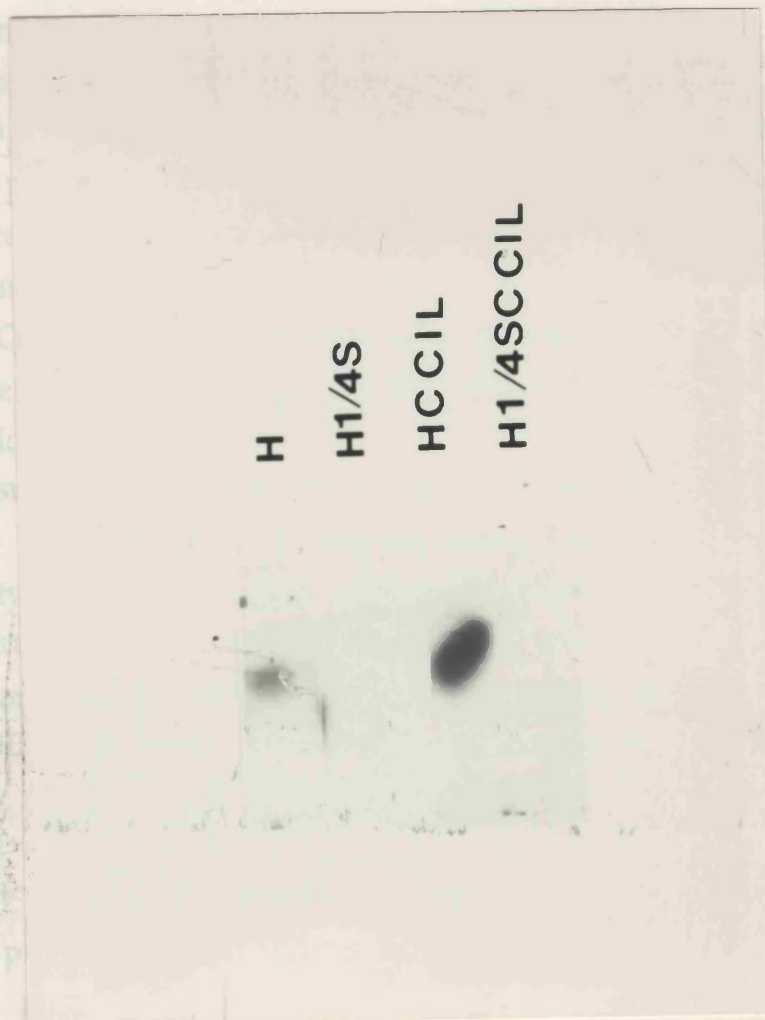


**C20 modified H-ras is palmitoylated.**

COS cells expressing H-ras proteins were labelled for 4hr with [$^3$H] palmitic acid. The cells were lysed in Triton X114, immunoprecipitated and resolved by SDS-PAGE. H and HCCIL both show incorporation of palmitic acid whereas the H1/4S and H1/4SCCIL mutants do not.

| | | |
|---|---|---|
| H | = | H-ras with wild type C-terminal motif |
| HCCIL | = | H-ras with a CCIL C-terminal motif |
| H1/4SCCIL | = | H-ras Cys181/184Ser with a CCIL C-terminal motif |
| H1/4S | = | H-ras Cys181/4Ser with a wild type C-terminal motif |

After the pulse label, wild type H-ras shows the majority of the [$^{35}$S] labelled protein in the aqueous phase **(Figure 2.5)**. Following the cold chase a significant amount of the protein partitioned into the detergent phase indicating that post-translational modifications of the CAAX motif have taken place. The $C_{20}$ H-ras mutant (HCCIL) shows a similar distribution pattern indicating that geranylgeranylation does not affect the step 1 processing of the protein apart from the prenylation being with a different length alkyl chain. The palmitoylation deficient mutants (H1/4S and H1/4SCCIL) also show the same distribution pattern. This is because these proteins still undergo CAAX processing but cannot undergo palmitoylation.

The subcellular localisation of the $C_{20}$ modified H-ras proteins was investigated by fractionation of electroporated COS cells. **Figure 2.6** shows that wild type H-ras is almost entirely membrane associated (p100). This was also true of the $C_{20}$ modified H-ras protein (HCCIL). When the palmitoylation sites of H-ras are mutated to serines (H1/4S), the protein is cytosolic (s100 associated). The same mutations in C20 modified H-ras (H1/4SCCIL) shows significant amounts are p100 associated although there is also some cytosolic protein. Therefore prenylation of H-ras by $C_{20}$ in the absence of palmitoylation leads to a membrane associated protein whereas farnesylation of H-ras without palmitoylation renders the protein cytosolic.

Salt wash experiments were performed on p100 fractions of the $C_{20}$ modified H-ras proteins to measure the strength of membrane association compared to the farnesylated H-ras proteins. Wild type H-ras showed only 30% of the protein entering the s100 fraction after a 1M salt wash due to the presence of the palmitate moiety holding the protein firmly in the membrane **(Table 2.3)**. The H-CCIL mutant showed 90% of the protein was still membrane bound after salt washing, the H1/4SCCIL protein showed the same pattern even though it has no palmitate residues to assist membrane attachment. These experiments show the stronger membrane association of $C_{20}$ modified H-ras compared to the farnesylated form. They also demonstrate the stronger membrane association of H-ras compared to K-ras.

The localisation of the $C_{20}$ modified H-ras proteins was further examined by immunofluorescence. The immunofluorescent analysis was performed using both standard post-fixation permeabilisation and pre-

Figure 2.5.
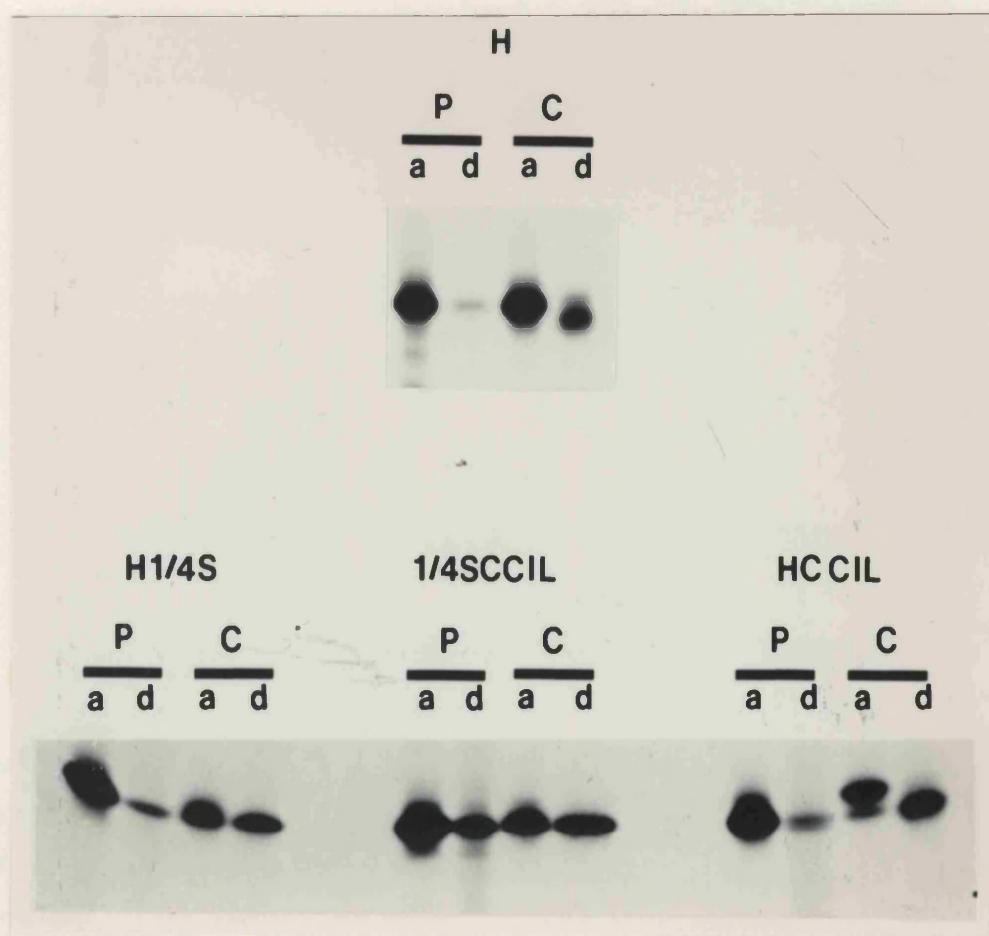


**Step 1 processing of $C_{20}$ modified H-ras.**
COS cells expressing Ras proteins were pulse labelled with [$^{35}$S] methionine and either lysed immediately in Triton X114 (P) or chased with cold methionine for 4hr before lysis (C). The lysates were partitioned into aqueous (a) and detergent (d) phases, immunoprecipitated and resolved by SDS-PAGE. Step 1 processing is accompanied by an increase in hydrophobicity and gel mobility of the Ras protein; compare each P(a) lane with each C(d) lane. Palmitoylation site mutations (Cys 181 and 184--->Ser) have no effect on step 1 processing of the CAAX motif.

H          =    H-ras with wild type C-terminal motif
HCCIL      =    H-ras with a CCIL C-terminal motif
H1/4SCCIL  =    H-ras Cys181/184Ser with a CCIL C-terminal motif
H1/4S      =    H-ras Cys181/4Ser with a wild type C-terminal motif

## Figure 2.6.
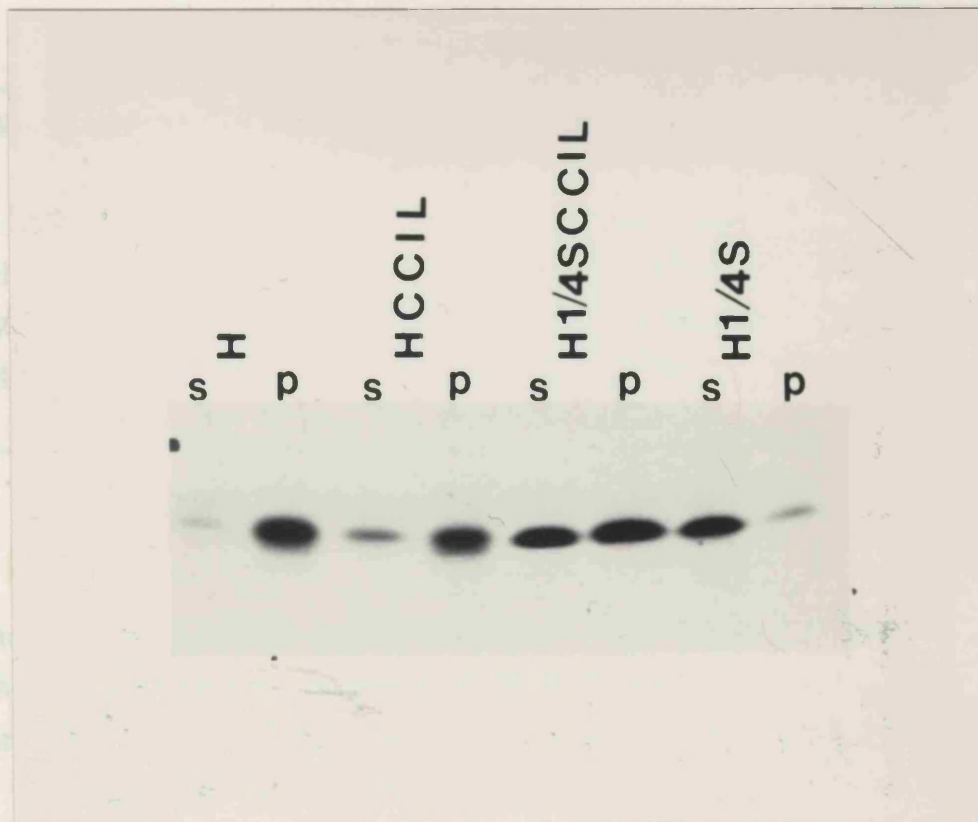


**COS cell fractionation of C$_{20}$ modified H-ras proteins.**

COS cells expressing H-ras proteins were metabolically labelled for 18hr with [$^{35}$S] methionine and after a 5hr cold chase were fractionated into p100(p) and s100(s) fractions. Equal proportions of each fraction were immunoprecipitated, resolved by SDS-PAGE and autoradiographed.

| | | |
|---|---|---|
| H | = | H-ras with wild type C-terminal motif |
| HCCIL | = | H-ras with a CCIL C-terminal motif |
| H1/4SCCIL | = | H-ras Cys181/184Ser with a CCIL C-terminal motif |
| H1/4S | = | H-ras Cys181/4Ser with a wild type C-terminal motif |

**Table 2.3.**

**Salt wash of H-ras COS cell membranes.**

| Ras protein | percentage P100 washout |
|---|---|
| | 1M NaCl |
| H-ras | 30 |
| HCCIL | 10 |
| H1/4SCCIL | 12 |

[35S] methionine labelled p100 fractions were prepared from COS cells expressing H-ras proteins. Aliquots of these fractions were washed with 1M NaCl for 30min at 20°C and the membranes repelleted at 120,000g. H-ras protein was immunoprecipitated from the new s100 and p100 fractions, resolved by SDS-PAGE and cut out of the gel following autoradiography. The amount of H-ras protein remaining in the new pellet and the amount washed out into the s100 fraction was then quantified by scintillation counting pronase digests of the gel slices. Results (mean of two experiments) are presented as percentage p100 washout, calculated as (c.p.m. in Ras in the s100 fraction x 100% / total c.p.m. in Ras) The mutant H-ras proteins are described in the legend to **Figure 2.4**.

fixationpermeabilisationThe pre-fixationpermeablisationtechnique washes free cytosolic proteins out of the cell. Cells were permeablised with a solution of 0.5% saponin-0.2% bovine serum albumin in PBS for 3min and fixed in 4% formaldehyde in PBS for 20min. **Figure 2.7** shows that $C_{20}$ modified H-ras (HCCIL) that is also palmitoylated, is plasma membrane associated and is not removed by pre-fixationpermeabilisation The geranylgeranylated H-ras protein that does not undergo palmitoylation (H1/4SCCIL) is cytosolic and is washed out of the cell by pre-fixation permeabilisation.

These results taken together with the fractionation data indicate that plasma membrane targeting of H-ras requires prenylation together with palmitoylation. The prenylation can be with either a $C_{15}$ or a $C_{20}$ moiety. Geranylgeranylation of H-ras in the absence of palmitoylation whilst producing a membrane (p100) associated protein does not lead to plasma membrane association.

### 2.2.9. Transforming efficiency of C20 modified H-ras

NIH 3T3 focus assays were performed to see if the presence of a geranylgeranyl moiety affected the biological activity of oncogenic (Val 12) H-ras. **Table 2.4** shows that the transforming efficiency of the oncogenic (Val 12) geranylgeranylated proteins in NIH 3T3 focus assays was only slightly reduced compared to that of the farnesylated proteins. Similarly the activity of the geranylgeranylated H1/4S mutants was not found to be compromised compared to that of the farnesylated H1/4S mutant. Prenylation by a $C_{20}$ alkyl chain does therefore not significantly affect the transforming ability of H-ras.

### 2.2.10.Protein A chimeras

The plasma membrane association of K-ras requires the presence of both the CAAX motif and the polybasic domain (Hancock *et al*, 1990). In order to see whether these are the only regions required to achieve plasma membrane targeting, a polybasic domain and a CAAX motif were cloned onto the C-terminus of protein A. This protein is normally cytosolic and the effect of the addition of these Ras targeting sequences on its subcellular localisation were examined.

The coding sequence for the C-terminal 17 amino acids of K-ras was amplified from a K-ras cDNA by polymerase chain reaction (PCR) and

**Figure Legend 2.7.**
**Subcellular localisation of $C_{20}$ modified H-ras.**

Immunofluorescence of cells expressing $C_{20}$ modified H-ras proteins. (a) H-CCIL shows plasma membrane localisation. (b) H1/4SCCIL shows some plasma membrane staining. (c) HCCIL remains associated with the plasma membrane when the cells are subjected to pre-permeablisation fixation. (d) H1/4SCCIL is washed out of the cell when the cells are subjected to pre-permeablisation fixation.

**Figure 2.7.**
**Subcellular localisation of C20 modified H-ras.**
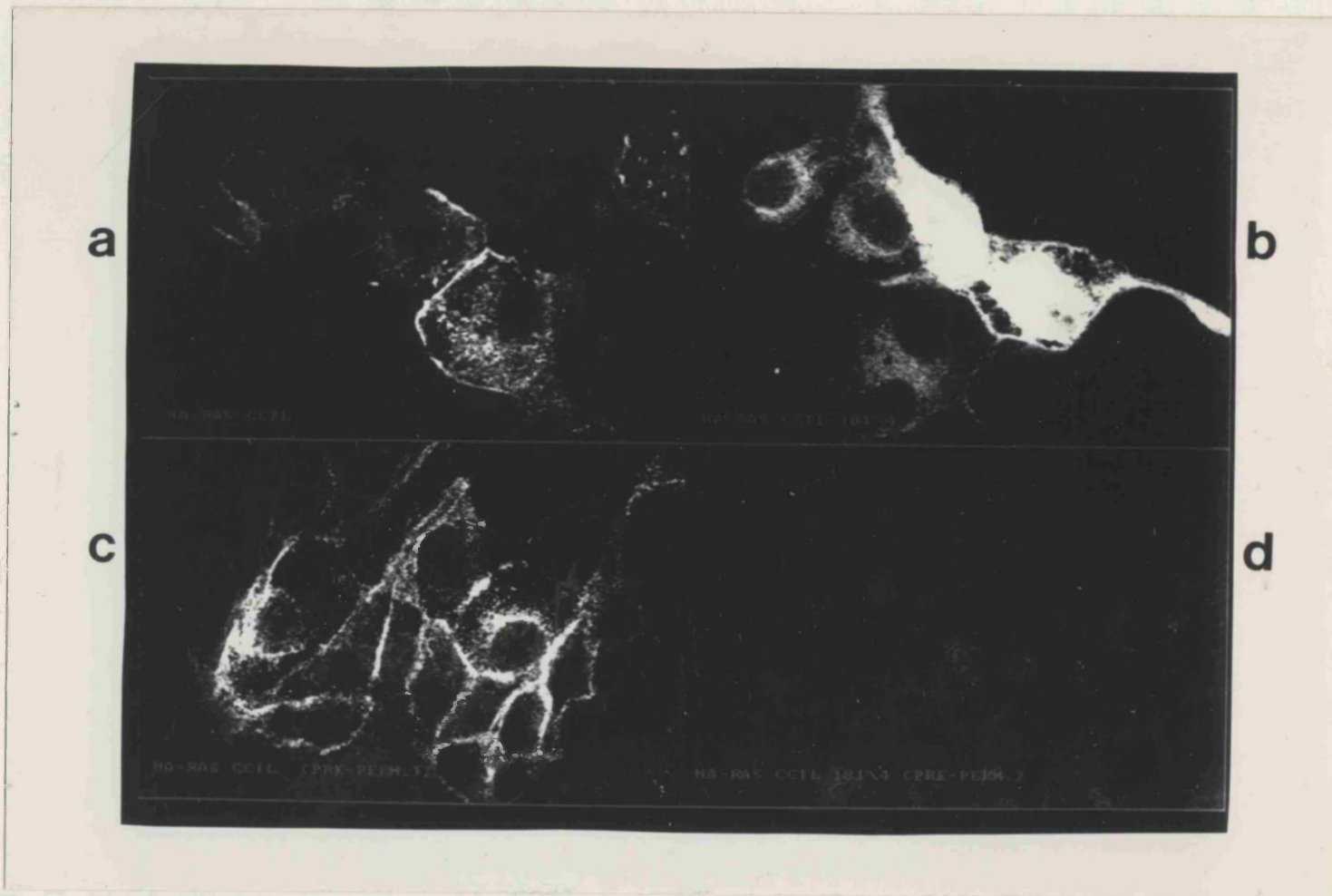
**Table 2.4.**

**NIH 3T3 focus assays of C20 modified H-ras proteins.**

| ras construct | Relative activity |
|---|---|
| H-ras | 1.00 |
| H1/4S | 0.38 |
| HCCIL | 0.76 |
| H1/4SCCIL | 0.29 |
| H186S | 0.00 |

Relative transforming efficiencies of H-ras (Val 12) cDNAs with altered C-termini. 2ng of each EXV plasmid was transfected with 20ug of normal human DNA as carrier onto $1.3 \times 10^5$ NIH 3T3 cells. Foci were scored 10 - 14 days following transfection. H-ras (Val 12) with a wild type C-terminus gave 20-30 foci/ng and results were expressed relative to this value.

cloned onto the C-terminus of protein A using a restriction site introduced into the K-ras coding sequence during amplification.

When the wild type protein A and the protein A/K-ras chimera (K) were expressed in COS cells which were subsequently fractionated into membrane (p100) and cytosolic (s100) fractions, it was seen that the protein A was predominantly (80%) localised to the cytosolic (s100) fraction (Figure 2.8). The chimeric protein, K, containing the polybasic domain and the CAAX motif of K-ras´ was by contrast almost entirely localised to the membrane (p100) fraction. Another chimera containing the CAAX motif and a polyglutamine domain (K6Q) instead of the normal polylysine domain, was found to be associated with the cytosolic (s100) fraction.

Further analysis of the subcellular localisation of these proteins was performed using immunofluorescence studies on MDCK cells transiently expressing the proteins following microinjection of the plasmid DNA. Figure 2.9 shows that the wild type protein A was localised to the cytosol and nucleus. The protein A/K-ras chimera with the polylysine domain and the CAAX motif was localised to the plasma membrane. The chimeric protein (K6Q) that had the polyglutamine domain was localised to the cytosol but was not found in the nucleus. The presence of the CAAX motif without a polybasic domain would therefore appear to have no effect on the localisation pattern of the wild type protein A other than to exclude it from the nucleus.

Previous work (Hancock et al, 1990) has shown that progressively decreasing the number of lysine residues in the polybasic domain of K-ras(B) and substituting them for glutamine residues decreases the extent of membrane association and the protein becomes increasingly cytosolic. When all the lysines are substituted for glutamines, the protein (K6Q) is entirely cytosolic. It was decided to examine this effect using protein A and cloning various K-ras C-termini that had increasing numbers of lysine to glutamine substitutions onto the C-terminus of protein A.

Immunofluorescent analysis of MDCK cells transiently expressing the protein A chimeras following microinjection of the plasmid DNA, showed that as the number of lysine residues was decreased the protein A chimeras became increasingly cytosolic (Figure 2.10). When there are fewer than two lysines in the polybasic domain no plasma membrane localisation takes place. Therefore the polybasic domain functions for a heterologous protein exactly as for K-ras.

**Figure 2.8.**
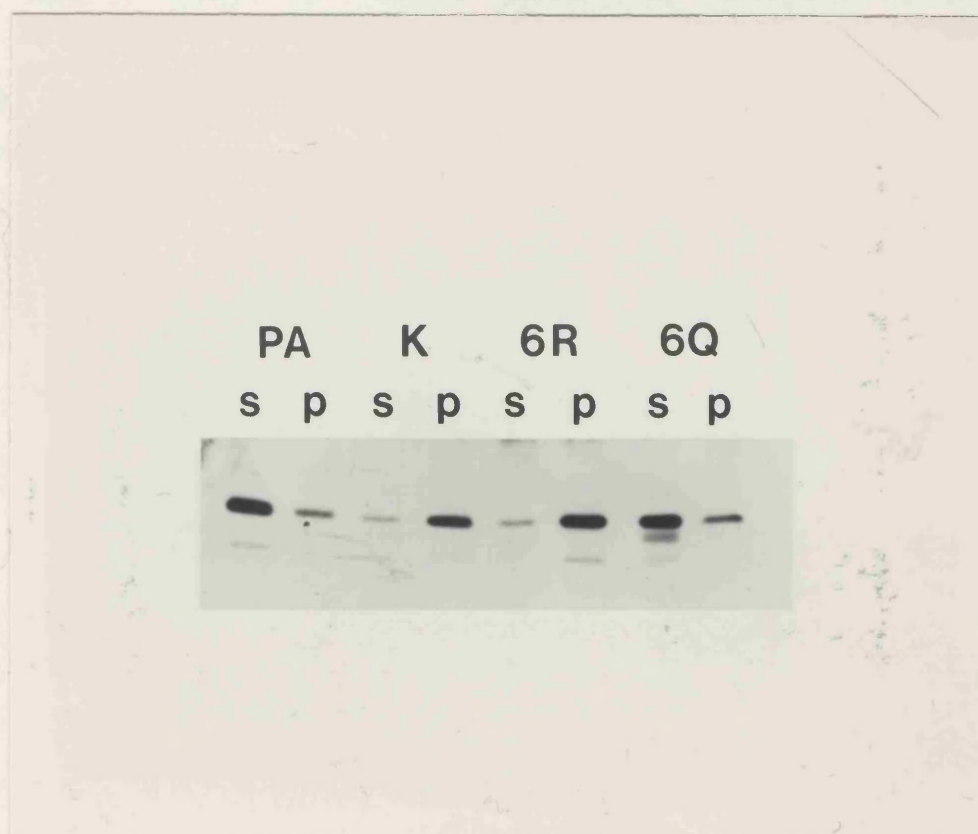


**Fractionation of COS cells expressing protein A : Ras chimeric proteins.**

COS cells expressing protein A (PA), or protein A chimeras with the C-terminal 17 amino acids from K-ras (K), were fractionated into p100 (p) and s100 (s) fractions. The polylysine domain of wild type K-ras is replaced with polyglutamine in the K6Q mutant and with polyarginine in the K6R mutant. Equal proportions of the s100 and p100 fractions were resolved by SDS-PAGE and Western blotted using an anti-protein A primary antibody.

**Figure Legend 2.9.**

**The CAAX motif together with the polybasic domain of K-ras is required to target protein A to the plasma membrane.**

Immunofluorescent analysis of MDCK cells transiently expressing protein A or protein A chimeric proteins following microinjection of plasmid DNA. (a) Protein A: cytoplasmic and nuclear staining. (b) Protein A with a CVLS C-terminal motif alone (from H-ras): cytoplasmic staining only. (c) Protein A with 17 C-terminal amino acids from wild type K-ras: plasma membrane staining. (d) Protein A with 17 C-terminal amino acids from the K-ras mutant, K6Q, which has the polylysine domain replaced with polyglutamine : cytoplasmic staining only.

**Figure 2.9.**

**The CAAX motif together with the polybasic domain of K-ras is required to target protein A to the plasma membrane.**
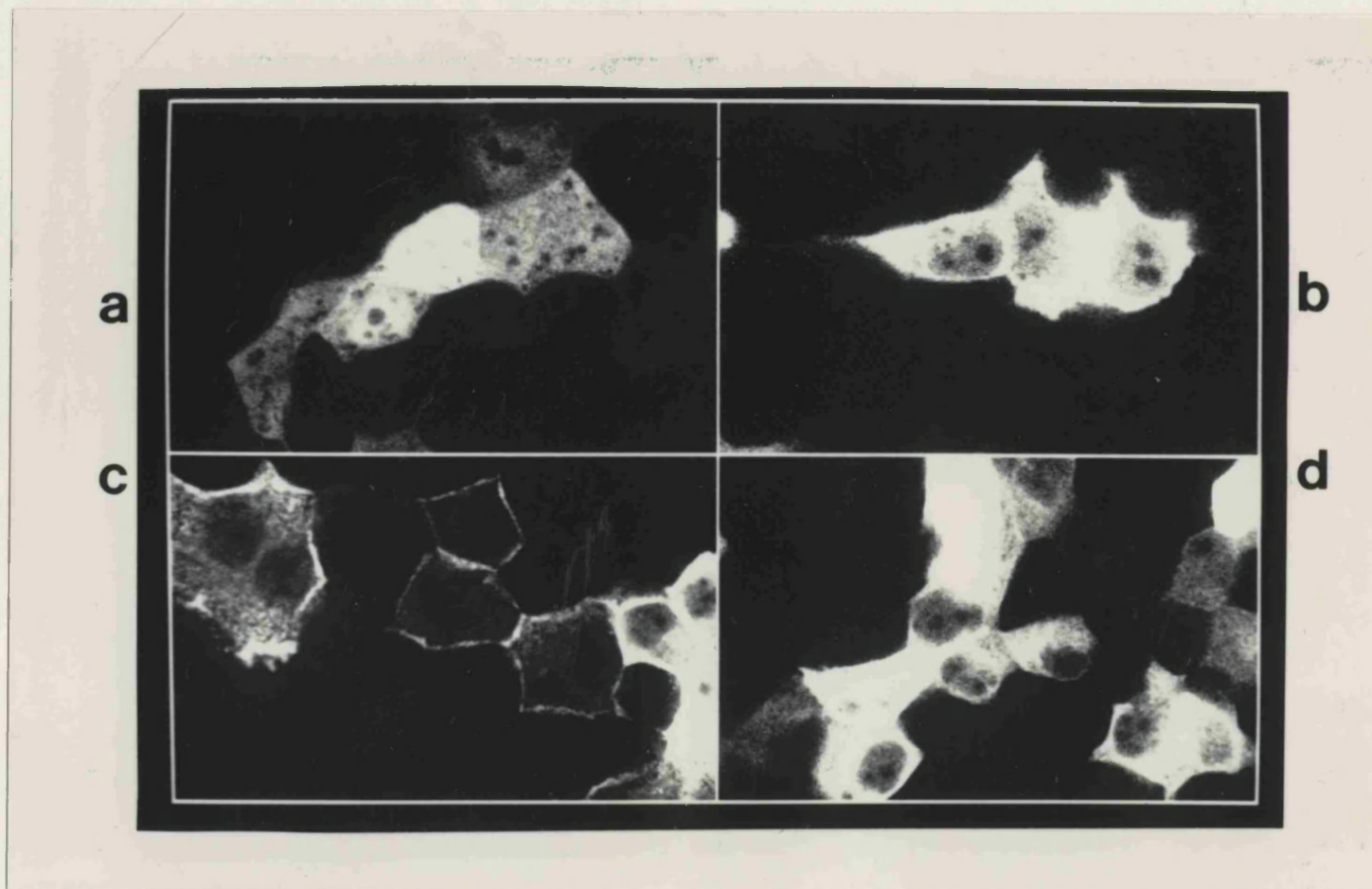
**Figure Legend 2.10.**

**The number of lysines present in the polybasic domain of K-ras affects its cellular location.**

Immunofluorescent analysis of MDCK cells transiently expressing protein A or protein A chimeric proteins following microinjection of plasmid DNA. (a) Protein A with the 17 C-terminal amino acids from a K-ras protein with three glutamine substitutions in the polybasic domain (K3Q) : combination of plasma and cytosolic staining. (b) Protein A with the 17 C-terminal amino acids from a K-ras protein with four glutamine substitutions in the polybasic domain (K4Q) : predominantly cytosolic with some plasma staining. (c) Protein A with the 17 C-terminal amino acids from a K-ras protein with five glutamine substitutions in the polybasic domain (K5Q) : cytosolic staining only. (d) Protein A with the 17 C-terminal amino acids from a K-ras protein with the polylysine domain replaced with polyarginine (K6R) : plasma membrane staining.

**Figure 2.10.**

**The number of lysines present in the polybasic domain of K-ras affects its cellular location.**
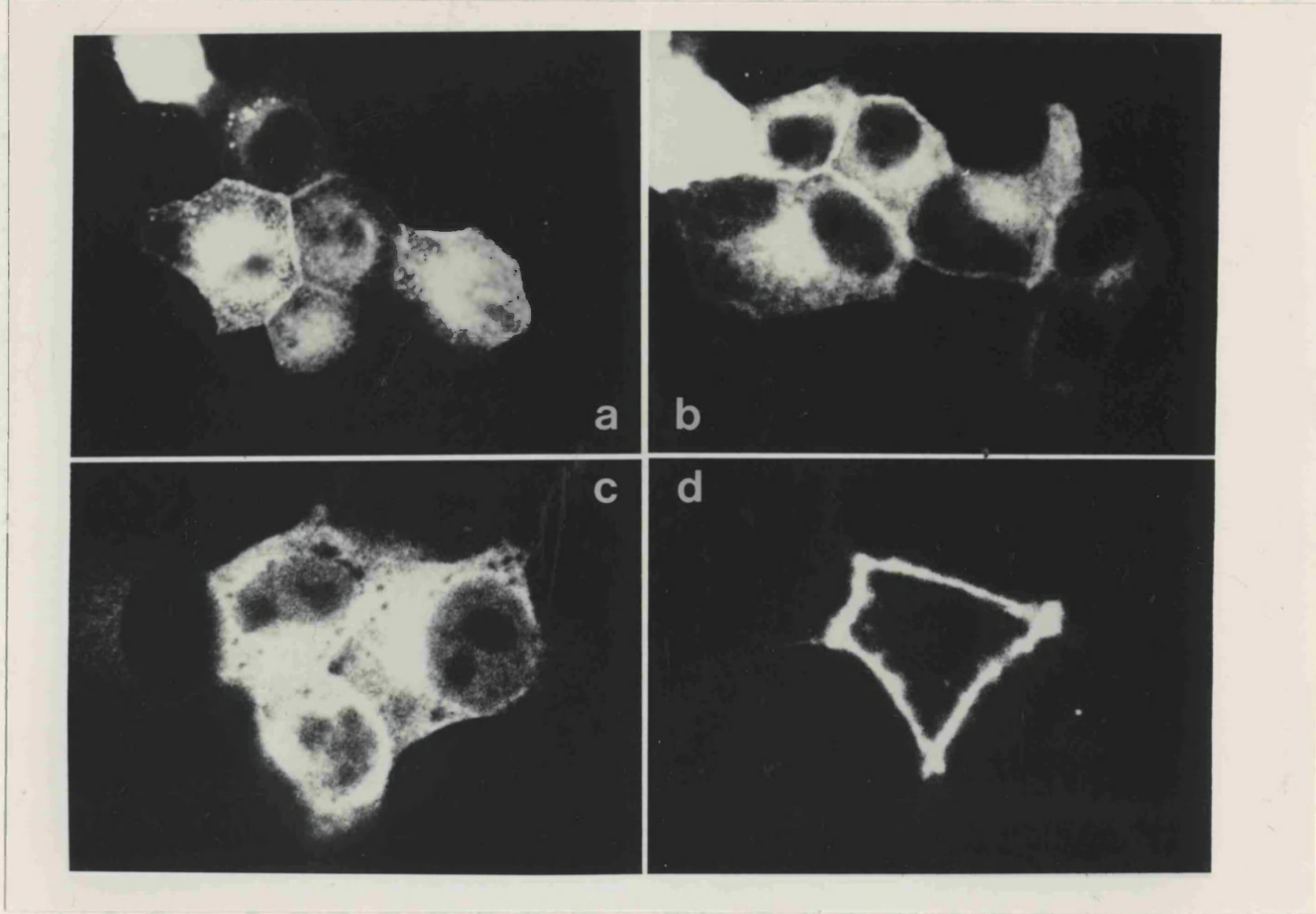
These experiments show that only the CAAX motif and the polybasic domain of K-ras are required to target a heterologous protein to the plasma membrane. They also show that a CAAX motif alone is insufficient to cause membrane association. The presence of the CAAX motif will lead to a farnesylated protein which in the absence of the polybasic domain remains cytosolic.

These results are identical to those obtained with protein A/H-ras chimeras in that membrane localisation of the heterologous protein requires the presence of the CAAX motif together with the upstream palmitoylation sites.

In **Figure 2.11** it can be seen that a protein A chimera with the C-terminal 10 amino acids of H-ras (CAAX motif together with the palmitoylation sites Cys181 and 184) is localised to the plasma membrane. When a similar chimera was made with the cysteine palmitoylation sites mutated to serines, the protein remained cytosolic. These results show that plasma membrane localisation of H-ras requires the CAAX motif modifications together with palmitoylation and that these signals appear to be the only ones necessary to alter the localisation of a normally cytosolic protein.

Protein A was also used to study the C-terminal requirements of the geranylgeranylated K-ras proteins. The 17 C-terminal amino acids of the $C_{20}$ K-ras mutants (CCIL and K6QCCIL) were cloned onto protein A and the subcellular localisation of these proteins was examined by fractionation of electroporated COS cells followed by western blotting. **Figure 2.12** shows that whilst the native protein A is cytosolic (s100) the CCIL and 6CCIL chimeras are both membrane (p100) associated. This experiment shows that whilst farnesylation alone is insufficient to target a heterologous protein to the membrane (p100) fraction, geranylgeranylation is sufficient whether there is a polybasic domain present or not.

## 2.2.11.Polyarginine can substitute for polylysine as a plasma membrane targeting motif for K-ras

The above experiments and previous work (Hancock *et al*, 1990) showed that in order for K-ras to be plasma membrane targeted there is a specific requirement for the presence of both the CAAX motif and the polybasic domain. The polybasic domain consists of six consecutive lysine residues (175 - 180). When all the lysines are mutated to glutamines the protein (K6Q) becomes cytosolic. The question remains as to whether the

**Figure 2.11.**
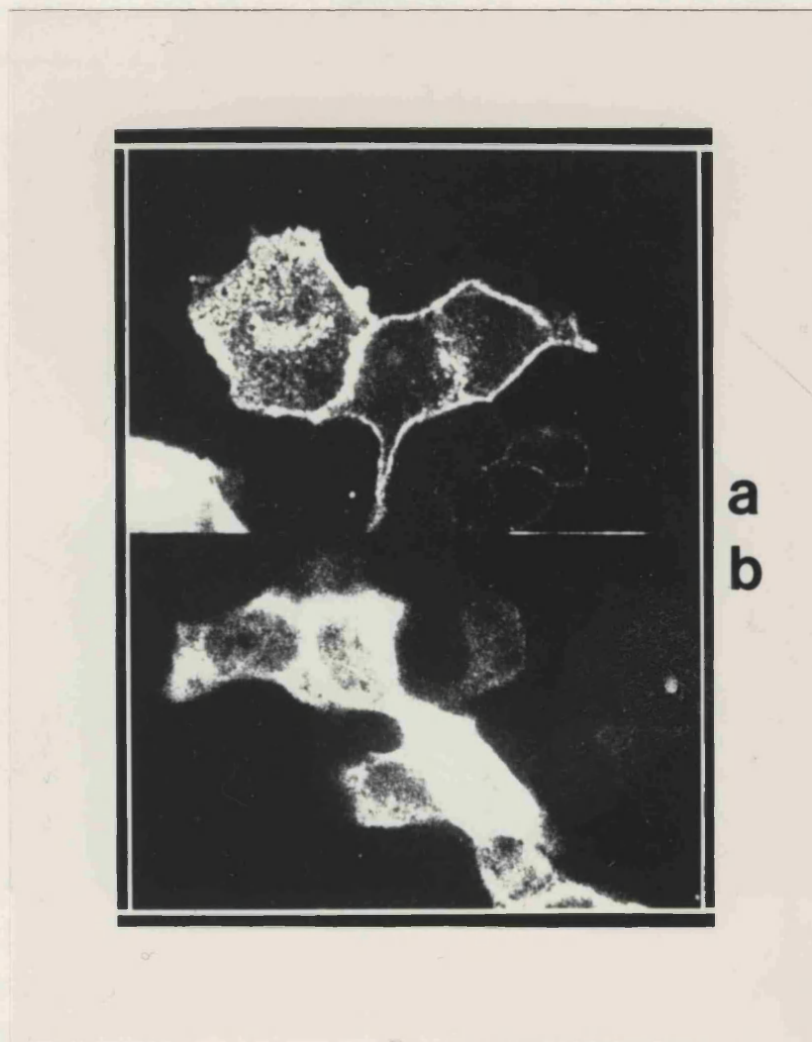


**The CAAX motif together with the upstream palmitoylation sites of H-ras is required for the plasma membrane targeting of protein A.**

Immunofluorescent analysis of MDCK cells transiently expressing protein A chimeric proteins following microinjection of plasmid DNA. (a) Protein A with the C-terminal 10 amino acids from H-ras : plasma membrane staining. (b) Protein A with the C-terminal 10 amino acids from H-ras Cys181/184Ser : cytoplasmic staining only.

**Figure 2.12.**



**COS cell fractionation of protein A and protein A : C20 modified K-ras chimeras.**

COS cells expressing protein A or protein A chimeras with the 17 C-terminal amino acids from K-ras mutant proteins with CCIL C-terminal motifs were fractionated into s100(s) and p100(p) fractions. Equal proportions of each fraction were resolved by SDS-PAGE and Western blotted with an anti-protein A primary antibody. The protein A-CCIL chimera (CCIL) has a wild type polylysine domain, whereas the protein A-6QCCIL chimera (6QCCIL) has had the lysines of this region replaced with glutamines.

polybasic domain interacts with the plasma membrane through a charge/charge interaction or does a 'docking' protein exist in the plasma membrane that recognises this polylysine sequence.

In order to attempt to answer this question, it was decided to mutate the lysines of the polybasic domain to arginines (K6R). This would keep the positive charge of the region intact since arginine is also positively charged and would therefore help to distinguish between a charge/charge interaction and a protein/protein interaction since the latter might be expected to be disrupted by the arginine replacement.

A protein A chimera was made that contained the CAAX motif and the polyarginine domain. **Figure 2.8** shows that when expressed in COS cells that are subsequently fractionated, the 6R protein is membrane (p100) associated. The biological activity of the full length K6R mutant was also tested in NIH 3T3 transformation assays and was found to be identical to K-ras (Val 12) **(Table 2.2)**.

These experiments showed that a polyarginine domain can substitute for the polylysine domain of K-ras in terms of membrane targeting and biological activity. Since substituting one positively charged amino acid for another does not affect the localisation of the protein, this would indicate that the interaction between K-ras and the plasma membrane involves an electrostatic interaction between the positively charged polybasic domain and the negatively charged phospholipid head groups of the plasma membrane. These experiments would appear to discount the theory that there is a specific docking protein that recognises the polylysine domain of K-ras since substituting arginine for lysine did not compromise the function of the domain. Of course there is still the possibility that a docking protein does exist that recognises a region of positive charge rather than a specific amino acid sequence.

## 2.3. Discussion

The Ras proteins form a small group within the Ras superfamily of proteins that are prenylated by the addition of farnesyl ($C_{15}$) rather than the more common method of prenylation, geranylgeranylation with a $C_{20}$ group. Since other proteins are associated with membranous structures other than the plasma membrane, it was of interest to investigate whether the addition of a $C_{20}$ group to K-ras or H-ras would alter its cellular location. To address this question, mutants of K-ras and H-ras were made with CAAX motifs that would signal for the addition of geranylgeranyl rather than farnesyl. The subcellular localisation of these mutant proteins was then examined by fractionation of COS cells expressing these mutant proteins and by immunofluorescence.

Fractionation of electroporated COS cells expressing $C_{20}$ modified K-ras proteins showed that prenylation by a geranylgeranyl group was sufficient to lead to membrane association of the protein. This was the case whether the polybasic domain was present or not. The addition of a $C_{20}$ CAAX motif with and without the polybasic domain of K-ras to protein A also lead to the chimeric proteins becoming p100 associated. With H-ras, the addition of a $C_{20}$ moiety lead to membrane association with or without the upstream palmitoylation sites. This indicates that modification by geranylgeranylation without the second signals of the polybasic domain or palmitoylation is sufficient to lead to membrane association.

The strength of membrane binding of these $C_{20}$ modified proteins was studied by performing 1M salt washes on membrane fractions. It was found that the geranylgeranylated proteins were bound much tighter to the membrane pellet than the farnesylated forms. However farnesylated H-ras binds to membranes with an avidity comparable to that of geranylgeranylated proteins due to the presence of an additional lipid, palmitic acid, near the C-terminus. K-ras binds to membranes with a much lower avidity than H-ras. The differences in strength between H-ras, K-ras and $C_{20}$ modified Ras-related proteins may reflect differences in the functional requirements of the proteins.

The immunofluorescence results show that in order to achieve specific plasma membrane targeting of the $C_{20}$ modified K-ras and H-ras proteins there is requirement for sequences upstream of the CAAX motif. K-ras requires the polybasic domain whilst H-ras requires the presence of the cysteine palmitoylation sites. Therefore although geranylgeranylation of Ras produces a membrane associated protein, without the second signals of

a polybasic domain or palmitoylation sites, these proteins bind nonspecifically to intracellular membranes. This is also suggested by the fact that more than 90% of the geranylgeranylated and polybasic mutated K-ras p100 protein could be solubilised by 0.5% Triton X-100. Since immunofluorescence of these $C_{20}$ modified K-ras and H-ras proteins lacking the second signals fails to associate them with a particular intracellular membrane it may be a non-specific interaction with all available intracellular membranes. These proteins could be associated with the endoplasmic reticulum in a similar manner to Rap2 (Beranger et al, 1991b) however this has not been convincingly established.

It was originally hoped that the geranylgeranylated K-ras with an intact polybasic domain would be localised not to the plasma membrane but to the Golgi since the Golgi localised Rap1 protein is geranylgeranylated and contains a polybasic domain (Beranger et al, 1991a). However the $C_{20}$ modified K-ras mutant proteins with a polybasic domain remained targeted to the plasma membrane. This suggests that there are possibly other domains in Rap1A which direct Golgi localisation or that the spatial relationship between the CAAX motif and the polybasic domain determines the subcellular localisation of the protein. In Rap1A there are two small polybasic domains separated by one amino acid which are only one base away from the CAAX motif. The polybasic domain of K-ras is by contrast, continuous and four residues from the CAAX motif. This led to the idea that by altering the distance between the CAAX motif and the polybasic domain of K-ras, the protein may become Golgi localised or associated with another membranous structure within the cell. The results of these experiments will be discussed in a later chapter. The Golgi localisation of Rab6 has recently been shown to require N-terminal sequences that comprise or include the effector domain (Beranger et al, 1994).

Geranylgeranylated K-ras and H-ras mutants were found to be biologically active in focus assays indicating that the transforming activity of Ras can be supported by farnesyl or geranylgeranyl. It has been observed that geranylgeranylated Gly12 H-ras was a potent inhibitor of cellular proliferation suggesting that normal Ras function is dependent on the presence of a farnesyl moiety (Cox et al, 1992).

To exclude the idea that other domains of Ras besides the CAAX motif and the polybasic domain are involved in membrane targeting, the effect of cloning these regions onto the C-terminus of a heterologous protein, protein A was examined. These experiments showed that the

addition of a CAAX motif together with the polybasic domain of K-ras(B) was sufficient to target the normally cytosolic protein A to the plasma membrane. A CAAX motif in isolation was not sufficient. When similar experiments were performed with H-ras, it was found that the CAAX motif and the palmitoylation sites were the only regions of H-ras required to target protein A to the plasma membrane.

The C-termini of H-ras and K-ras have also been found to target the normally cytoplasmic protein RasGAP (GTPase activating protein) to the plasma membrane (Huang *et al*, 1993a).

To establish a requirement for the polybasic domain in membrane localisation of the protein A/K-ras chimera, the lysines of the polybasic domain were mutated to glutamines. Localisation experiments showed that as the number of glutamines increased, the amount of protein that was membrane associated decreased. These results suggested that the plasma membrane localisation of K-ras was due to a charge/charge interaction between the positively charged lysines of the polybasic domain and the plasma membrane. Alternatively there could be a direct interaction between the polylysine region and a docking protein in the plasma membrane that specifically recognises this region. The construction of a mutant protein that had all of the lysines within the polybasic domain changed to arginines, another positively charged amino acid, helped to answer this question.

The mutant (K6R) was found to be associated with the p100 fraction and had the same biological activity as wild type K-ras (Val 12). This indicates that there is an electrostatic interaction between the positively charged polybasic domain and the negatively charged phospholipid head groups of the plasma membrane rather than there being a specific membrane protein that recognises the polybasic domain unless there is a docking protein that recognises a positively charged sequence within the K-ras protein.

## 2.4. Conclusions

In summary, the results presented in this chapter are that a geranylgeranyl group can substitute for the farnesyl group that normally prenylates K-ras and H-ras in terms of membrane targeting and biological activity. A $C_{20}$ moiety leads to membrane association of the proteins without the need for a second signal. However to achieve plasma membrane targeting rather than non-specific intracellular membrane

association, there is a requirement for a polybasic domain or palmitoylation sites to be present. The CAAX motif and either of these signals are all that is required to target the normally cytosolic protein A to the plasma membrane. The targeting of K-ras to the plasma membrane requires the presence of the polybasic domain. Decreasing the number of lysine residues in this region leads to a cytosolic protein. Substituting the lysines for another positively charged amino acid still leads to membrane association indicating that K-ras associates with the plasma membrane through electrostatic interaction.

# Chapter 3

## Myristoylation of H-ras and K-ras

### 3.1. Introduction

Eukaryotic cellular proteins are frequently modified by the covalent attachment of long chain fatty acids (reviewed in Towler *et al*, 1988b; Grand, 1989). Among the common methods of fatty acid linkage to proteins are the addition of palmitic acid (n-hexadecanoic acid, $CH_3 [CH_2]_{14}CO_2H$) via an ester or thioester bond to serine, threonine or cysteine residues, addition of myristic acid (n-tetradecanoic acid, $CH_3[CH_2]_{12}CO_2H$) to an N-terminal glycine residue via an amide bond or the carboxy terminal addition of a phosphatidyl inositol-containing glycan moiety.

### 3.1.1. Myristoylation

Myristoylated proteins include the product of the c-*src* proto-oncogene, $p60^{c-src}$ (Buss and Sefton, 1985), the catalytic subunit of the cAMP-dependent protein kinase (Carr *et al*, 1982), calcineurin - a protein phosphatase (Aitken *et al*, 1982), various gag proteins (Henderson *et al*, 1983) and the picornavirus family (Chow *et al*, 1987). The ARF proteins, a group of at least 14 Ras-related proteins, are also myristoylated (Kahn *et al*, 1992). The Ras gene product of the Rasheed retrovirus, p29ras, has an extra 59 amino acids at the N-terminus. These amino acids are derived from the helper virus *gag* gene p15 protein (Rasheed *et al*, 1983) and are responsible for the myristoylation of the protein.

Myristoylation occurs on the α amino group of an amino-terminal glycine via an amide bond (Aitken *et al*, 1982; Carr *et al*, 1982; Henderson *et al*, 1983; Ozols *et al*, 1984; Schultz *et al*, 1985). The addition of myristic acid is thought to be a co-translational event as drugs that inhibit protein synthesis completely inhibit myristoylation without a detectable time lag (Magee and Courtneidge, 1985; Olson and Spizz, 1986; Wilcox *et al*, 1987). Myristoylation is a stable protein modification having the same half life as the protein itself (Buss *et al*, 1984).

### 3.1.2. Myristoyl CoA : protein N-myristoyl transferase

The enzyme responsible for myristoylation - myristoyl CoA : protein N-myristoyl transferase (NMT) has a specific requirement for myristoyl CoA produced from myristate by acyl -CoA synthetase in the presence of ATP (Towler and Glaser, 1986). The initiating methionine is removed prior to

acylation by a methionine aminopeptidase (Towler *et al*, 1987a) leaving an N-terminal glycine which has been shown to be an absolute requirement for myristoylation (Towler *et al*, 1987b). The amino acid (position 2) following the glycine (position 1) is generally an uncharged residue (Ala, Ser, Asn, Gln, Val, Leu) whereas positions 3 and 4 have less stringent requirements and can be acidic, basic, bulky, hydrophobic or small, uncharged residues. Position 5 has to be a small uncharged residue (Ala, Ser, Thr, Cys, Asn and Gly) but marked preference is shown for Ser or Thr (Towler *et al*, 1988b).

NMT has been isolated from yeast (Towler *et al*, 1987a) and rat tissue (Towler *et al*, 1988b). The yeast NMT is a single polypeptide chain of 55KDa, has an optimum pH of 7.5 - 8.0 and does not require metal cations (Towler *et al*, 1987a). The yeast and rat enzymes appear to have different specificities which presumably reflects the different substrates encountered *in vivo* in higher and lower eukaryotes. NMT has been isolated in both cytoplasmic and membrane fractions of yeast and mammalian cells (Towler and Glaser, 1986; Towler *et al*, 1987a; 1988a).

### 3.1.3. Myristoylation leads to different intracellular locations

Acylation by palmitoylation, myristoylation or phospholipid addition can lead to membrane associated proteins. Myristoylation of p60$^{src}$ has been shown to be essential for both membrane association and transforming ability (Buss *et al*, 1986; Cross *et al*, 1984; Kamps *et al*, 1986). However not all myristoylated proteins are membrane associated, a number of them are soluble and cytoplasmic (Magee and Courtneidge, 1985; McIlhinney *et al*, 1985; Olson *et al*, 1985). For example the catalytic subunit of cAMP-dependent protein kinase behaves as a soluble protein once released from its regulatory subunits (Nairn *et al*, 1985).

Different myristoylated proteins localise to different cellular membranes. p60$^{v-src}$ is plasma membrane associated (Garber *et al*, 1985; Krueger *et al*, 1983) whereas cytochrome b5 reductase is bound to the endoplasmic reticulum (Ozols *et al*, 1984). The ARF proteins are involved in membrane trafficking (Kahn *et al*, 1991; Tsuchiya *et al*, 1991) and would therefore be expected to have a wide range of cellular locations implying that other regions of the protein besides the N-terminal myristate, determine localisation. The N-terminal domain adjacent to the myristoylation site shows significant divergence between ARFs (Tsuchiya *et al*, 1991). This region forms an amphipathic helix whose folding is potentiated by the myristate group (Kahn *et al*, 1992). Co-operation between a myristate group and nearby protein sequences could lead

to membrane association by simple partitioning into the membrane or these signals could be recognised by a membrane receptor protein.

Myristoylated proteins therefore contain other regions which are responsible for determining the cellular location of the protein although myristoylation does play a role in the membrane association of some proteins. Three N-terminal lysine residues have been identified in pp60$^{v\text{-}src}$ which along with myristoylation are crucial for membrane association (Silverman and Resh, 1992; Silverman et al, 1993). These lysines were also found to be conserved among several Src family members.

Several membrane proteins (protein kinase C, HIV gag) have been shown to have clusters of basic amino acids that form electrostatic interactions with negatively charged phospholipids (Taniguchi and Manenti, 1993; Zhou et al, 1994). The myristic acid could co-operate with the basic amino acids to promote binding of pp60$^{src}$ to the lipid bilayer.

Schultz and co-workers (1985) proposed that specific membrane receptors existed for myristoylated proteins. Resh (1989) identified a receptor that bound to the extreme amino-terminal domain of a pp60$^{v\text{-}src}$ polypeptide and was specific for p60$^{src}$ rather than being a 'myristate receptor'. However further experiments identified this 'p60$^{src}$ receptor' as the mitochondrial ADP/ATP carrier (AAC) (Sigal and Resh, 1993). This protein specifically binds a myristoyl-src peptide both in vitro and in vivo, however it is localised to the inner surface of the mitochondria and it is therefore not a receptor for the plasma membrane localised pp60$^{src}$.

The Gibbs free energy for the binding of a myristoylated peptide to a phospholipid vesicle has been calculated to be 8kcal/mol (Peitzsch and McLaughlin, 1993), equivalent to an apparent $K_D$ of $10^{-4}$M which is insufficient to anchor a myristoylated protein to the lipid bilayer. Therefore myristoylation alone is insufficient to bring about membrane association and other processes or interactions must be involved.

It has now been shown that Src-related proteins can undergo palmitoylation at N-terminal cysteine residues following N-terminal myristoylation. This has been shown for p56$^{lck}$, p57$^{fyn}$ and myristoylated $\alpha$ subunits of heterotrimeric G proteins (Linder et al, 1993; Mumby et al, 1994; Paige et al, 1993; Parenti et al, 1993; Shenoy-Scaria et al, 1993; Wedegaertner et al, 1993). The sequence for palmitoylation appears to be Myr - Gly - Cys and this is found in seven out of nine Src family members (Resh, 1994). Since palmitoylation is a reversible process this may account for the ability of Src

proteins to interact with multiple membrane bound targets and to be involved in multiple signal transduction pathways.

### 3.1.4. Directing localisation of Ras by myristoylation

The p60$^{v\text{-}src}$ protein and several gag proteins are associated with the plasma membrane. The addition of myristic acid to their N-termini has been shown to be essential for this localisation (Buss et al, 1984; Cross et al, 1984). Fusion of the first 14 amino acids of p60$^{v\text{-}src}$ to the amino-terminus of two heterologous proteins, Fujimami Sarcoma Virus p93$^{fps}$ and Chimpanzee α-globin produced membrane associated proteins (Pellman et al, 1984). The addition of myristic acid to a normally non-myristoylated protein can therefore alter its cellular location and lead to its membrane association.

Several workers have investigated the effect of N-terminal myristoylation on H-ras (Buss et al, 1988; 1989; Lacal et al, 1988). At the time of these studies H-ras was known to be associated with the plasma membrane and that this association was necessary for biological function. Membrane localisation was thought to involve the addition of palmitic acid to the cysteine at position 186 towards the C-terminus of the protein. Mutation of this residue to serine produced a non palmitoylated, non transforming, cytosolic protein (Willumsen et al, 1984a; 1984b). Hence it was assumed that membrane association of H-ras was due to the addition of palmitic acid to the cysteine at position 186.

Addition of N-terminal sequences from p60$^{src}$ or Rasheed Ras produced myristoylated H-ras. The myristoylation sequences were also added to the Ser186 mutants and it was found that the myristoylated Ser186 mutants were now membrane (p100) associated and were also biologically active. It was also found that myristoylated forms of wild type H-ras became transforming. These experiments therefore suggested that H-ras could be directed to the plasma membrane without C-terminal processing provided that there was N-terminal myristoylation.

It is now known that H-ras undergoes other post-translational processing besides palmitoylation to achieve its plasma membrane localisation. Palmitoylation of cysteines 181 and 184 only occurs after the CAAX processing events have taken place at cysteine 186. In the light of this, it was decided to construct a variety of myristoylated mutants with different C-termini mutations to investigate the involvement or any co-operation that might exist between myristoylation, CAAX processing and/or palmitoylation.

In the previous chapter it was shown that geranylgeranylation could replace the need for farnesylation in targeting Ras to the plasma membrane

only in the presence of a polybasic domain or palmitoylation. It was therefore of interest to determine whether the correct targeting of myristoylated Ras was also dependent on the presence of the signals that are located in the hypervariable region. Geranylgeranylation was also shown to produce biologically active Ras. The ability of myristoylation to support Ras biological activity was also examined. This was of special interest since it had previously been reported that myristoylation restores focus forming activity to biologically inactive H-ras G12V, C186S and surprisingly oncogenically activates H-ras Gly12, C186S (Buss *et al*, 1989; Lacal *et al*, 1988).

During the course of this work an assay was developed that used MAP kinase activation as a read out for Ras activity. This assay relies on the activation of Raf by activated Ras (Moodie *et al*, 1993; Vojtek *et al*, 1993; Warne *et al*, 1993; Zhang *et al*, 1993). Activated Raf then phosphorylates and activates MEK which in turn phosphorylates and activates MAP kinase (Macdonald *et al*, 1993). p42 MAP kinase has been found to be the major MEK kinase activity (>90%) in the cytosol of Ras expressing serum starved COS cells (Macdonald and Hancock, unpublished data). By using kinase dead MEK as a MAP kinase substrate, the phosphorylation of MEK kinase can be measured and used as a readout for Ras activity. The radioactivity incorporated into MEK is measured after incubating cytosolic fractions from electroporated COS cells with kinase dead MEK immobilised on beads and $[^{32}P]\gamma$ ATP. This assay was used to attempt to define a relationship between biological activity, the type of lipid modification undergone and the localisation of the protein.

The effect of myristoylation on K-ras(B) was also studied to see if the polybasic domain had any effect on the localisation pattern shown by these mutants.

## 3.2. Results

### 3.2.1. Construction of myristoylated H-ras mutants

Myristoylated H-ras was made by using the polymerase chain reaction (PCR) to introduce a myristic acid coding sequence onto the N-terminus of H-ras. The myristoylation signal used to make the H-ras mutants consisted of the first 6 amino acids (MGQSLT) from Rasheed Ras, a myristoylated Ras protein (Schultz and Oroszlan, 1984), linked to the second amino acid of H-ras (**Table 3.1**). There was also a Sac 1 restriction site engineered onto the PCR oligonucleotide in order to make later cloning easier.

A PCR was performed using an oligonucleotide containing a myristic acid coding sequence at the N-terminus of Ras together with an anti-sense C-terminal H-ras oligo and H-ras cDNA as the template. After the

**Table Legend 3.1.**

**Co- and post-tramslational processing of myristoylated H-ras mutant proteins.**

Table 1 shows the nomenclature and C-terminal sequences of the mutant H-ras proteins used in this study. The cysteine palmitoylation sites are residues 181 and 184. The lipid modifications on each protein are indicated with + signs.

# Table 3.1.

**Co- and post-translational processing of myristoylated H-ras mutant proteins.**

The myristic acid signal cloned onto the N-terminus of H-ras had the following amino acid sequence:

M - G - Q - S - L - T

The PCR oligo used to generate these mutants had the following sequence:

5'GAGCTCACCATGGGTCAATCTCTTACAACCGAATACAAGCTTGTTGTT'3

| H-ras mutant | C-terminal sequence | | | | | | | | | | | | | Processing | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | myristoyl | palmitoyl | farnesyl |
| H-ras | S | G | P | G | C | M | S | C | K | C | V | L | S | - | + | + |
| C181S,C184S | - | - | - | - | S | - | - | S | - | - | - | - | - | - | - | + |
| C186S | - | - | - | - | - | - | - | - | - | S | - | - | - | - | - | - |
| MH-ras | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | + |
| MC186S | - | - | - | - | - | - | - | - | - | S | - | - | - | + | + | - |
| MC181S,C184S | - | - | - | - | S | - | - | S | - | - | - | - | - | + | - | + |
| MC181S,C184S,C186S | - | - | - | - | S | - | - | S | - | S | - | - | - | + | - | - |

PCR was performed the PCR fragment was digested with Sac 1 and Nco 1 and cloned into the EXV-3 mammalian expression vector together with the C-terminus of H-ras. After cloning and detecting correct recombinants by diagnostic restriction digests, the presence of the in frame myristic acid sequence and the C-terminus of H-ras was confirmed by sequencing.

Having made a myristoylated Val12 H-ras mutant it was then possible to make myristoylated H-ras mutants with different C-terminal mutations which affect Ras processing and therefore Ras localisation. One construct (myristoylated H-ras C181S,C184S,C186S) had all of the C-terminal processing sites of H-ras removed by mutating the cysteine (186) of the CAAX motif and the upstream cysteines (181 and 184) that are normally palmitoylated, to serines. This mutant could therefore only undergo myristoylation and no CAAX processing or palmitoylation could occur. Other mutants had either a CAAX mutation (C186S) or had the palmitoylation sites mutated (C181S, C184S). By using this panel of mutants it was therefore possible to determine if myristoylation of H-ras in the absence of Ras post-translational modifications was sufficient to target plasma membrane localisation or whether myristoylation combined with farnesylation and/or palmitoylation was required.

The original myristoylated H-ras (Val12) DNA was also used to construct wildtype (Gly12) myristoylated mutants.

### 3.2.2. COS cell studies on myristoylated H-ras

The myristoylated H-ras mutants and the non-myristoylated controls were electroporated into COS cells and various experiments were performed to examine the subcellular distribution of the proteins and acylation events.

### 3.2.2.1. Co-translational versus post-translational modifications

To confirm that the myristoylated constructs were capable of being N-terminally myristoylated, transfected COS cells were labelled with [3H] myristic acid, lysed in Triton X114, immunoprecipitated and resolved by SDS-PAGE. **Figure 3.1** shows that all of the myristoylated constructs incorporated [3H] myristic acid whereas the non-myristoylated controls did not.

As myristoylation had previously been shown to be a co-translational modification, the effect of myristoylation on the distribution of Triton X114 lysed Ras protein between the aqueous and detergent fractions was

**Figure Legend 3.1.**

**Processing of myristoylated H-ras proteins.**

COS cells expressing H-ras proteins were pulse labelled with [$^{35}$S] methionine and either lysed immediately in Triton X114 (P) or chased for 4 hours with cold methionine before lysis (C). The lysates were partitioned into aqueous (a) and detergent (d) phases, immunoprecipitated and resolved by SDS-PAGE. As myristoylation is a co-translational event, the myristoylated proteins show detergent partitioning after pulse labelling. The post-translational processing of H-ras results in the protein fractionating into the detergent phase only after a cold chase. The H-ras C186S mutant remains in the aqueous phase even after the cold chase because CAAX processing has been abolished.

An aliquot of cells was labelled with [$^3$H] myristic acid and then lysed in Triton X114 before being immunoprecipitated and resolved by SDS-PAGE. Myristic acid labelling is shown by all the myristoylated mutants whereas no incorporation is observed in the non-myristoylated proteins (lane m).

| | | |
|---|---|---|
| H | = | H-ras |
| MH | = | myristoylated H-ras |
| H1/4S | = | H-ras C181S,C184S |
| M1/4S | = | myristoylated H-ras C181S,C184S |
| M1/4/6S | = | myristoylated H-ras C181S,C184S,C186S |
| H186S | = | H-ras C186S |
| M186S | = | myristoylated H-ras C186S |

# Figure 3.1.
## Processing of myristoylated H-ras proteins.
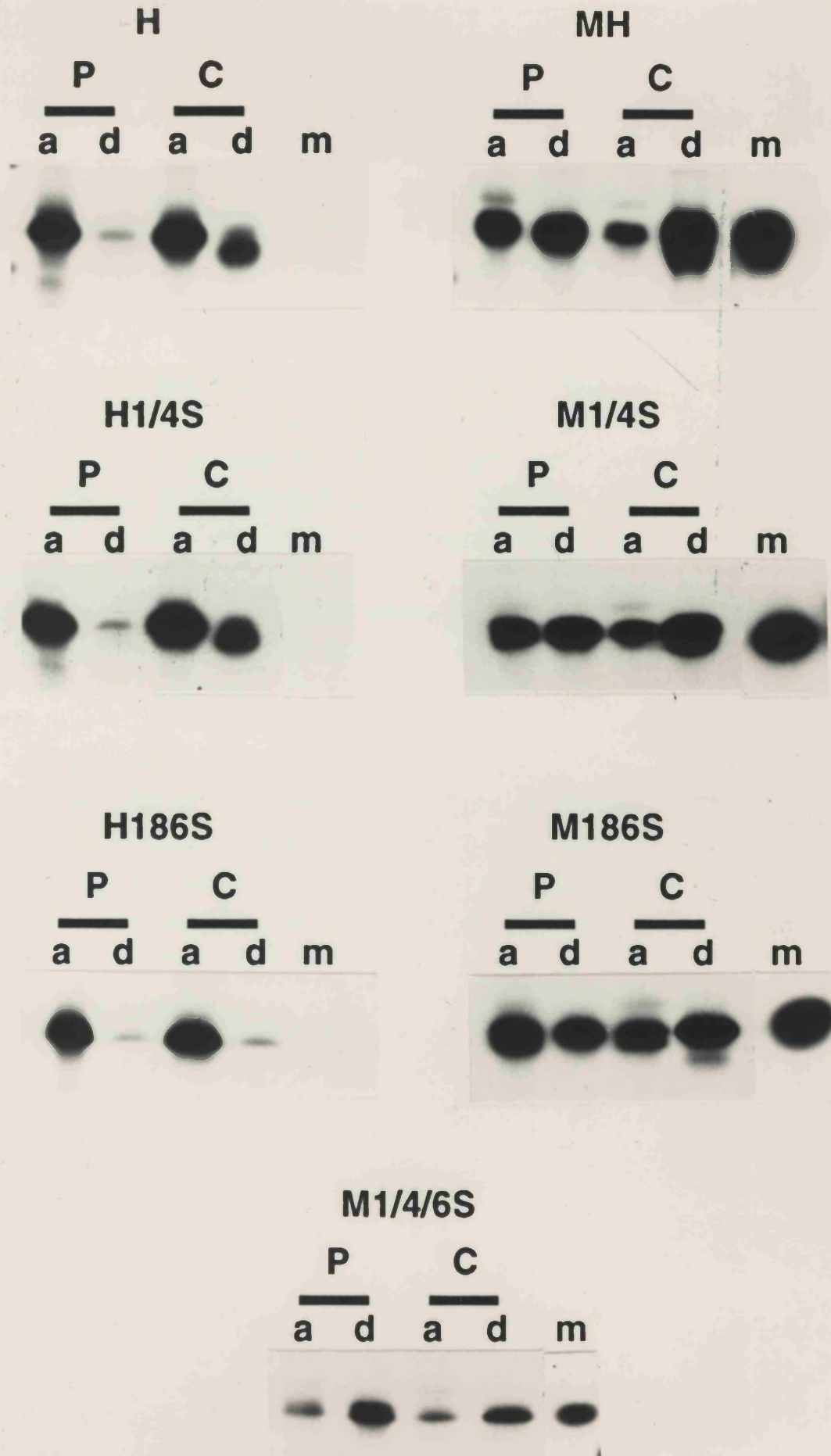
examined. COS cells expressing the proteins were pulse labelled with [$^{35}$S] methionine and lysed in Triton X114. The lysates were then partitioned into aqueous and detergent fractions by heating. The fractions were immunoprecipitated and resolved by SDS-PAGE. Another sample of cells from the same transfection was incubated in cold methionine containing medium (DMEM) for 4 hours after the pulse label. These samples were also lysed in Triton X114 and separated into aqueous and detergent phases. Hydrophilic proteins partition into the aqueous phase of Triton X114 whereas lipidated, hydrophobic proteins partition into the detergent phase.

Figure 3.1 shows that there was a marked difference in the distribution of the myristoylated and non-myristoylated proteins when labelled with [$^{35}$S] methionine and harvested in Triton X114 with and without a 4 hour cold chase. All the myristoylated proteins irrespective of the type of C-terminal present showed detergent partitioning after a pulse label. This indicated that myristoylation was occurring co-translationally and the addition of myristic acid leads to detergent partitioning of the protein after a pulse label.

The experiment in Figure 3.1 shows that H-ras proteins with an N-terminal myristoylation sequence were 50 - 80% co-translationally lipidated. Therefore the N-terminal MGQSLT sequence results in efficient myristoylation of H-ras.

In contrast, the non-myristoylated proteins only show detergent partitioning after the 4 hour cold chase. This is because Ras processing events occur post-translationally as opposed to co-translationally. The Ser186 mutant does not show detergent partitioning even after a chase period because this protein can not undergo lipidation and the protein therefore remains in the aqueous phase.

### 3.2.2.2.Fractionation of myristoylated H-ras

Transfected COS cells were harvested after an 18 hour label with [$^{35}$S] methionine and fractionated into s100 and p100 fractions. Equal proportions of each fraction were immunoprecipitated and separated by SDS-PAGE. Figure 3.2 shows that when the myristoylated H-ras C181S, C184S, C186S protein was fractionated, 90% of the protein was associated with the s100 (cytosolic) fraction. As this protein has all the CAAX processing (Cys186) and the palmitoylation sites (Cys181 and 184) mutated to serines, the only modification that can occur is myristoylation. This

**Figure Legend 3.2.**

**Subcellular fractionation of myristoylated H-ras proteins.**

COS cells expressing Ras proteins were metabolically labelled for 18 hours with [$^{35}$S] methionine and fractionated into p100 (p) and s100 (s) fractions. Equivalent proportions of each fraction were immunoprecipitated, resolved by SDS-PAGE and visualised by autoradiography.

| | | |
|---|---|---|
| H | = | H-ras |
| MH | = | myristoylated H-ras |
| H1/4S | = | H-ras C181S,C184S |
| M1/4S | = | myristoylated H-ras C181S,C184S |
| M1/4/6S | = | myristoylated H-ras C181S,C184S,C186S |
| H186S | = | H-ras C186S |
| M186S | = | myristoylated H-ras C186S |

**Figure 3.2.**
**Subcellular fractionation of myristoylated H-ras proteins.**
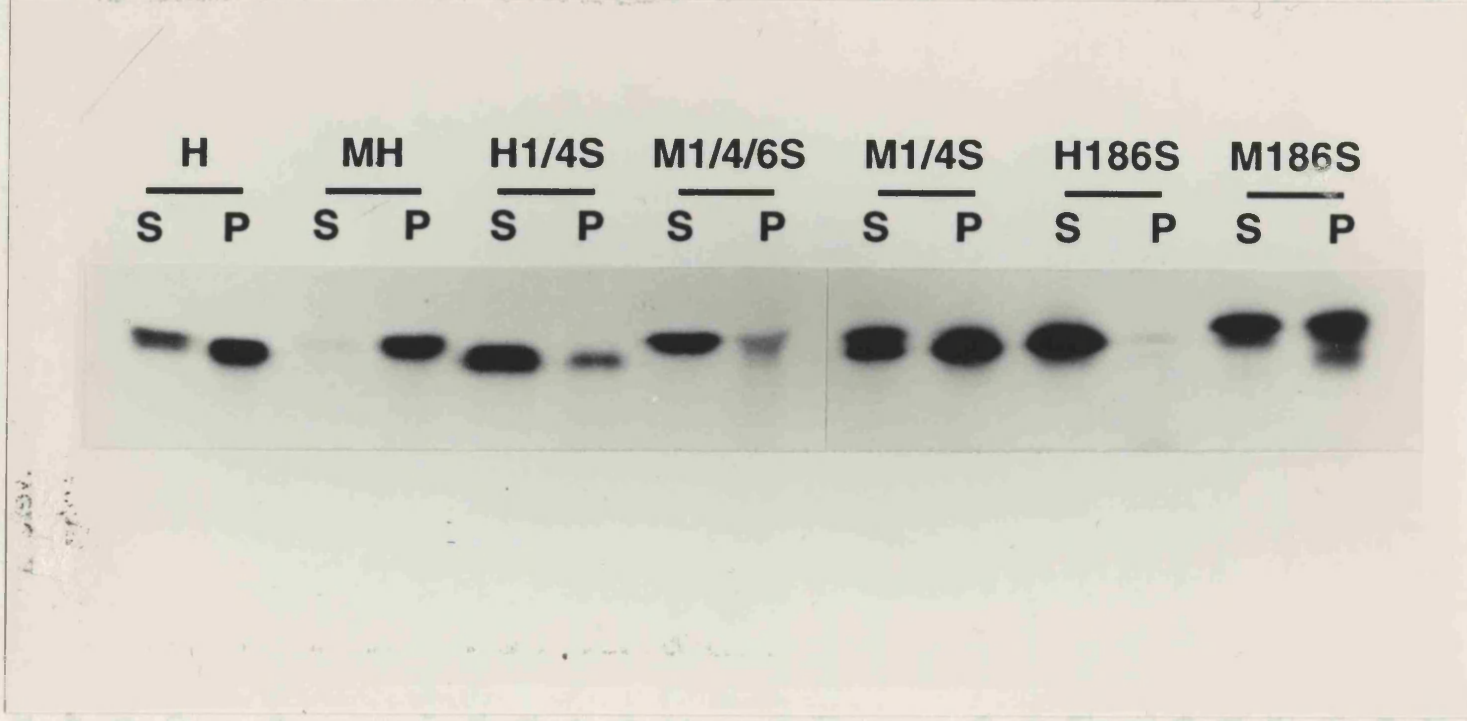
showed that myristoylation alone was insufficient to target H-ras to the membrane fraction and that other processing events or localisation signals are required.

All the other myristoylated constructs showed p100 association. This was in marked contrast with the non-myristoylated parent proteins. Both H-ras C186S and H-ras C181S,C184S were cytosolic because either CAAX processing and therefore palmitoylation was blocked (H-ras C186S), or CAAX processing could take place but palmitoylation could not (H-ras C181S,C184S). Myristoylation of both of these proteins appeared to restore p100 association.

As expected normal H-ras and its myristoylated counterpart were both p100 associated.

### 3.2.2.3.Myristoylation can replace the need for farnesylation to occur before palmitoylation of H-ras can take place

The myristoylated H-ras C181S,C184S,C186S and the myristoylated H-ras C186S mutants were expected to show the same partitioning pattern in that they were both expected to be cytosolic whereas in fact the myristoylated H-ras C186S mutant was membrane associated. Normally the H-ras C186S protein is cytosolic as not only is CAAX processing abolished by this mutation but palmitoylation cannot occur since farnesylation is a prerequisite for palmitoylation (Hancock et al, 1989). It was therefore a possibility that the addition of myristic acid at the N-terminus might functionally replace the need for C-terminal prenylation and allow palmitoylation at cysteines 181 and 184 in the myristoylated H-ras C186S mutant, hence its membrane localisation.

To test this theory, transfected COS cells were labelled with [$^3$H] palmitic acid before being immunoprecipitated and resolved by SDS-PAGE. **Figure 3.3** shows that both normal H-ras and the myristoylated H-ras C186S proteins could be labelled with palmitic acid whereas the myristoylated H-ras C181S,C184S,C186S protein remained unlabelled. This suggested that N-terminal myristoylation can replace the requirement for C-terminal prenylation to occur before palmitoylation of H-ras can take place. The extent of palmitoylation of the myristoylated H-ras C186S is less than that seen with wild type H-ras suggesting that the acyltransferase works less efficiently on myristoylated H-ras than on farnesylated H-ras.

**Figure 3.3.**



**Myristoylated H-ras C186S undergoes palmitoylation.**
COS cells expressing H-ras proteins were labelled for 4 hours with [$^3$H] palmitic acid, lysed in Triton X114, immunoprecipitated and resolved by SDS-PAGE.

| | | |
|---|---|---|
| H | = | H-ras |
| M186S | = | myristoylated H-ras C186S |
| M1/4/6S | = | myristoylated H-ras C181S,C184S,C186S |

These myristoylated H-ras proteins therefore become membrane (p100) associated by virtue of N-terminal myristoylation together with either CAAX processing or palmitoylation.

### 3.2.3. Immunofluorescence studies in MDCK cells to determine the precise subcellular location of the myristoylated H-ras proteins

Immunofluorescence experiments using MDCK cells expressing the mutant proteins following microinjection of plasmid DNA showed that the myristoylated proteins were only plasma membrane associated if the protein underwent palmitoylation as well as myristoylation. **Figure 3.4** shows that both myristoylated H-ras (which is additionally palmitoylated and farnesylated) and myristoylated H-ras C186S (which is additionally palmitoylated) are plasma membrane localised whereas myristoylated H-ras C181S,C184S (which is additionally farnesylated) binds almost exclusively to intracellular membranes including the nuclear membrane. The myristoylated H-ras C181S,C184S,C186S mutant showed a classic soluble staining pattern although when highly expressed some protein appears bound to cellular membranes including the plasma and nuclear membrane (**Figure 3.4**). However this must represent a very weak interaction since >90% of the myristoylated H-ras C181S,C184S,C186S protein fractionates to the s100 fraction when cells are lysed (**Figure 3.2**).

It therefore seems that palmitoylation is essential to target H-ras to the plasma membrane whereas other lipid modifications will lead to nonspecific membrane association.

### 3.2.4. Construction of myristoylated K-ras mutants

A strategy similar to that used to produce the myristoylated H-ras mutants was used to make the myristoylated K-ras mutants. The same myristoylation sequence was introduced onto the N-terminus of K-ras using PCR and the PCR product was then cloned into the EXV-3 mammalian expression vector along with different C-terminal K-ras mutants (**Table 3.2**).

Again versions were made that would not undergo CAAX processing (C186S) or did not possess the polybasic domain (K[175 - 180]Q or K6Q mutation). Wild type (Gly12) and activated (Val12) versions of all the different mutants were made.

**Figure Legend 3.4.**

**Subcellular location of myristoylated H-ras proteins as determined by immunofluorescence.**

Confocal images of MDCK cells transiently expressing myristoylated H-ras proteins following microinjection of plasmid DNA.

A. Myristoylated H-ras : plasma membrane staining
B. Myristoylated H-ras C181S,C184S : practically all plasma membrane staining is lost and replaced with staining of intracellular membranes including the nuclear membrane
C. Myristoylated H-ras C181S,C184S,C186S : extensive cytoplasmic staining consistent with predominant cytosolic localisation, there is however some highlighting of plasma and nuclear membranes
D. Myristoylated H-ras C186S : plasma membrane staining

Figure 3.4.

Subcellular location of myristoylated H-ras proteins as determined by immunofluorescence.
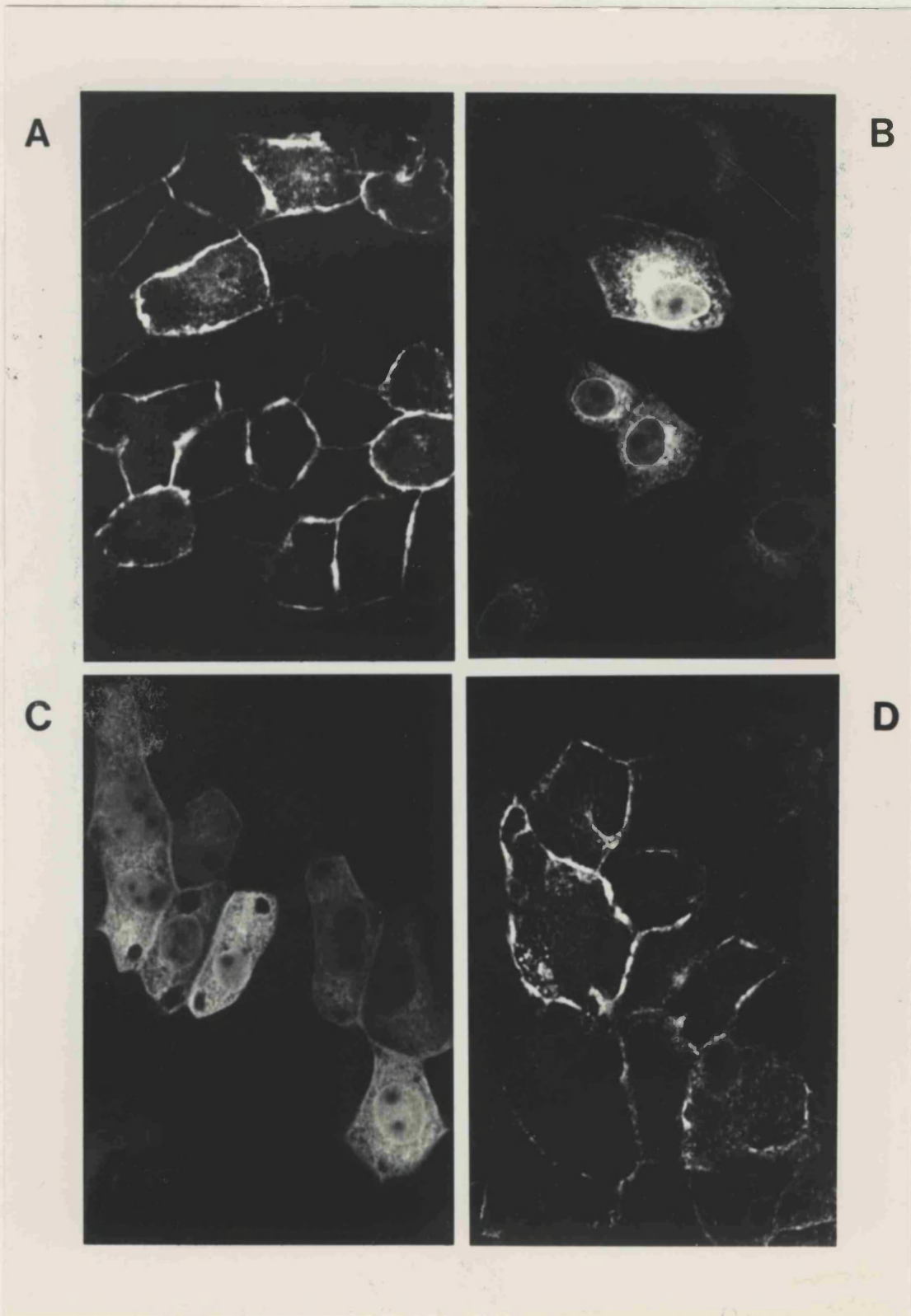
**Table Legend  3.2.**

Co-  and post-translational processing  of  myristoylated  K-ras  mutant
proteins.

Table 2 shows the nomenclature and C-terminal sequences of the mutant K-
ras proteins used in this study. The lipid modifications on each protein are
indicated by a + sign. The polybasic domain comprises residues 175 - 180.

# Table 3.2.

**Co- and post-translational processing of myristoylated K-ras mutant proteins.**

The myristic acid signal cloned onto the N-terminus of K-ras had the following amino acid sequence :

M - G - Q - S - L - T

The PCR oligo used to generate these mutants had the following sequence:

5'GAGCTCACCATGGGTCAATCTCTTACAACTGAATATAAACTTGTGGTA'3

| K-ras mutant | C-terminal sequence | | | | | | | | | | | | | | | | Processing myristoyl | farnesyl |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| K-ras | E | G | K | K | K | K | K | K | S | K | T | K | C | V | I | M | - | + |
| K6Q | - | - | Q | Q | Q | Q | Q | Q | - | - | - | - | - | - | - | - | - | + |
| KC186S | - | - | - | - | - | - | - | - | - | - | - | - | S | - | - | - | - | - |
| MK | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + |
| MK6Q | - | - | Q | Q | Q | Q | Q | Q | - | - | - | - | - | - | - | - | + | + |
| MKC186S | - | - | - | - | - | - | - | - | - | - | - | - | S | - | - | - | + | - |

### 3.2.5. Cellular distribution of myristoylated K-ras

### 3.2.5.1.The mutants are myristoylated

A Triton X114 assay was performed on the myristoylated and non-myristoylated K-ras proteins to examine differences between co-translational and post-translational lipid modifications that may occur on these proteins. Electroporated COS cells were pulse labelled with [$^{35}$S] methionine and lysed immediately in Triton X114 or chased with cold methionine for 4 hours before lysis. The Triton X114 lysates were heated to separate the detergent and aqueous phases and these were then immunoprecipitated and resolved by SDS-PAGE. **Figure 3.5** shows that the myristoylated K-ras proteins are co-translationally lipidated since the proportion partitioning into the detergent phase after a pulse label is much higher (80%) than for non-myristoylated K-ras. The non-myristoylated K-ras proteins show a greater proportion of detergent partitioning protein after a pulse label than H-ras. This is due to K-ras being a better substrate for farnesyltransferase than H-ras (Reiss et al, 1990). The K-ras C186S mutant remains aqueous following the cold chase since this protein is blocked for C-terminal farnesylation whereas the myristoylated K-ras C186S protein is 80% detergent partitioning due to myristoylation.

All the myristoylated K-ras proteins showed incorporation of label from [$^{3}$H] myristic acid (**Figure 3.6**) confirming that the co-translational modification of these proteins is myristoylation.

### 3.2.5.2.Fractionation of myristoylated K-ras mutants

Transfected COS cells were fractionated into s100 and p100 fractions and equal proportions were immunoprecipitated before being resolved by SDS-PAGE. **Figure 3.7** shows that all the myristoylated K-ras mutants were p100 associated. The non-myristoylated K-ras C186S and the K[175-180]Q mutants were associated with the s100 fraction. This showed that with the non-myristoylated proteins, CAAX processing is required together with the polybasic domain in order to achieve membrane localisation. When K-ras is myristoylated, the presence of the polybasic domain or the cysteine at position 186 is required to achieve p100 association.

**Figure Legend 3.5.**
**Processing of myristoylated K-ras proteins.**

Triton X114 partitioning assays were carried out as described in **Figure 3.1.** K-ras is a considerably better substrate for farnesyltransferase than H-ras (Reiss *et al*, 1990). Some post-translational processing of newly synthesised K-ras takes place during the 10 minute pulse accounting for the presence of some detergent partitioning K-ras and K-ras K6Q protein in the pulse fractionation. The proportion of MK and MK6Q that partition into the detergent phase of the pulse sample is much higher (80%), consistent with efficient myristoylation. The MK186S protein is 80% myristoylated.

|         |   |                                |
|---------|---|--------------------------------|
| K       | = | K-ras                          |
| MK      | = | myristoylated K-ras            |
| K186S   | = | K-ras C186S                    |
| MK186S  | = | myristoylated K-ras C186S      |
| K6Q     | = | K-ras K[175 - 180]Q            |
| MK6Q    | = | myristoylated K-ras K[175 - 180]Q |

# Figure 3.5.
## Processing of myristoylated K-ras proteins.
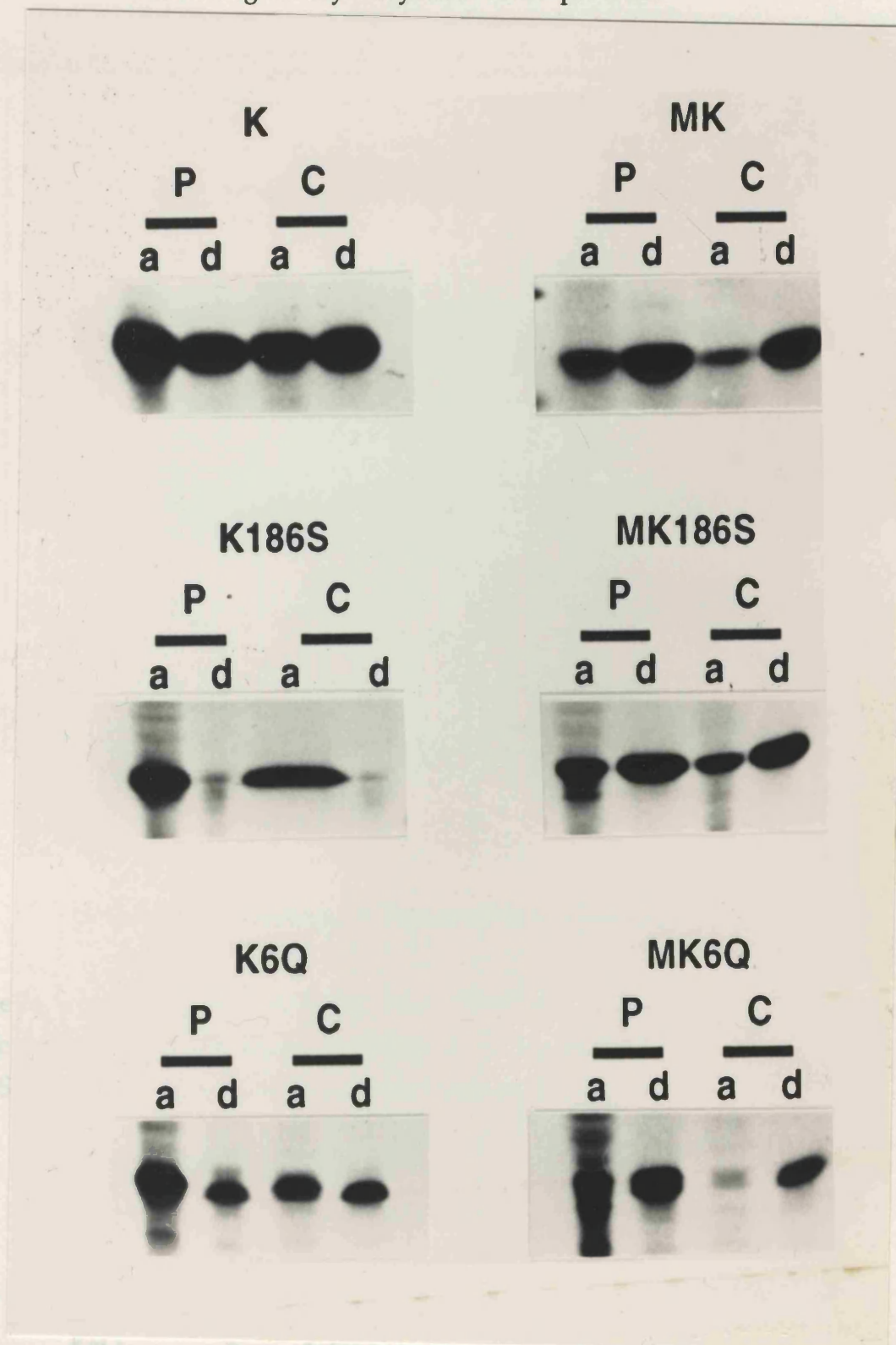
Figure 3.6.
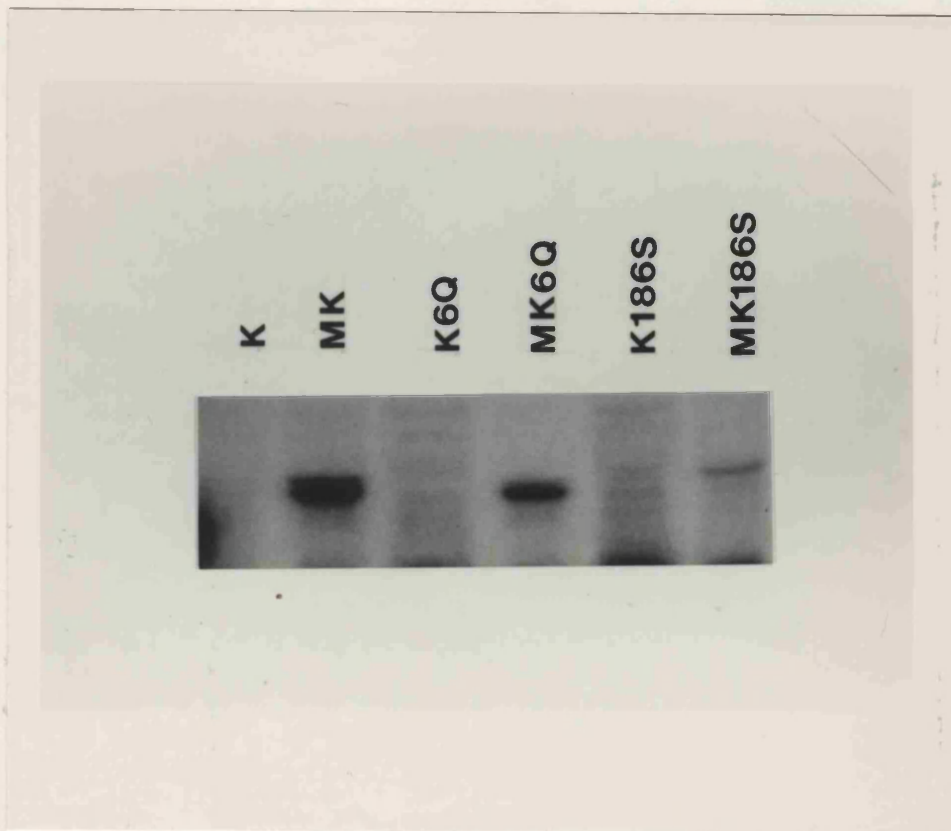


**Myristic acid labelling of myristoylated K-ras mutants.**

COS cells expressing ras proteins were labelled for 4 hours with [$^3$H] myristic acid. Cells were lysed in Triton X114, immunoprecipitated, resolved by SDS-PAGE and visualised by autoradiography.

| K | = | K-ras |
|---|---|---|
| MK | = | myristoylated K-ras |
| K186S | = | K-ras C186S |
| MK186S | = | myristoylated K-ras C186S |
| K6Q | = | K-ras K[175 - 180]Q |
| MK6Q | = | myristoylated K-ras K[175 - 180]Q |

**Figure Legend 3.7.**

**Subcellular distribution of myristoylated K-ras proteins.**

COS cells expressing K-ras proteins were metabolically labelled for 18 hours with [$^{35}$S] methionine and fractionated into p100 (p) and s100 (s) fractions. Equivalent proportions of each fraction were immunoprecipitated, resolved by SDS-PAGE and visualised by autoradiography.

| | | |
|---|---|---|
| K | = | K-ras |
| MK | = | myristoylated K-ras |
| K186S | = | K-ras C186S |
| MK186S | = | myristoylated K-ras C186S |
| K6Q | = | K-ras K[175 - 180]Q |
| MK6Q | = | myristoylated K-ras K[175 - 180]Q |

Figure 3.7.
Subcellular distribution of myristoylated K-ras proteins.
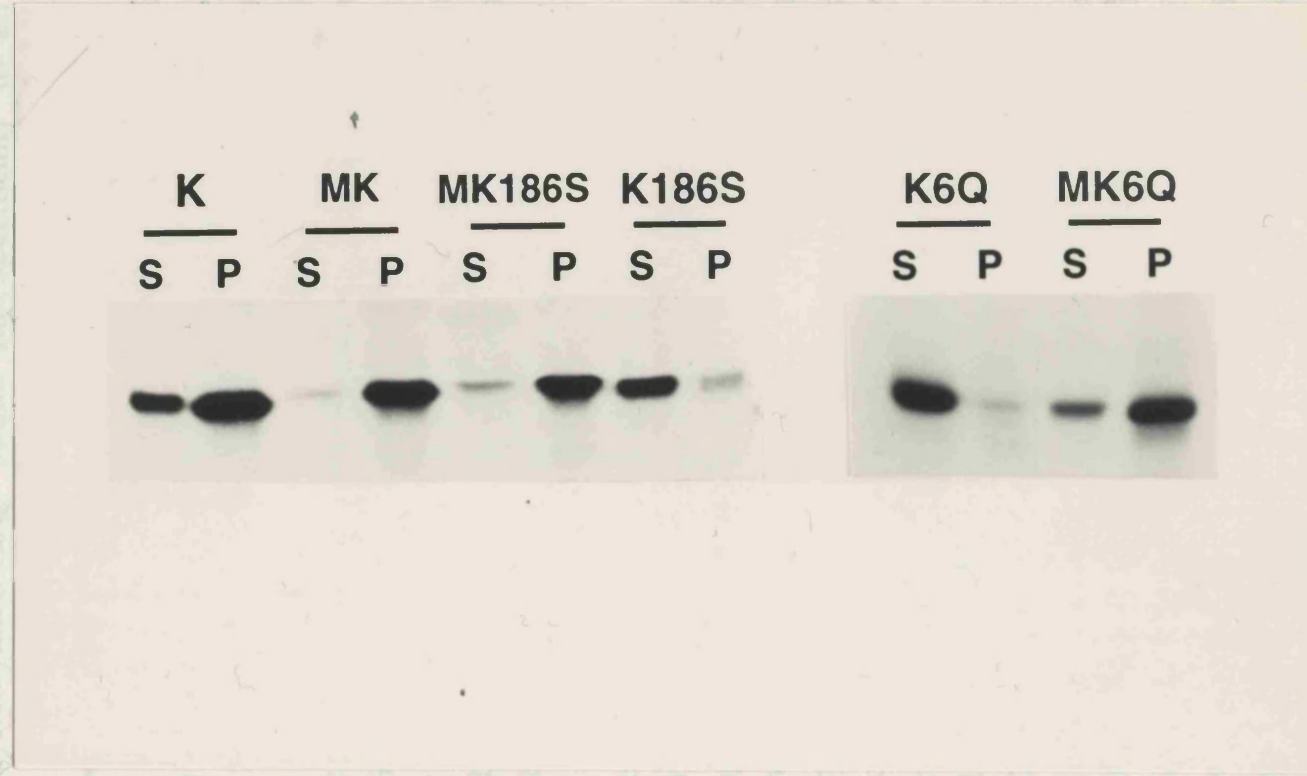
### 3.2.5.3.Plasma membrane association requires the presence of the polybasic domain

Immunofluorescence studies in MDCK cells transiently expressing the myristoylated K-ras proteins showed that plasma membrane association required the presence of the polybasic domain. **Figure 3.8** shows that myristoylated K-ras and myristoylated K-ras C186S were plasma membrane localised whereas myristoylated K6Q bound non specifically to all available cellular membranes including the nuclear envelope, Golgi and endoplasmic reticulum. There was also some very weak plasma membrane staining. This distribution pattern is very similar to that of myristoylated, farnesylated H-ras C181S,C184S (compare **figures 3.8 and 3.5**). Some myristoylated K6Q does bind to the plasma membrane. It is possible that the two remaining lysine residues at the C-terminus of K6Q (residues 182 and 184) could account for the small residual plasma membrane binding of myristoylated K6Q.

The polybasic domain therefore appears to be essential in the specific targeting of farnesylated or myristoylated K-ras to the plasma membrane as opposed to any intracellular membrane.

### 3.2.6. Biological activity of myristoylated H- and K-ras

Myristoylation has previously been reported to restore focus forming activity to the biologically inactive H-ras G12V,C186S (Buss *et al*, 1989; Lacal *et al*, 1988). The normally non-transforming Gly12 C186S H-ras mutant was also found to become oncogenically active when it was myristoylated. The biological activity of Ras proteins with different lipid modifications and different subcellular localisation patterns was examined.

The biological activity of the myristoylated H-ras and K-ras proteins was tested in NIH 3T3 focus assays (**Table 3.3**). Myristoylation in isolation was found to produce a biologically inactive protein whilst myristoylation in combination with farnesylation or the membrane targeting signals of palmitoylation or the polybasic domain produces active proteins. Therefore myristoylation alone produces a biologically inactive protein which is in contrast to farnesylation which in isolation leads to a biologically active protein.

The GDP/GTP loading state of each of the myristoylated proteins was examined to verify that different C-terminal modifications did not affect the proportion of GTP bound to the mutant proteins. All of the oncogenic mutant (G12V) proteins were 60-70% GTP bound (not shown) whilst the Gly12

**Figure Legend 3.8.**
**Immunofluorescent analysis of myristoylated K-ras proteins.**

Confocal images of MDCK cells transiently expressing myristoylated K-ras proteins following microinjection of plasmid DNA.

A. Myristoylated K-ras : plasma membrane staining
B. Myristoylated K-ras C186S: predominantly plasma membrane staining   with some cytosolic staining
C. Myristoylated K-ras K[175 -180]Q : significant reduction in plasma
   membrane staining compared with A and B. Staining of nuclear and
   other intracellular membranes in a similar manner to **Figure 3.4B**

**Figure 3.8.**

Immunofluorescent analysis of myristoylated K-ras proteins.
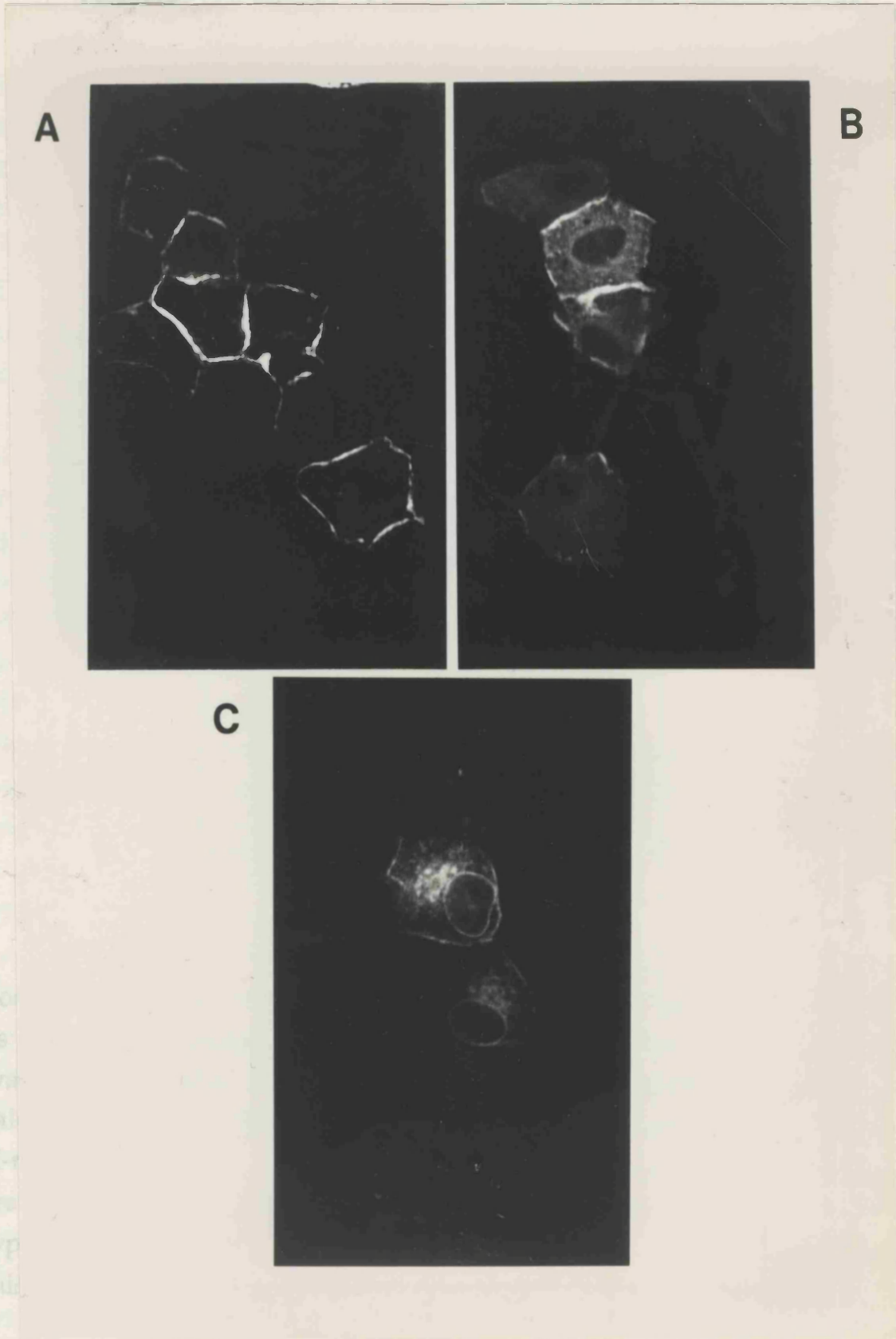
**Table 3.3.**

**Transforming activity of oncogenic myristoylated H-ras and K-ras proteins.**

| protein | relative activity |
|---|---|
| Val12 H-ras | 1.0 |
| MH | 0.36 |
| 181/4S | 0.46 |
| M181/4S | 0.3 |
| H186S | 0.0 |
| MH186S | 0.38 |
| M1/4/6S | 0.0 |
| | |
| Val12 K-ras | 1.0 |
| MK | 0.5 |
| K6Q | 0.33 |
| MK6Q | 0.32 |
| K186S | 0.0 |
| MK186S | 0.45 |

Focus assays were performed with the myristoylated H-ras and K-ras DNA as described (Materials and Methods) and the number of transformed foci was counted after 10-20 days. Relative transforming activities were calculated by dividing each absolute value by the control value (H-ras or K-ras). H-ras (Val 12) with a wild type C-terminus gave 20-30 foci/ng and results were expressed relative to this value. K-ras (Val 12) with a wild type C-terminus gave 1 - 1.9 foci / ng and results were expressed relative to this value.

versions of the same proteins were 4 - 6% GTP bound when expressed in COS cells (**Figure 3.9**).

A novel assay that measured MAP kinase activity in COS cells using kinase dead MEK as a MAP kinase substrate was used as a read out for Ras activity. COS cells, electroporated with Ras plasmids, or empty vector as a baseline control, were harvested three days later following 18 hours of serum starvation. Aliquots of cytosol, normalised for protein content, were incubated with kinase dead MEK immobilised on beads in the presence of $[^{32}P]\gamma$ ATP. The radioactivity incorporated into MEK was measured by direct β-scanning (see materials and methods). Western blots were performed on all samples to confirm that Ras expression was equivalent in all cases.

**Figure 3.10** shows that oncogenic H-ras increased MAP kinase activity 7-fold over the control. The cytosolic proteins H-ras C186S and myristoylated H-ras C181S,C184S,C186S were completely inactive whereas the cytosolic, farnesylated H-ras C181S,C184S was equipotent in activating MAP kinase. Farnesylation is therefore required for cytosolic Ras to activate the MAP kinase pathway and myristoylation cannot functionally substitute for farnesylation. Myristoylation significantly reduced MAP kinase activation by H-ras (with a wild type C-terminus) and farnesylated H-ras C181S,C184S. The plasma membrane localised myristoylated, palmitoylated H-ras C186S had an activity intermediate between wild type H-ras and myristoylated wild type H-ras. This suggests that providing Ras is plasma membrane localised, myristoylation can replace farnesylation to produce an active protein.

MAP kinase activities for the K-ras mutants are also shown in **Figure 3.10**. Again farnesylation leads to MAP kinase activation whereas cytosolic K-ras C186S is inactive. Myristoylation reduced the activity of both K-ras and the K6Q mutant but rescued the activity of K-ras C186S. Therefore myristoylation can functionally replace farnesylation only when the protein is plasma membrane localised.

It was decided to analyse MAP kinase activation of the geranylgeranylated ($C_{20}$) modified H-ras mutants described in the previous chapter. The $C_{20}$ modified H-ras (H-CCIL) is plasma membrane associated whereas the H181/4CCIL mutant whilst being p100 associated, is localised nonspecifically to intracellular membranes. This is a similar to the myristoylated H-ras proteins which with palmitoylation are plasma

Figure 3.9.
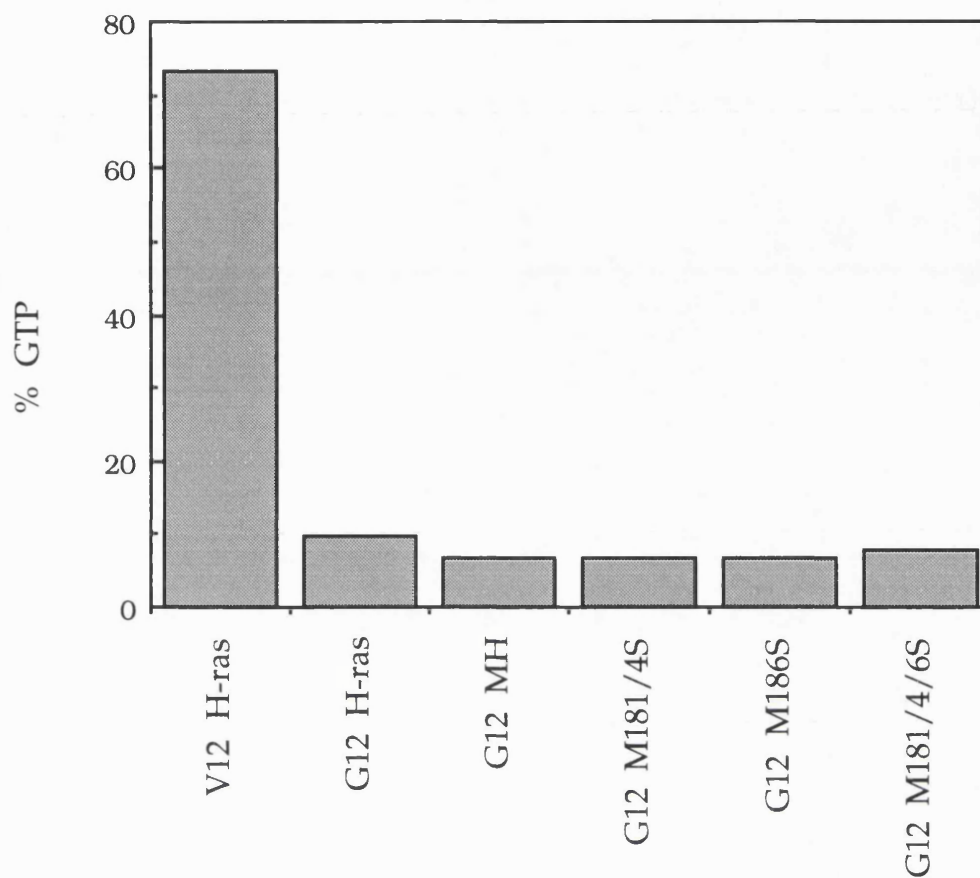


**GTP loading of myristoylated Gly12 H-ras proteins.**

Electroporated COS cells were labelled with [$^{32}$P] orthophosphate and immunoprecipitated. The eluted nucleotides were separated by TLC and the plate was then scanned by an Ambis β-scanner. The GTP ratios were then calculated [0.66GTPcpm /GDPcpm + 0.66GTPcpm].

**Figure 3.10.**
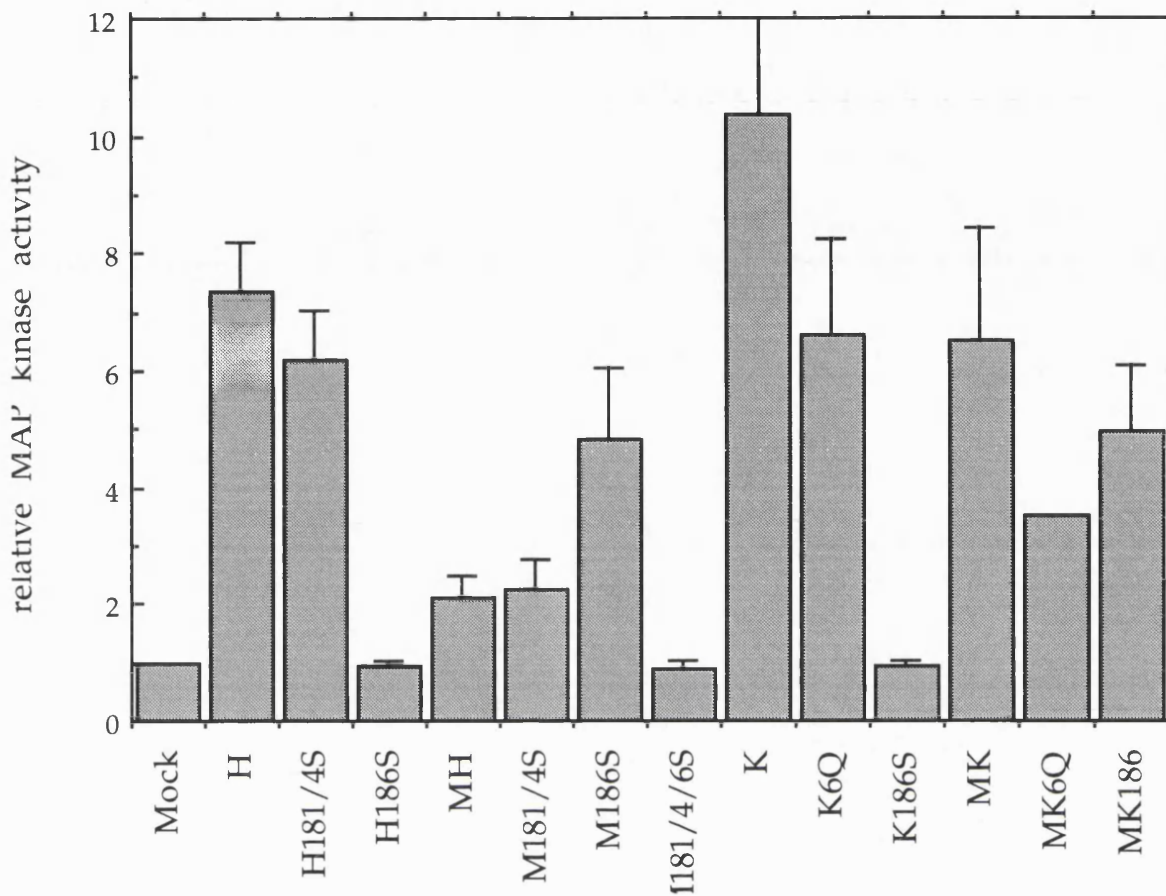


**Relative MAP kinase activity of myristoylated Ras proteins expressed in COS cells.**

MAP kinase activity present in 50µg cytosol was assayed (as described in materials and methods) and expressed relative to the activity present in cells transfected with empty vector. Results are the mean of four independent experiments. Equivalent expression of the different Ras proteins was verified by western blotting s100 and p100 fractions from each transfection with Y13-259.

membrane localised but without palmitoylation associate with intracellular membranes.

**Figure 3.11** shows that the MAP kinase activity of the plasma membrane localised mutant, H-CCIL (geranylgeranylated and palmitoylated), was found to be less than that of H-ras and unpalmitoylated H-ras (C181/184S). The geranylgeranylated but nonpalmitoylated mutant (H1/4CCIL) had significantly reduced MAP kinase activity. This reduction in MAP kinase activation seen with $C_{20}$ modified H-ras may be due to the stronger membrane binding of these proteins compared to farnesylated H-ras.

**Figure 3.11.**
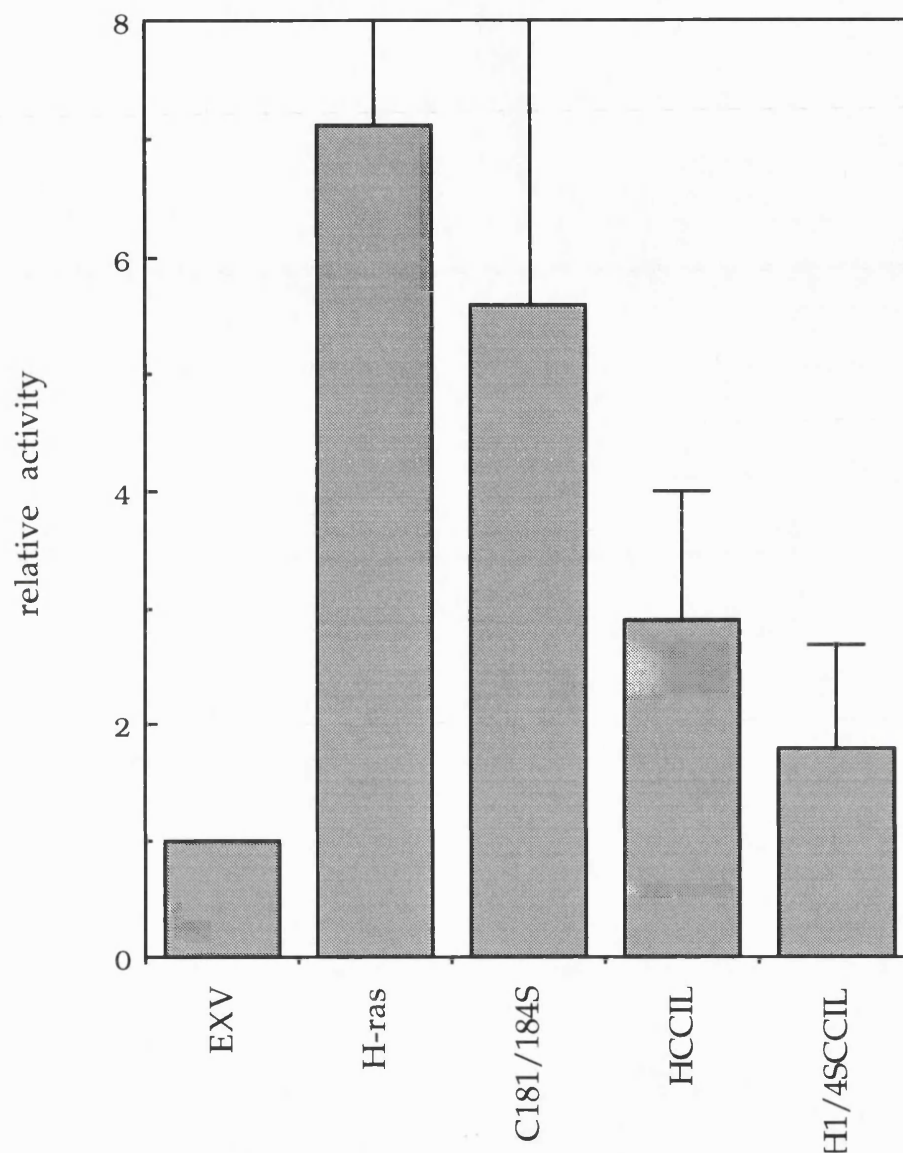


**Relative MAP kinase activity of $C_{20}$ modified H-ras proteins expressed in COS cells.**

MAP kinase activity present in 50µg cytosol was assayed (as described in materials and methods) and expressed relative to the activity present in cells transfected with empty vector. Results are the mean of four independent experiments. Equivalent expression of the different Ras proteins was verified by western blotting s100 and p100 fractions from each transfection with Y13-259.

## 3.3. Discussion

Many proteins achieve their specific cellular location by acylation. This can involve palmitoylation, myristoylation or the addition of a phosphatidyl inositol - containing glycan moiety. H-ras and K-ras are localised to the plasma membrane after the C-terminal CAAX motif has undergone a complex series of post-translational modifications. These include prenylation, proteolysis and methylation. H-ras then undergoes palmitoylation whereas for K-ras the interaction of the polybasic domain with the plasma membrane is essential.

The effect of N-terminal myristoylation on H-ras localisation was previously studied (Buss et al, 1988; 1989; Lacal et al, 1988) and was found to lead to the membrane association of the protein. These studies failed to identify whether myristoylation of H-ras without the normal Ras post-translational modifications was sufficient to target the protein to the plasma membrane and if not, what Ras processing events were essential to work in co-operation with N-terminal myristoylation in order to achieve plasma membrane localisation.

By making a series of myristoylated H-ras and K-ras mutants with different C-termini, I have shown that myristoylation of H-ras (myristoylated H-ras C181S,C184S,C186S) in the absence of any Ras post-translational events does not lead to plasma membrane association and the protein remains cytosolic. The membrane association of myristoylated H-ras requires other membrane localisation signals besides the addition of myristic acid. This is not surprising given that it is now known that myristoylation of several proteins is accompanied by palmitoylation to achieve membrane localisation.

Myristoylated H-ras proteins with an intact CAAX motif are p100 associated but without the palmitoylation sites (Cys181S,C184S), the protein (myristoylated H-ras C181S,C184S) binds non-specifically to all intracellular membranes including the nuclear membrane. Myristoylated H-ras that is also palmitoylated (myristoylated H-ras and myristoylated H-ras C186S) is not only p100 associated but is also plasma membrane associated. This occurs whether the CAAX motif is intact or mutated as in the myristoylated H-ras C186S mutant.

With K-ras, myristoylation leads to the p100 association of mutants with either the CAAX motif mutation (C186S) or a defective polybasic domain (K[175 - 180]Q). However in order to achieve specific localisation to

the plasma membrane, the presence of an intact polybasic domain is essential (myristoylated K-ras and myristoylated K-ras C186S) whereas the CAAX motif is not (myristoylated K-ras K[175 - 180]Q). Myristoylation can therefore replace farnesylation and target Ras to the plasma membrane only in the presence of a polybasic domain or cysteine palmitoylation sites.

Farnesylation of H-ras is a prerequisite for Ras palmitoylation but here it has been shown that myristoylated H-ras C186S is palmitoylated. Thus it would appear that there is a requirement for a lipid modification prior to palmitoylation but this is not restricted to farnesylation. The myristoylated H-ras C186S mutant showed that the addition of myristic acid to the N-terminus replaced the need for C-terminal prenylation to occur prior to palmitoylation. This could be explained by the fact that in the crystalline structure of Ras, the N- and C-termini are in close proximity. N-terminal myristoylation could possibly generate a suitable substrate for the palmitoyltransferase merely by its close position to the C-terminus where prenylation would normally occur. Several myristoylated proteins have now been found to be additionally palmitoylated at N-terminal cysteines so the palmitoylation of myristoylated H-ras is not so surprising.

Myristoylation can also produce a biologically active protein but only when other membrane targeting signals (farnesylation, palmitoylation or a polybasic domain) are present. The cytosolic, myristoylated H-ras C181S,C184S,C186S protein was found to biologically inactive. This contrasts with the cytosolic, farnesylated H-ras C181S,C184S which transforms.

The interaction between the plasma membrane and Ras appears to involve two components. The first is a nonspecific interaction between a hydrophobic lipid and the membrane lipid bilayer. This lipid could be farnesyl, geranylgeranyl or myristate. The second component is an interaction between a plasma membrane associated docking protein and the Ras hypervariable region. The docking protein may bind to Ras in a transient manner so that once in place, Ras remains plasma membrane bound through non-specific interactions. For H-ras, these interactions could involve the lipids farnesyl and palmitate inserting into the membrane lipid bilayer. The docking protein for H-ras could possibly be a plasma membrane localised palmitoyltransferase. The binding of K-ras would involve electrostatic interaction between the positively charged lysines of the polybasic domain and the negatively charged phospholipid head groups of the membrane together with the farnesyl group.

One puzzle that remains unresolved is whether it is possible to separate plasma membrane localisation from Ras biological activity. In other words, is farnesylation important for the interaction of Ras with its regulators and effectors independently of promoting membrane binding. Farnesylated Ras2 is a much more potent activator of adenyl cyclase *in vitro* than unmodified Ras2 (Kurodi *et al*, 1993). The specific isoprenoid used to prenylate Ras would also appear to have some effect on Ras biology since geranylgeranylated H-ras Gly12 is growth inhibitory at low levels of expression (Cox *et al*, 1992).

The interaction between Ras/Raf may be a method of moving Raf from the cytosol to the plasma membrane. Raf is predominantly cytosolic in quiescent cells but activated Raf co-localises with Ras in Ras-transformed HER313 cells (Traverse *et al*, 1993). By measuring the level of MAP kinase activity, plasma membrane localised Ras was found to activate the MAP kinase cascade independently of farnesylation. Cytosolic but farnesylated Ras (H-ras C181S,C184S and K-ras K6Q) was biologically active but unfarnesylated Ras (H-ras C186S and K-ras C186S) was inactive.

Farnesylated and myristoylated proteins showed reduced MAP kinase activation. The mislocalisation of the myristoylated H-ras C181S,C184S protein to intracellular membranes may sequester Raf away from potential plasma membrane localised activators. The myristoylated H-ras and K-ras proteins may be compromised for Raf binding. However since the binding sites of Raf and GAP to Ras show considerable overlap (Zhang *et al*, 1993) this is unlikely. The interaction of GAP with Ras is not affected since the Gly12 myristoylated proteins were not loaded with GTP.

An interesting idea is that full biological activity of Ras may require the protein to cycle on and off the membrane. Since palmitoylation is a reversible process this is possible for H-ras but K-ras/membrane interaction may be irreversible. Ras proteins that are modified with two irreversible lipid modifications, farnesylation and myristoylation, would presumably remain permanently membrane associated. However Ras that is myristoylated and palmitoylated should be able to cycle on and off the membrane. The finding that myristoylated H-ras C186S was more active than myristoylated H-ras would appear to support this hypothesis since myristoylated H-ras being myristoylated, farnesylated and palmitoylated would presumably be unable to cycle off the plasma membrane.

K-ras was a more potent activator of MAP kinase than H-ras. The presence or absence of the polybasic domain affects the activity of K-ras in a similar manner to that of palmitoylation on H-ras in that the polybasic domain is not required for activity providing that the protein is farnesylated. Myristoylation reduced the activity of K-ras presumably because it increases the strength of membrane association. Again the myristoylated K-ras proteins need to be plasma membrane associated to be active in these assays.

Geranylgeranylated H-ras also shows reduced MAP kinase activity. The decrease in activity of the $C_{20}$ mutant that is unpalmitoylated may again be due to mislocalisation to other intracellular rather than the plasma membrane. The salt wash experiments of $C_{20}$ modified H-ras showed that geranylgeranylated H-ras was bound to membranes more tightly than farnesylated H-ras. This tighter association may lead to reduced activity by preventing or slowing down the normal cycling of the protein on and off the plasma membrane.

This cycling of proteins on and off membranes has been considered for other proteins. It has been suggested that GDIs (guanine nucleotide dissociation inhibitors) and GDS (guanine nucleotide dissociation stimulators) proteins are involved in releasing prenylated Rab proteins from membranes and recycling them via the cytosol (Magee and Newman, 1992). Rhodopsin kinase translocates from the cytosol to the retinal membranes after photon stimulation (Inglese et al, 1992). This translocation is dependent on farnesylation and the cytosolic rhodopsin kinase may form a complex with a GDI-like molecule. Interestingly a geranylgeranylated rhodopsin mutant remains membrane associated and does not dissociate from the membrane after light stimulation. Following activation of the neutrophil superoxide-generating system, p47 and p67 translocate to the membrane (Abo et al, 1991). This translocation could possibly be associated with the release of Rac from the cytoplasmic Rac/RhoGDI complex. The myristoylated ARF proteins may cycle on and off membranes simply by GTP hydrolysis inducing a conformational change which leads to membrane dissociation (Kahn et al, 1991). It is therefore possible that Ras cycles on and off the plasma membrane by a mechanism that involves GDP/GTP exchange.

## 3.4.    Conclusions

In the previous chapter I showed that prenylation of H-ras and K-ras by geranylgeranylation instead of farnesylation also lead to plasma membrane associated proteins provided that the protein could be palmitoylated (H-ras) or had an intact polybasic domain (K-ras). Geranylgeranylation in the absence of these localisation signals lead to non specific membrane association of the proteins. In this chapter I have shown that H-ras and K-ras can be targeted to the membrane fraction by myristoylation but in order for the proteins to be localised to the plasma membrane, there is again an absolute requirement for the presence of a polybasic domain (K-ras) or the addition of palmitic acid (H-ras).

It would therefore seem that the addition of a farnesyl, geranylgeranyl or even a myristate group to H-ras or K-ras can lead to plasma membrane association provided that the signalling regions upstream of the CAAX motif are intact. The addition of palmitic acid to H-ras and the presence of the polybasic domain in K-ras is essential to target Ras specifically to the plasma membrane.

Myristoylation in isolation cannot produce a transformation competent protein unlike farnesylation.

By using a MAP kinase assay to measure the activity of Ras with different lipid modifications, it has been possible to attempt to rationalise lipidation and localisation with biological activity. It appears that farnesylation leads to MAP kinase activation without the need for plasma membrane localisation since farnesylated, cytosolic proteins are active (H-ras C181S,C184S and K-ras K6Q). In MAP kinase assays geranylgeranylation cannot replace farnesylation of H-ras to produce active proteins without palmitoylation and plasma membrane association. Myristoylation can replace farnesylation to produce biologically active proteins only when the protein can also be palmitoylated (H-ras) or contains a polybasic domain (K-ras) and is targeted to the plasma membrane.

# Chapter 4

# The role of the hypervariable domain

## 4.1. Introduction

The three human *ras* gene sequences are identical for the first 80 amino acids. The C-termini of the proteins show significant divergence with one region (residues 165 - 180) showing less than 20% homology between any two *ras* genes . This region is often referred to as the hypervariable region/domain (the HVR). The size of this region appears to be maintained even though the amino acid sequence is not (Shih and Weeks, 1984; Taparowsky *et al*, 1983). There is also another smaller hypervariable region between amino acid residues 121 and 132 (Dhar *et al*, 1982; McGrath *et al*, 1983; Shimizu *et al*, 1983; Tsuchida *et al*, 1982). The extreme C-terminal amino acids (the CAAX motif) are conserved and these are involved in Ras localisation.

## 4.1.2. The hypervariable region and Ras transforming activity

Removal of the hypervariable region (165 - 184) has been shown to have no effect on the transforming ability of H-ras provided that the CAAX motif is present (Lacal *et al*, 1986; Willumsen *et al*, 1985; 1986). Increasing the length of the hypervariable domain to almost double its size was also found to have little effect on transforming efficiency (Willumsen *et al*, 1985). Replacing the C-terminus (121 - 189) of H-ras (Val12) with that of Dras 3 resulted in a protein that was still capable of transforming rat-1 cells (Schetjer and Shilo, 1985) suggesting that this hypervariable domain although conserved for a particular species is interchangeable between species.

The conservation of this region implies that it serves a specific function in the physiological role of Ras proteins and that in some way it is responsible for the possible different functions of the Ras family. On the other hand it may simply serve to connect the N - terminus with the C - terminus and hence the need to retain this region (Willumsen *et al*, 1985). There does however appear to be a limit to the extent to which this region can be lengthened. Mutants with inserts larger than 30 amino acids fail to transform cells (Willumsen *et al*, 1985). There also appears to be some constraint on the flexibility of this region since substituting the 80 C-terminal residues (138 -218) of R-ras p23 for residues 112 - 189 of H-ras leads to an inactive Ras protein (Lowe *et al*, 1988). The R-ras N-terminus/ H-ras C-terminus chimera was also found to be transformation incompetent. It was

suggested that the inability of these chimeras to transform was due to the fact that R-ras is evolutionarily distant from the rest of the Ras family and may have a different function.

### 4.1.3. The function of the C-terminus in classical G proteins

The C-terminus domain of classical G proteins binds to activated receptors resulting in nucleotide exchange. Transducin 1 and Gi (the inhibitory subunits of adenyl cyclase) are ADP-ribosylated at a cysteine towards the C-terminus by pertussis toxin. The ADP-ribose group sterically hinders interaction with photorodopsin and inhibitory adenoreceptors (Ui et al, 1984; Van Dop et al, 1984). Transducin and arrestin both bind to photoactivated photorhodopsin. The C-terminal 25 amino acids of these proteins are highly homologous (Wistow et al, 1986) and this region appears to be the receptor binding domain since in vivo competition between the two proteins terminates signal transduction through this pathway (Stryer, 1986).

Adenyl cyclase cannot be activated by hormonal agonists acting on prostaglandin $E_1$ receptors or adenoceptors in S49 unc cells. However it is activated by cholera toxin and AlF4 directly activating $G_S$ (Bourne et al, 1982). S49cyc cells that do not express $G_S$ cannot complement the unc deficiency in somatic cell hybrids (Bourne et al, 1982) whereas addition of purified $G_S$ to S49unc cell membranes restores the response to agonists (Sternweiss and Gilman, 1979). The unc mutation would therefore appear to be in $G_S$ and interferes with G-protein receptor binding. The unc $G_S$ contains an Arg ---> Pro372 mutation (Sullivan et al, 1986) near the C-terminus of the protein. This region is thought to be involved in receptor-G-protein coupling since a $G_i/G_S$ chimera containing the N-terminal 60% of the $G_i$ protein and the C-terminal 40% of the $G_S$ protein was able to mediate β-adrenoceptor stimulation of adenylate cyclase when introduced into S49 cyc cells (Masters et al, 1988).

### 4.1.4. RAS C-terminal domain function

CDC25 is a RASGEF (guanine nucleotide exchange factor) for S.cerevisiae (Broek et al, 1987; Gibbs et al, 1989; Robinson et al, 1987) whose action leads to adenylate cyclase activation (Camonis et al, 1986; Martegani et al, 1986; Tripp and Pinon, 1986). CDC25 is required for normal RAS function but temperature sensitive mutations within the gene or gene deletions can be overcome by a RAS1 or RAS2 valine 19 mutation (Marshall et al, 1987; Robinson et al, 1987). Deletion of the C-terminal 174-300 amino acids of RAS2 can rescue lethal

CDC25 deletions whilst overexpression of full length RAS2 cannot rescue viability (Marshall *et al*, 1987).

RAS2Δ expression leads to increased adenylate cyclase levels suggesting that the C-terminal domain has a negative regulatory role. GTP loading of RAS2Δ was found to be 6% whereas no detectable GTP was found on RAS2 (Gibbs *et al*, 1987), Val19 RAS1 had 16% GTP bound and Val19 Ras2Δ had 33%, whereas wildtype H-ras expressed in *S.cerevisiae* was found to be 38% GTP bound. Mammalian Ras contains 110 less C-terminal amino acids than yeast RAS. In RAS this region appears to be a negative regulator of RAS activity. Whether there is co-operation between the C-terminal domain and IRA1 and IRA2, the GAP genes of *S.cerevisiae* since they both act as negative regulators is not known.

### 4.1.5. Possible function of the Ras hypervariable domain

The hypervariable region may confer functional differences on the Ras proteins by allowing different protein/protein interactions. The region may also be involved in exchange factor interaction. Since the Val19 oncogenic mutant of H-ras is resistant to GTPase action, it remains in the GTP state. Deletion of the hypervariable domain should not affect transforming activity since exchange factor interaction is not required. However hypervariable deletions in wild type Ras would be expected to inactivate the protein since if this region is involved in exchange factor interaction RasΔ would remain in the GDP form. However, when oncogenic (Val12) hypervariable region deletion mutants (166 - 180) were tested for transforming ability in NIH 3T3 cells, they were all found to have significantly reduced activity compared to the full length protein (Hancock and Marshall, unpublished data). These results are at variance with those reported by Willumsen and co-workers (1985).

The hypervariable region of Rab6 has recently been shown to be required for RabGDI binding and geranylgeranylation of the protein by RabGGTase (Beranger *et al*, 1994). In the light of these results it was decided to re-examine the role of the hypervariable domain of H-ras and K-ras using several different approaches.

### 4.2. Results
### 4.2.1. K-spacers

In order to investigate whether there was a spatial relationship between the relative positions of the polybasic domain and the C-terminus CAAX motif

of K-ras, several mutants were made containing extra amino acids inserted between these two important membrane localisation signalling regions. It was hoped that by increasing the distance between the polybasic domain and the CAAX motif, a different localisation pattern would be seen.

The original K-Spacer was made by linker insertion using two oligonucleotides in the ligation mix. When ligated into K-ras these would produce an Xcm 1 site in the mutant progeny. The resulting mutant had positions 180 and 181 of K-ras removed and these were replaced by six new amino acids. This meant that one of the lysines of the polybasic domain was lost so all of the spacer mutants only have five lysines in their polybasic domain.

To make K-spacer mutants of longer length, the original K-spacer was digested with Xcm 1, treated with alkaline phosphatase and then ligated with phosphorylated C and G oligonucleotides in a 3M excess. This led to a series of four different spacer mutants being produced with inserts of 6, 9, 12 and 18 amino acids (Figure 4.1).

## 4.2.2. Localisation of K-spacer mutants

COS cells were transfected with K-spacer DNA and 72hr afterwards were labelled overnight with [$^{35}$S] methionine (50μCi/ml). After a six hour cold chase, the cells were fractionated into s100 and p100 fractions. Equal proportions of both fractions were immunoprecipitated and resolved by SDS PAGE. Figure 4.2 shows that as the number of amino acids inserted between the polybasic domain and the CAAX motif was increased, the amount of protein localising to the membrane (p100) fraction remained constant. Spacers 3 and 4 also showed significantly less expression of protein despite repeating the transfection several times, trying different DNA preparations, increasing the amount of DNA used per transfection and increasing the number of cells used in the transfection.

Figure 4.3 shows confocal microscope studies of MDCK cells transiently expressing the spacer proteins. Spacers 1, 2 and 3 show significant plasma membrane localisation however spacer 4 does not appear to be targeted to the plasma membrane. The immunofluorescence data also suggested that spacers 1 and 2 transformed the cells whereas spacer 3 and 4 did not.

## 4.2.3. Transforming ability of the K-spacer mutants

The transforming efficiency of these K-spacer mutants was formally examined using the NIH 3T3 CaCl$_2$ transfection method. As shown in Table 4.1,

**Figure 4.1.**

**Sequences of K-ras and the K-spacer mutants.**

The sequence of wild type K-ras is shown across the top of the table. The amino acids inserted into this sequence to produce the K-spacer mutants are shown below.

**Figure 4.1.**

**Sequences of K-ras and the K-spacer mutants.**

K-ras      K K K K K S K T K C V I M

S1      K K K K K <u>N H E L I G</u> S K T K C V I M      6 amino acid insert

S2      K K K K K <u>N H E L I G L I G</u> S K T K C V I M      9 amino acid insert

S3      K K K K K <u>N H E L H E L I G L I G</u> S K T K C V I M      12 amino acid insert

S4      K K K K K <u>N H E L H E L I G L H E L I G L I G</u> S K T K C V I M      18 amino acid insert

**Figure 4.2.**
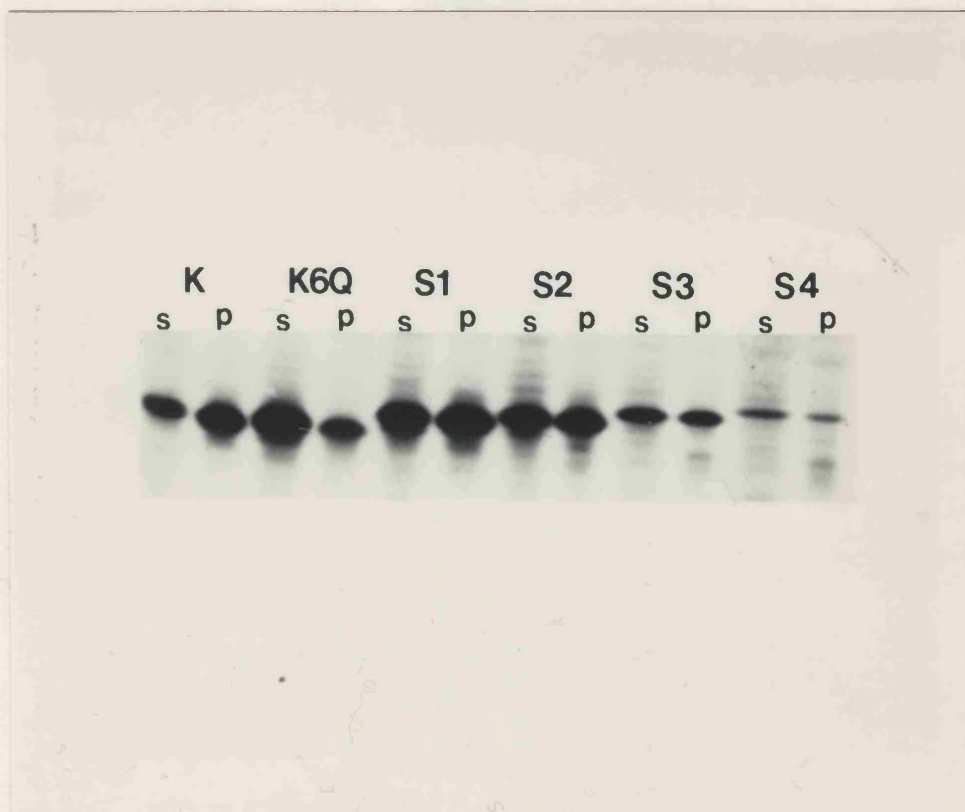


**Subcellular Fractionation of K-ras spacer proteins.**

COS cells transiently expressing K-ras mutants were labelled with [$^{35}$S] methionine 72hr after transfection. Following a cold chase of 6hr, cells were fractionated into s100 and p100 fractions, immunoprecipitated and resolved by SDS PAGE.

| | | |
|---|---|---|
| K | = | K-ras |
| K6Q | = | K-ras K[175 - 180]Q |
| S1 | = | K-ras with 6 extra amino acids |
| S2 | = | K-ras with 9 extra amino acids |
| S3 | = | K-ras with 12 extra amino acids |
| S4 | = | K-ras with 18 extra amino acids |

**Figure Legend 4.3.**

**Immunofluorescence of MDCK cells transiently expressing K-ras spacer proteins.**

MDCK cells were microinjected with plasmid DNA and examined by immunofluoresence. Spacers 1, 2 and 3 all show plasma membrane localisation similar to K-ras. However spacer 4 shows significantly weaker plasma membrane association.

K-ras control  =  K-ras
K-ras spacer 1  =  K-ras with 6 extra amino acids
K-ras spacer 2  =  K-ras with 9 extra amino acids
K-ras spacer 3  =  K-ras with 12 extra amino acids
K-ras spacer 4  =  K-ras with 18 extra amino acids

**Figure 4.3.**

Immunofluorescence of MDCK cells transiently expressing K-ras spacer proteins.
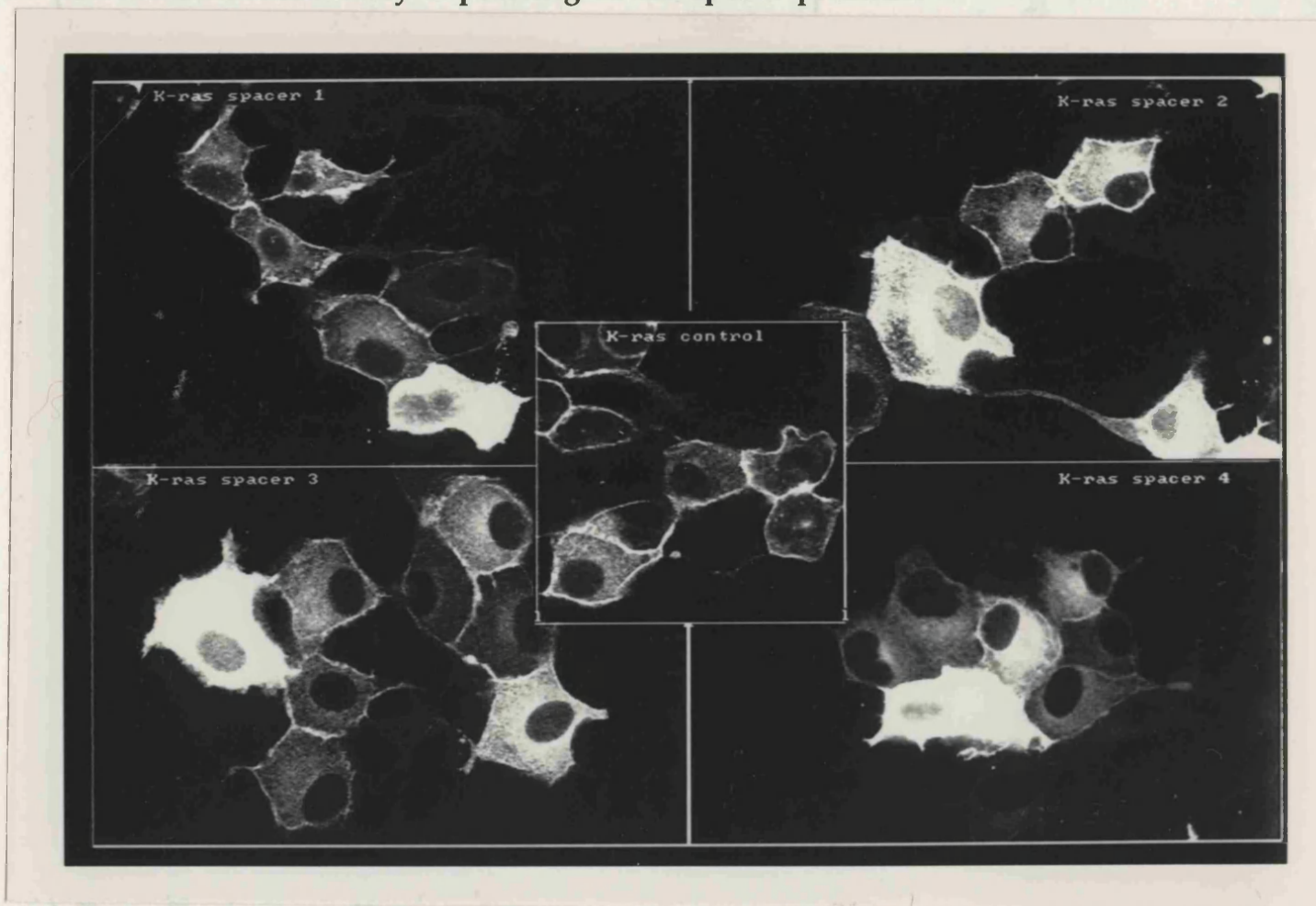
**Table 4.1.**

Transforming activity of oncogenic mutant K-ras proteins with C-terminal insertions.

| protein | mutation | relative activity |
|---|---|---|
| K-ras | - | 1.0 |
| K6Q | polybasic removed | 0.1 |
| K186S | Ser186 | 0.0 |
| S1 | +6 amino acids | 1.0 |
| S2 | +9 amino acids | 0.6 |
| S3 | +12 amino acids | 0.1 |
| S4 | +18 amino acids | 0.0 |

Focus assays were performed with the K-spacer DNA as described (Materials and Methods) and the number of transformed foci was counted after 15-20 days. Relative transforming activities were calculated by dividing each absolute value by the control value (K-ras).

the spacer 1 mutant transformed with the same efficiency as Val12 K-ras. However as the number of amino acids inserted between the polybasic region and the CAAX motif was increased there was a corresponding decrease in transforming efficiency until with the addition of 18 amino acids (spacer 4) the protein was biologically dead / non transforming.

### 4.2.4. H-ras hypervariable deletion mutants

Previously it has been shown that deletion of amino acids 165 - 184 does not affect the transforming ability of H-ras (Lacal *et al*, 1986; Willumsen *et al*, 1985; 1986). In initial experiments using a series of hypervariable deletion mutants they were all found to be severely compromised for transformation (Hancock and Marshall, unpublished data). Since this region has remained throughout evolution even though some workers have found it to have no biological function, it was decided to re-examine these mutants and to try to establish a role for the hypervariable domain. Possible functions of this region are that it plays a role in effector binding, it could determine the different functional roles of the Ras proteins or it could merely serve as a connecting region, joining the N-terminus of the protein to the C-terminus.

### 4.2.5. Construction of the H-ras deletion mutants

A series of H-ras hypervariable deletion mutants was made by site-directed mutagenesis and cloned into the pEXV-3 mammalian expression vector. Wild type (Gly12), oncogenic (Val12) and dominant negative (Asp17) versions of all the mutants were made. The region deleted from each mutant is shown in **Figure 4.4**.

In order to see if the sequence of the hypervariable region of H-ras was in itself significant, a mutant was made that had the amino acids at positions 171 - 178 (equivalent to those deleted in the Δ12 mutant) replaced with amino acids of similar size and charge to those of the original H-ras sequence. The sequence of this mutant, **insert**, is shown in **Figure 4.4**.

### 4.2.6. COS cell fractionation

COS cells were electroporated with the mutant DNA and labelled overnight with |50uCi/ml of [$^{35}$S] methionine 72 hours after transfection. Cells were fractionated into membrane (p100) and cytosolic fractions (s100). Equal proportions of each fraction were immunoprecipitated with Y13-259, resolved by SDS-PAGE and autoradiographed.

**Figure Legend 4.4.**
**H-ras hypervariable deletion mutants.**

The sequence of wild type H-ras is given across the top of the figure. The regions deleted from each mutant is shown below. The sequence replaced in the 'insert' mutant is also shown.

**Figure 4.4.**

H-ras hypervariable deletion mutants.
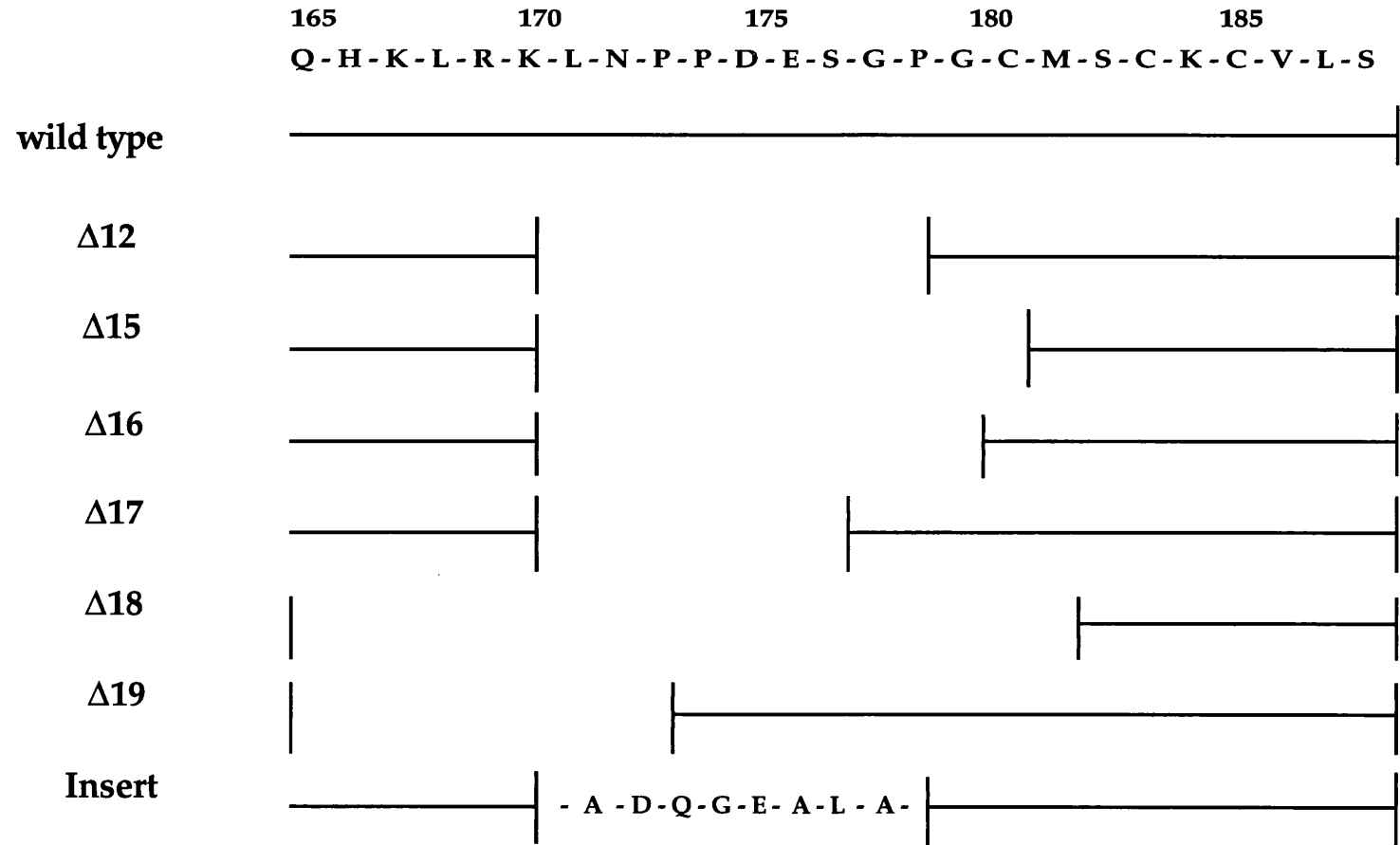
Figure 4.5 shows that all of the deletion mutants were distributed between the membrane (p100) and cytosolic (s100) fractions in a similar way to H-ras, that is they all showed predominantly p100 or membrane association. The Cys181,184Ser mutant (palmitoylation deficient) showed 90% s100 association upon fractionation. This figure also shows the different mobilities of the deletion mutant proteins due to the different sizes of the proteins.

### 4.2.7. The deletion mutants are palmitoylated

H-ras is plasma membrane localised after the CAAX modifications together with palmitoylation have occurred. In order to confirm that the normal Ras post-translational processsing of these mutants was unaffected, electroporated COS cells were labelled for 4 hours with 0.75mCi/ml [$^3$H] palmitic acid. After labelling, the cells were lysed in Triton X114, immunoprecipitated with Y13-259, resolved by SDS-PAGE and autoradiographed.

Figure 4.6 shows that all of the deletion mutants incorporated significant amounts of [$^3$H] palmitate. The post-translational processing of these mutants was unaffected by the deletions within the hypervariable domain. This was expected since none of the deletions involved the Ras processing regions of the CAAX motif and the palmitoylation sites at cysteine 181 and 184. The H-ras Cys181,184Ser mutant showed no incorporation of [$^3$H] palmitic acid since the palmitoylation sites (Cys 181 and 184) have been mutated to serines.

### 4.2.8 Immunofluorescence studies of the H-ras deletion mutants

Figure 4.7 shows the precise cellular location of some of the deletion mutants by immunofluorescence of cells that were microinjected with the mutant DNA. Mutants D16 and D19 showed plasma membrane localisation in a similar manner to wild type H-ras. The Δ12 mutant showed some plasma membrane localisation together with possibly some Golgi staining.

### 4.2.9. Biological activity of the H-ras deletion mutants in NIH 3T3 transfections

The biological activity of the deletion mutants was also tested in NIH 3T3 transformation assays. All of the deletion mutants were found to be severely compromised for transformation (**Table 4.2**) implying that in

**Figure 4.5.**
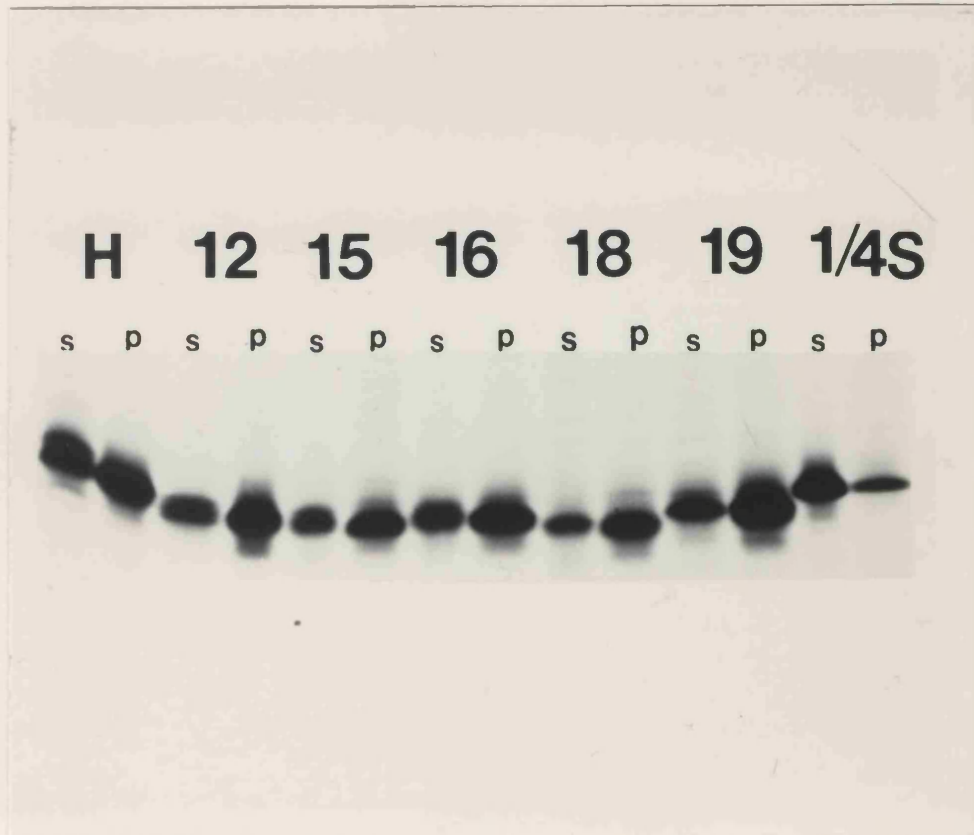


**Subcellular fractionation of H-ras deletion mutants.**

COS cells were electroporated, labelled with [$^{35}$S] methionine, fractionated into s100 (s) and p100 (p) fractions, immunoprecipitated, resolved by SDS-PAGE and visualised by autoradiography.

| | | |
|---|---|---|
| H | = | H-ras |
| 12 | = | Δ12 mutant (missing 171 - 178) |
| 15 | = | Δ15 mutant (missing 171 - 180) |
| 16 | = | Δ16 mutant (missing 171 - 179) |
| 18 | = | Δ18 mutant (missing 166 - 181) |
| 19 | = | Δ19 mutant (missing 166 - 172) |
| 1/4S | = | H-ras Cys181,184Ser |

**Figure 4.6.**
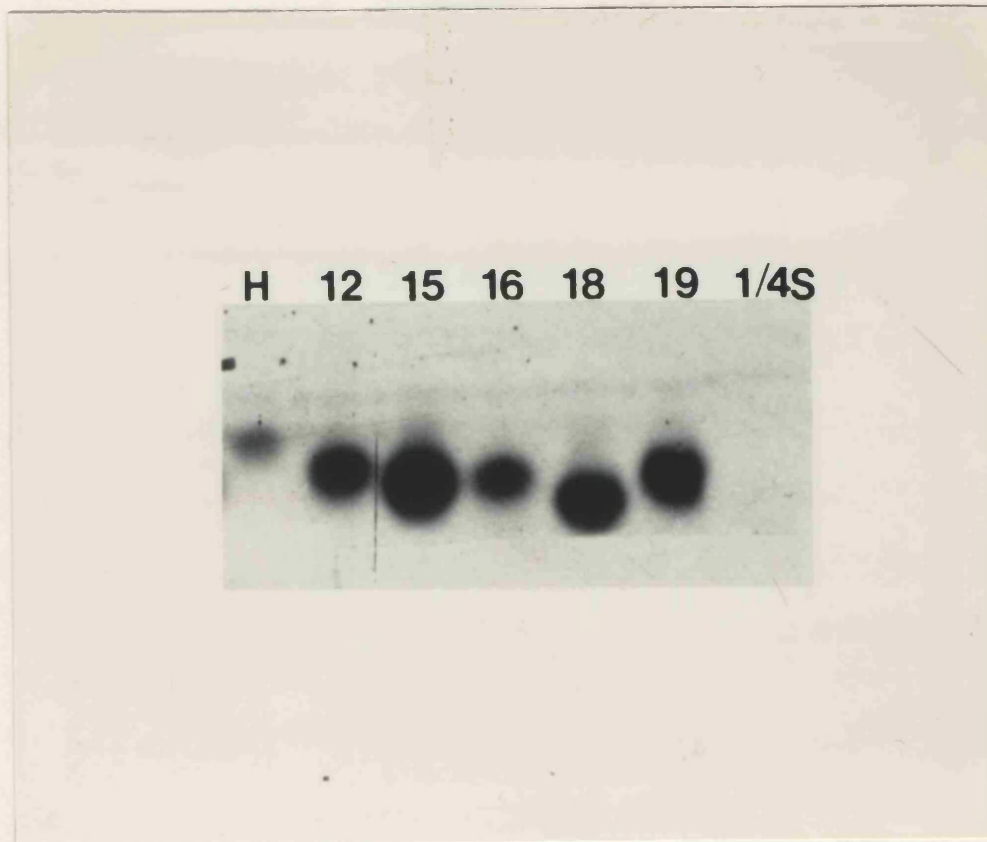


**Palmitoylation of the H-ras deletion mutants.**

Electroporated COS cells were labelled with [$^3$H] palmitic acid, lysed in Triton X114, immunoprecipitated and resolved by SDS-PAGE.

H     =     H-ras
12    =     Δ12 mutant (missing 171 - 178)
15    =     Δ15 mutant (missing 171 - 180)
16    =     Δ16 mutant (missing 171 - 179)
18    =     Δ18 mutant (missing 166 - 181)
19    =     Δ19 mutant (missing 166 - 172)
1/4S  =     H-ras Cys181,184Ser

**Figure Legend 4.7.**
**Subcellular localisation of the H-ras deletion mutants as determined by immunofluorescence.**

Confocal images of MDCK cells transiently expressing proteins following microinjection of plasmid DNA.

a)$\Delta$12 mutant (missing 171 - 178) : Golgi and plasma membrane staining

b)$\Delta$16 mutant (missing 171 - 179) : plasma membrane staining

c)$\Delta$19 mutant (missing 166 - 172) : plasma membrane staining

d)H-ras : plasma membrane staining

**Figure 4.7.**

Subcellular localisation of the H-ras deletion mutants as determined by immunofluorescence.
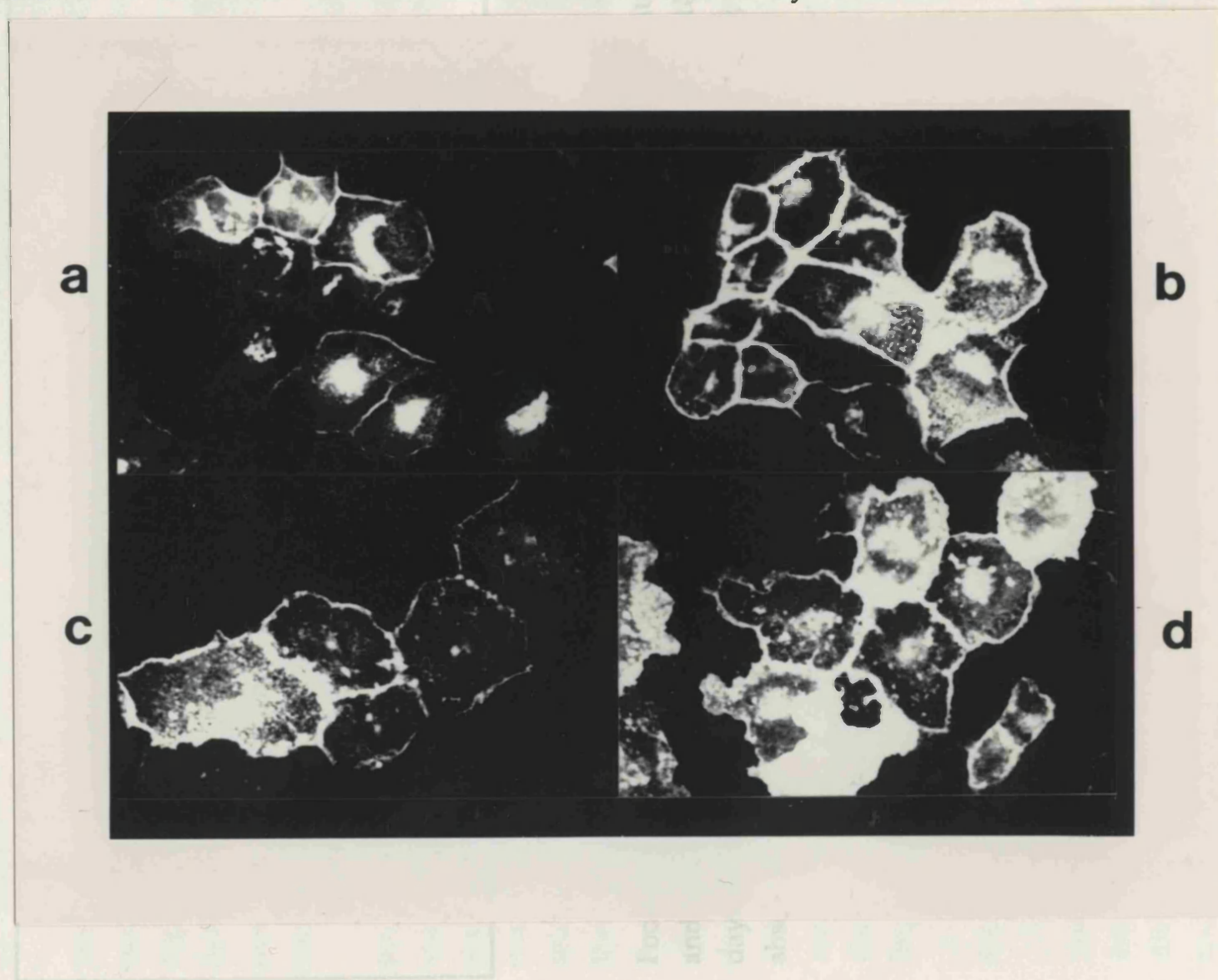
**Table 4.2.**

Transforming activity of oncogenic H-ras hypervariable deletion mutants.

| mutant | relative activity |
|---|---|
| H-ras | 100 |
| Δ12 | 0.0 |
| Δ15 | 0.05 |
| Δ16 | 0.03 |
| Δ17 | 0.01 |
| Δ18 | 0.04 |
| Δ19 | 0.04 |
| C186S | 0.01 |
| C181/184S | 20 |
| **insert** | 50 |

Focus assays were performed with the H-ras mutants as described (Materials and Methods). The number of transformed foci was counted after 10 - 15 days and the relative transforming activity was calculated by dividing each absolute value by the control value (H-ras).

these experiments the HVR is essential for transformation. The **insert** mutant was found to transform with half the efficiency of H-ras which suggested that the sequence of amino acids within the hypervariable domain is not important.

#### 4.2.10.Rescue of transforming activity with N17 deletion mutants

A H-ras mutation, Asp 17 (N17), generates a dominant negative phenotype. This mutant has increased affinity for GDP and is unable to release GEF and bind GTP (Feig and Cooper, 1988). If the hypervariable region of H-ras is involved in effector interaction, it is possible that deleting this region may allow GEF release and GTP binding. In order to test this theory, N17 versions of all of the hypervariable deletion mutants were made.

The dominant negative mutant, Asp 17 (N17), has been shown to inhibit proliferation of NIH 3T3 cells in co-transfections with Geneticin sulphate selection (Feig and Cooper, 1988). It was decided to test the activity of the deletion mutants in these assays. NIH 3T3 cells were transfected with 5µg of DNA together with 1ng of pSV2neo and grown in selection medium (G418) for ∼10 - 14 days. The number of foci per plate was then counted. **Figure 4.8** shows that all of the deletion mutants were able to rescue transforming activity although some were more efficient than others. The mutants all rescued transformation to between 84 and 75% of the pSV2neo control except for the Δ17 mutant showed only 37% rescue. Val 12 H-ras was toxic in these experiments. The mutant, **insert** (Val 12) was also found to be toxic in co-transfection experiments again suggesting that the specific sequence of the hypervariable region of H-ras is of limited importance.

#### 4.2.11.GDP/GTP loading of the H-ras deletion mutants

Since the Val12 deletion mutants failed to transform and the N17 mutants could rescue proliferation of NIH 3T3 cells in co-transfection assays, it is possible that the nucleotide binding of these mutants is disrupted due to inefficient GEF interaction. The Val12 mutants might be transformation deficient due to an inability to bind GTP. Therefore the GDP/GTP loading state of the Gly12 and Val12 versions of the deletion mutants was examined. Electroporated COS cells were incubated with phosphate free medium for 1 hour before being labelled with [$^{32}$P] orthophosphate for 4 hours. After lysis, cells were immunoprecipitated with

**Figure 4.8.**
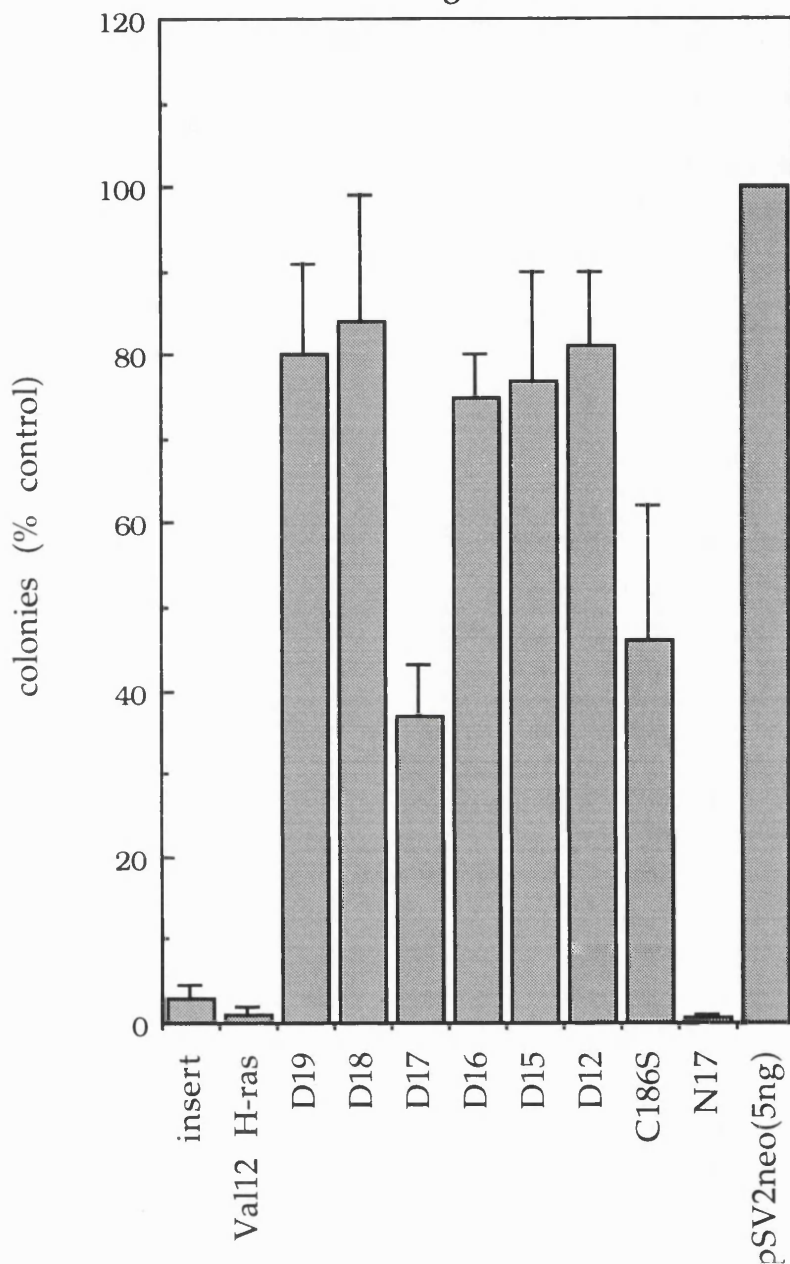
**Biological effect of H-ras hypervariable deletion mutants in NIH 3T3 co-transfections.**

NIH 3T3 fibroblasts were co-transfected with pSV2neo (1ng) and various H-ras deletion mutants (5μg); the transfectants were selected with Geneticin sulphate and the number of colonies formed were scored after 10 to 14 days. The graph shows the numbers of neomycin-resistant colonies arising in co-transfections relative to those with the control transfection with pSV2neo alone.

Y13-259. The bound nucleotide was eluted from the beads used in the immunoprecipitation reactions and separated by TLC. The separated nucleotides were then quantitated by scanning the TLC plate with an Ambis β-scanner.

Figure 4.9 shows that the Gly12 and Val12 versions of the deletion mutants (Δ12, Δ15 and Δ16) are appropriately loaded, that is the GTP ratio for the wild type or Gly12 versions was between 2 and 5% whereas for the oncogenic or Val12 mutants this increased to 75 - 79%.

Figure 4.10 shows a similar experiment for the mutants Δ17, Δ18 and Δ19. The wild type mutants showed a GTP ratio of 1 - 3%, whilst the oncogenic mutants had ratios of 74 - 84%.

Figure 4.11. shows the results of these GTP loading experiments expressed in graph form.

## 4.2.12. Val12 H-ras deletion mutant MAP kinase activation

As discussed in the previous chapter, the activation of MAP kinase can be used as a measure of Ras activity by using kinase dead MEK as a substrate for MAP kinase. Since the GDP/GTP loading experiments showed that the Val12 deletion mutants were appropriately loaded with GTP, it was decided to test the activity of the Val12 deletion mutants in MAP kinase assays. The MAP kinase activating ability of these mutants may be reduced since these mutants were unable to transform, possibly due to inability or reduced ability to activate this kinase pathway.

The MAP kinase activating ability of all of the Val12 deletion mutants was found to be significantly reduced (Figure 4.12) compared to that of Val12 H-ras. The hypervariable region could therefore play some role in activation of the kinase pathway and when removed leads to a protein which is unable to activate the kinase cascade and is therefore nontransforming.

The MAP kinase activating ability of the Val12 insert mutant was found to be similar to that of Val12 H-ras.

**Figure 4.9.**
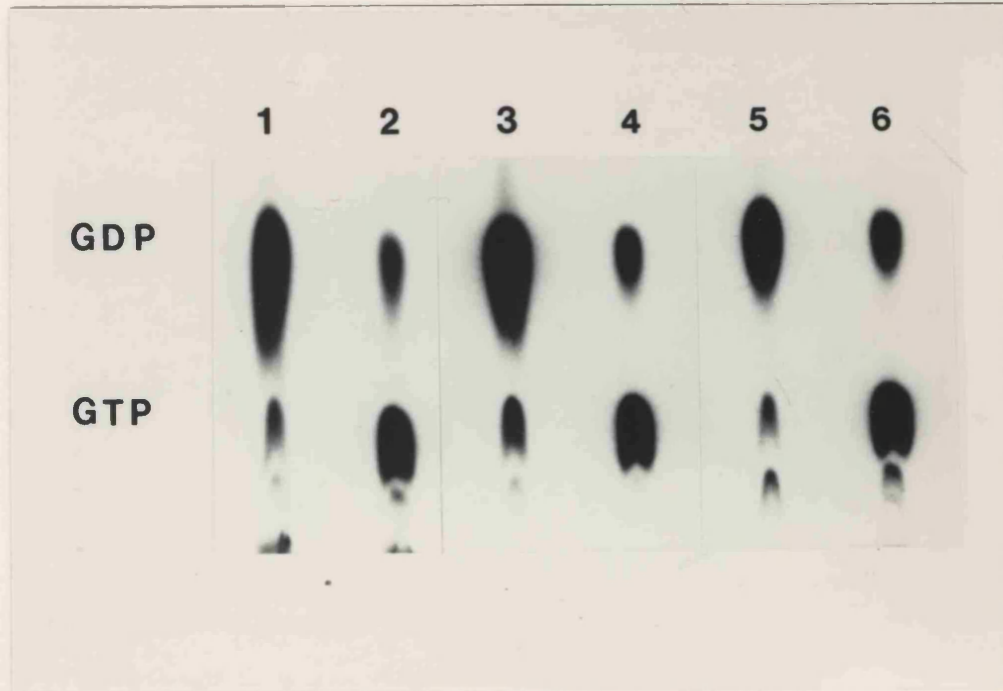


**GDP/GTP loading of Δ12, 15 and 16 H-ras mutants.**

Electroporated COS cells were labelled with [$^{32}$P] orthophosphate and immunoprecipitated. The eluted nucleotides were separated by TLC and the plate was then scanned by an Ambis β-scanner. The GTP ratios were then calculated [0.66GTPcpm/GDPcpm + 0.66GTPcpm].

|   |   |   | %GTP loaded |
|---|---|---|---|
| 1 | = | Gly 12 Δ12 mutant (missing 171 - 178) | 4.8 |
| 2 | = | Val 12 Δ12 mutant (missing 171 - 178) | 78.1 |
| 3 | = | Gly 12 Δ15 mutant (missing 171 - 180) | 2.8 |
| 4 | = | Val 12 Δ 15 mutant (missing 171 - 180) | 76.8 |
| 5 | = | Gly 12 Δ 16 mutant (missing 171 - 179) | 3.8 |
| 6 | = | Val 12 Δ 16 mutant (missing 171 - 179) | 78.6 |

# Figure 4.10.



**GDP/GTP loading of Δ17, 18 and 19 H-ras mutants.**

Electroporated COS cells were labelled with [$^{32}$P] orthophosphate and immunoprecipitated. The eluted nucleotides were separated by TLC and the plate was then scanned by an Ambis β-scanner. The GTP ratios were then calculated [0.66GTPcpm/GDPcpm + 0.66GTPcpm].

|    |   |                                             | %GTP loaded |
|----|---|---------------------------------------------|-------------|
| 7  | = | Val 12 Δ17 mutant (missing 171 - 176)       | 75.4        |
| 8  | = | Gly 12 Δ17 mutant (missing 171 - 176)       | 1.2         |
| 9  | = | Gly 12 Δ18 mutant (missing 166 - 181)       | 3.2         |
| 11 | = | Val 12 Δ18 mutant (missing 166 - 181)       | 74.3        |
| 10 | = | Gly 12 Δ19 mutant (missing 166 - 172)       | 1.1         |
| 12 | = | Val 12 Δ19 mutant (missing 166 - 172)       | 84.4        |

**Figure 4.11.**
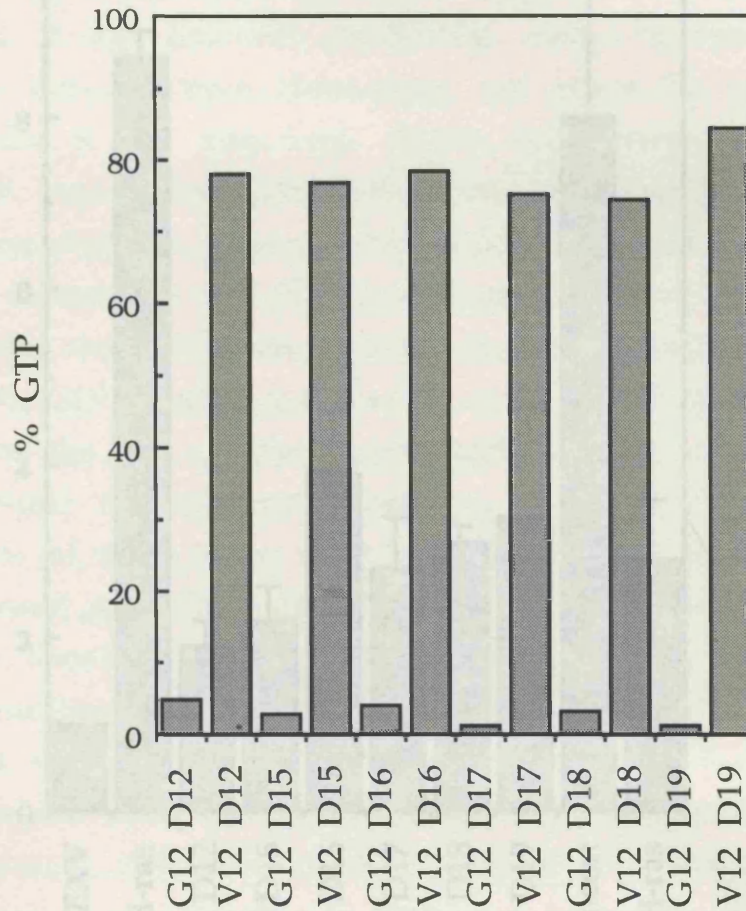


**The GTP loading of the Gly12 and Val12 hypervariable deletion mutants.**

Elecroporated COS cells were labelled with [$^{32}$P] orthophosphate and immunoprecipitated. The eluted nucleotides were separated by tlc and the plate was then scanned by an Ambis β-scanner. The GTP ratios were then calculated [0.66GTPcpm/GDPcpm + 0.66GTPcpm].

Figure 4.12.
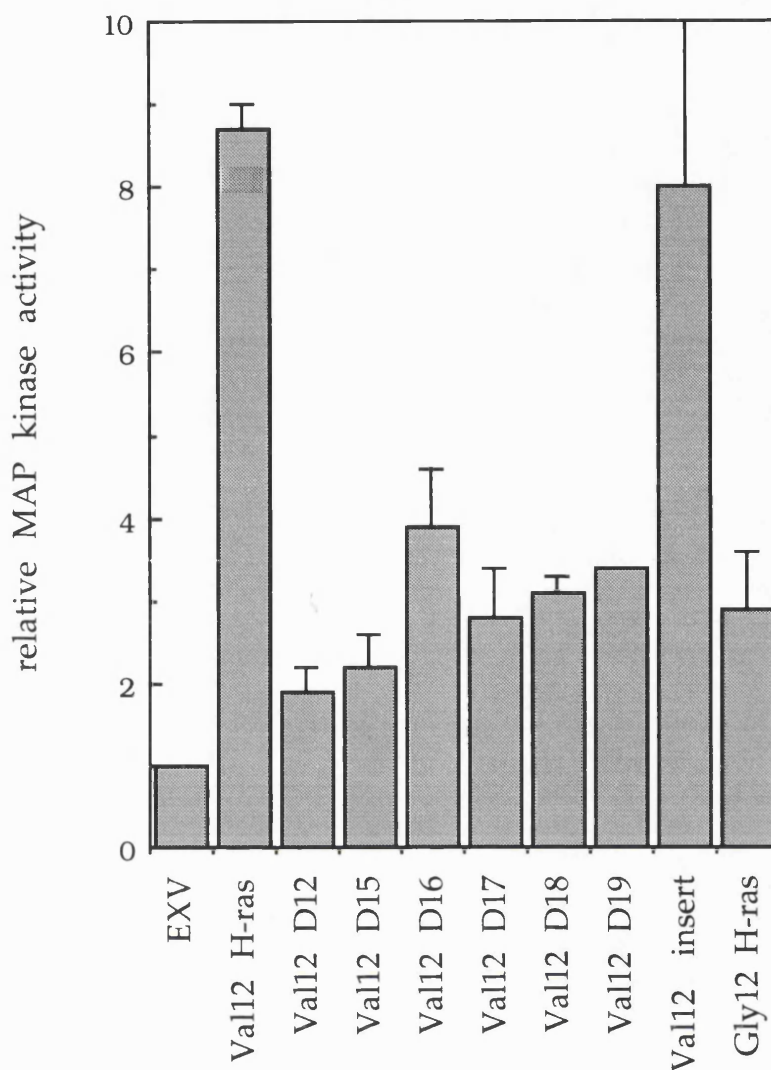
Relative MAP kinase activity of Val12 H-ras deletion mutants expressed in COS cells.

MAP kinase activity present in 50μg cytosol was assayed (as described in materials and methods) and expressed relative to the activity present in cells transfected with empty vector. Results are the mean of four independent experiments. Equivalent expression of the different Ras proteins was verified by western blotting s100 and p100 fractions from each transfection with Y13-259.

## 4.3. Discussion

The role of the hypervariable region of Ras has yet to be defined. The fact that it is evolutionarily conserved suggests that it plays a role in Ras function. It has however previously been reported that deletion of amino acids 165 - 184 from H-ras does not affect the transforming ability of the protein. It has also been shown that increasing the size of the hypervariable region has little effect on transforming efficiency. These results suggest that the hypervariable region has no function other than to connect the N-terminus and the C-terminus. Since the hypervariable region of Rab6 has recently been shown to be important for RabGGTase interaction, RabGDI binding and specific membrane targeting, it was decided to re-examine the role of the hypervariable domain in both H-ras and K-ras using several different approaches.

Mutants of K-ras were made that had amino acids inserted between the CAAX motif and the polybasic domain. It was wondered if increasing the distance between the polybasic domain and the CAAX motif would alter the localisation pattern of the protein to another membrane compartment within the cell such as the Golgi or if the protein would become cytosolic and biologically inactive. The relative spacing of the polybasic domain and the CAAX motif of K-ras was found to be fairly flexible in that it was possible to insert an extra 12 amino acids into this connecting region and the proteins remained plasma membrane localised although expression of these proteins was reduced. A protein with an insertion of 18 amino acids showed some p100 association when cells were fractionated but very little plasma membrane staining was seen by immunofluorescence. The reduction in protein expression seen with insertions of 12 and 18 amino acids may be due to a toxic effect. The ability to cause transformation was more limited since mutants with more than 7 amino acids inserted were non - transforming.

The role of the hypervariable region of H-ras was studied by making a series of mutations with various sizes of deletions in this domain. All of the mutants had intact CAAX motifs and at least one of the cysteine palmitoylation sites present. The post-translational processing of these mutants should therefore be unaffected and this was found to be the case. All of these mutants showed p100 association by COS cell fractionation and they also showed incorporation of [$^3$H] palmitic acid. One interesting finding was the possible Golgi localisation of the Δ12 mutant whilst all other mutants studied showed the familiar Ras plasma membrane localisation pattern. The only difference between this mutant, Δ12, and the

plasma membrane localised Δ16 was the presence of amino acid 179 in the Δ12 mutant.

When tested in NIH 3T3 focus assays, the relative transforming activity of all the deletion mutants was found to be severely reduced compared to H-ras. This is in contrast to the results obtained previously by other groups. The reason for this discrepancy is not clear, the DNA used in earlier work (Lacal et al, 1986; Willumsen et al, 1985; 1986) contained two activating mutations whilst the mutants used in my work were only activated at position 12. Different expression vectors were also used however it is not understood how these differences would explain the contrasting results presented here.

The dominant negative mutation (N17) of H-ras has increased affinity for GDP and inhibits proliferation of NIH 3T3 cells. N17 versions of the hypervariable deletion mutants were made and tested in NIH 3T3 co-transfection experiments to see if the inhibition of cell proliferation was reduced with these mutants. It was found that all of the deletion mutants showed significant rescue of cell proliferation. This implies that the N17 deletion mutants no longer bind irreversibly to GEF and are able to bind GTP which N17 H-ras is unable to do. Deletions within the HVR may affect the three dimensional structure of Ras in its effector binding domain and alter GEF interactions. The HVR contains an α helix which when removed may distort other regions of the protein and could alter GEF interaction and/or affinity.

The GDP/GTP loading of the mutants was examined and the Gly12 and Val12 versions of the mutants were all found to be appropriately loaded with the Gly12 mutants having 1 - 5% GTP bound whilst the Val12 forms had 74 - 84% GTP loaded. The inability of the Val12 deletion mutants to transform was therefore not due to a lack of GTP binding. The low GTP association of the Gly12 deletion mutants showed that their ability to interact with GAP was unaffected. The Val12 deletion mutants may therefore be compromised for effector interaction whilst the GAP binding domain is unaffected by these deletions.

The ability of the Val12 deletion mutants to activate MAP kinase was measured since the lack of transformation observed may be due to the inability to activate this signal transduction pathway. All of the mutants showed a significant reduction in the ability to activate MAP kinase to approximately Gly12 H-ras levels. This suggests that the biological inactivity of these mutants is indeed due to their inability to sufficiently activate this kinase cascade to the level required for cellular transformation. Of course

other cellular pathways may also be involved in cell transformation but these have yet to be identified.

Another approach tried to determine the role of the hypervariable region, was to mutate part of this region to amino acids of similar size and charge to those that were deleted from the Δ12 mutant. This would hopefully determine whether there was a requirement for the specific amino acids that make up the hypervariable domain of H-ras or whether any sequence of amino acids would be able to function as a hypervariable region. This mutant **'insert'** when tested in MAP kinase activation assays, NIH 3T3 co-transfections and NIH 3T3 focus assays, was found to behave in a similar manner to H-ras. This suggests that the sequence of the hypervariable region in itself is not specific but from the deletion work, its presence is essential.

## 4.4. Conclusions

The mutants used in this study had hypervariable region deletions of various sizes, however long and short deletions in this region produced similar results suggesting that the whole region is important. The results in this chapter indicate that the hypervariable region is required for biological activity yet a specific function for this region remains undefined.

The distance between the polybasic domain and the CAAX motif of K-ras can be increased quite significantly without affecting the plasma membrane localisation or the biological activity of the protein. However increasing the distance between these two regions by more than 12 amino acids reduced plasma membrane association and rendered the protein biologically inactive.

Removing the hypervariable region of H-ras led to a complete inability to produce cell transformation. This was shown not to be a result of lack of GTP binding but was possibly due to a reduction in MAP kinase activity. This suggests that removal of the hypervariable region disrupts effector interaction whilst the rescue of NIH 3T3 cell proliferation seen with the N17 deletion mutants suggests that GEF interaction may involve the hypervariable region.

The specific sequence of the hypervariable region does not appear to be important since it can be replaced with a completely different sequence without affecting the activity of the protein. These data together with the K-ras spacer results suggest that the region serves to maintain a certain distance between the N-terminus and the C-terminus which is vital for the biological activity of the protein.

Although the results presented here imply that the hypervariable region may be involved in GEF interaction there are still many unanswered questions concerning the function of this region and these will be addressed further in Chapter 5.

# Chapter 5

# Discussion

## 5.1. Introduction

The research presented in this thesis has concentrated on the post-translational modifications of Ras, subsequent protein localisation and the effect that this has on biological activity. A study was made of the effect of geranylgeranylation and myristoylation, rather than farnesylation, on H-ras and K-ras. The membrane localisation of the $C_{20}$ modified and myristoylated proteins was studied by cell fractionation and immunofluorescence. The biological activity of these proteins in focus assays and MAP kinase experiments was also examined. The results of this work led to the proposal of a model where the biological activity of Ras is related to its cellular location and its potential ability to cycle on and off the plasma membrane.

I have also begun research to establish a role for the hypervariable region. The effect of increasing the distance between the polybasic domain and the CAAX motif of K-ras was examined. The role of the hypervariable region of H-ras was studied by deleting the region and examining the effect on localisation, focus formation and the ability to activate MAP kinase. A model is proposed whereby the hypervariable region is essential for maintaining the correct spatial relationship between the N-terminus and the C-terminus of Ras necessary to allow efficient GEF interaction and activation of the MAP kinase signal transduction pathway.

## 5.2. Plasma membrane localisation requires the second signals of palmitoylation or a polybasic domain.

The plasma membrane localisation of H-ras requires the presence of the CAAX motif together with the cysteine palmitoylation sites. The plasma membrane targeting signals of K-ras are the CAAX motif together with a polybasic domain. To exclude the possibility that other sequences of Ras are involved in plasma membrane targeting, various C-termini from H-ras and K-ras were cloned onto the C-terminus of protein A, a normally cytosolic protein.

It was found that the CAAX motif together with the polybasic domain of K-ras or the cysteine palmitoylation sites of H-ras were the only sequences necessary to produce plasma membrane localisation of protein A.

The CAAX motif in isolation was insufficient to lead to plasma membrane association.

In order to examine the requirement for these second signals when another processing event other than farnesylation occurs, the CAAX motif of H-ras and K-ras was mutated to represent the CAAX motif of Ral and the brain G-protein $\gamma$ subunit, both of which are modified by geranylgeranyl. The majority of Ras-related proteins are prenylated by a $C_{20}$ (geranylgeranyl) moiety rather than the farnesyl or $C_{15}$ group that is attached to the Ras proteins. The addition of $C_{20}$ to H-ras or K-ras was able to replace the need for farnesylation in directing membrane association. Upon fractionation, $C_{20}$ modified Ras proteins were found to be p100 associated even in the absence of the second signals of plasma membrane localisation (palmitoylation or a polybasic domain). This is in contrast to the situation that occurs with farnesylated Ras, H-ras or K-ras that is only farnesylated and has no palmitoylation sites or a polybasic domain, is cytosolic. From immunofluorescence studies it was discovered that in order for geranylgeranylation to lead to plasma membrane association of Ras, palmitoylation or the polybasic domain was required. Without these second signals, the geranylgeranylated Ras proteins were nonspecifically associated with intracellular membranes. The membrane association of the geranylgeranylated mutant Ras proteins was found to be more avid than the farnesylated Ras mutants. This stronger interaction of the geranylgeranyl group with membranes may account for the nonspecific membrane association of these mutant proteins in the absence of the plasma membrane targeting sequences of palmitoylation or a polybasic domain.

Several proteins are modified by myristoylation of an N-terminal glycine residue via an amide bond. Myristoylation is a co-translational processing event in contrast to the post-translational modifications undergone by the Ras proteins. Myristoylation aids the membrane association of some proteins notably p60[src]. A myristoylation coding sequence from Rasheed Ras was cloned onto the N-terminus of H-ras and K-ras and the effect of myristoylation in combination with various Ras membrane localisation signals was examined.

It was found that myristoylation in isolation was insufficient to lead to membrane association, whereas myristoylation in combination with either farnesylation, palmitoylation or a polybasic domain led to p100 association. However specific plasma membrane localisation required the targeting signals of the hypervariable region together with myristoylation. This is a similar

situation to the Src proteins where myristoylation in isolation is insufficient to bring about membrane localisation. Membrane targeting of $pp60^{v-src}$ appears to involve electrostatic interactions between N-terminal lysine residues and the negatively charged phospholipids of the membrane acting in combination with the myristate group (Silverman and Resh, 1992; Silverman et al, 1993).

Myristoylation was able to replace the need for farnesylation to occur prior to palmitoylation of H-ras and it has now been shown by several groups that a number of myristoylated proteins also undergo N-terminal palmitoylation in order to achieve membrane localisation. The sequence for N-terminal palmitoylation of the Src proteins, $p56^{lck}$ and $p57^{fyn}$, is Myr-Gly-Cys. (Paige et al, 1993; Shenoy-Scaria et al, 1993). The Src and Ras proteins could be said to be mirror images of one another with one group (the Src family) undergoing N-terminal processing events whilst the other group (the Ras family), is processed at the C-terminus. The three dimensional structure of Ras as we currently know it, positions the N- and C-terminus in close proximity to one another and this could enable N-terminal myristoylation to substitute for C-terminal farnesylation in providing a recognisable substrate for the Ras palmitoyltransferase. Therefore myristoylated proteins are recognised by palmitoyltransferases as substrates.

The plasma membrane localisation of Ras therefore involves two signals, alkylation and a second signal contained within the hypervariable region. Several models for membrane targeting have been proposed. There could be specifically localised membrane-bound receptors that direct prenylated carboxy-termini to intracellular compartments (Figure 5.1). These hypothetical prenyl-protein receptors would presumably recognise the prenyl moiety and some specific amino acid sequence or protein structure since proteins with identical lipid modifications can be localised to different membrane compartments within the cell. For example it has been reported that the prenylated mating pheromone a-factor interacts with a specific transmembrane receptor, STE3, to induce growth arrest and morphological differentiation in cells of the α mating type (Hagen et al, 1986). The lamin B receptor is also thought to be an integral membrane protein (Worman et al, 1990) that mediates the association of lamin B with the nuclear membrane, a process that is prenyl-dependent (Worman et al, 1988).

The 'dumb' lipid model (Schafer and Rine, 1992) speculates that the prenyl group provides a nonspecific lipid attachment to membranes but does not mediate targeting to a specific membrane (Figure 5.1). In contrast,

**Figure legend 5.1.**

**Suggested models for prenyl-dependent targeting.**

A. Prenyl-protein receptor model. The prenyl group and the protein structure are recognised by membrane localised receptors.

B. Dumb lipid model. Membrane association is the result of the nonspecific affinity of the lipid group for membranes. Protein-protein interactions mediate specific targeting.

C. Smart lipid model. Protein localisation is mediated by specific lipid-lipid interactions.

D. Secondary interaction model. A secondary modification that is dependent upon prenylation mediates protein targeting.

**Figure 5.1**

**Models for prenyl-dependent protein targeting.**
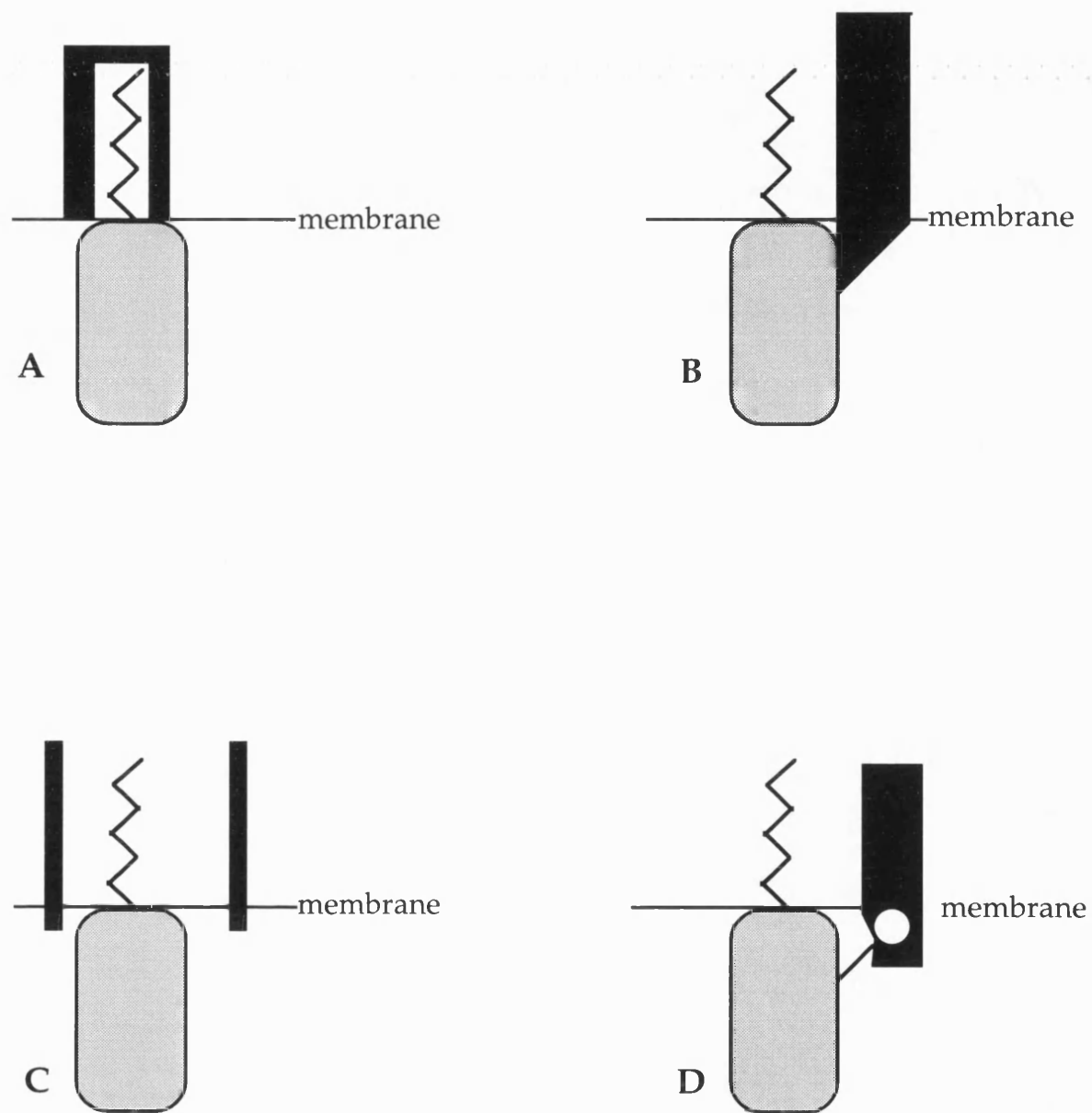
the 'smart' lipid model has the lipid modification directing the protein to a specific locale through a nonreceptor based mechanism (Figure 5.1).

Membrane association may also result from a secondary modification that is itself prenyl-dependent (Figure 5.1). For example the assembly of normal lamin A into the nuclear lamina requires farnesylation of prelamin A. However the essential step for localisation is not farnesylation but the removal of the prelamin A carboxyl-terminus which requires prenylation. Lamin A precursors lacking C-terminal amino acids have been shown to be assembled in the lamina without prenylation (Lutz et al, 1992).

The membrane association of Ras and Ras-related proteins suggests that their targeting to specific membrane compartments does not involve the prenyl group but rather amino acid sequences of the proteins. The hypervariable region directs membrane localisation since Ras can be modified by farnesyl, geranylgeranyl or myristate and is still plasma membrane localised provided that the hypervariable region is intact. Prenylation may be a prerequirement for a secondary modification that actually directs protein localisation. This appears to be the case for H-ras where farnesylation has to occur before palmitoylation take place.

Other Ras-related proteins also have at least a two signal requirement to achieve membrane localisation. The hypervariable region of Rab proteins has been shown to be critical to the localisation of these proteins to specific membrane compartments but targeting clearly involves other protein sequences in addition (Chavrier et al, 1991). The C-terminal modifications together with a polybasic domain may also be necessary for the Golgi localisation of Rap1A. The nuclear membrane localisation of lamin proteins requires CAAX motif modifications in conjunction with a nuclear localisation signal. The membrane association of the Src proteins requires N-terminal myristoylation together with N-terminal palmitoylation or N-terminal lysine residues.

This two signal method of membrane localisation is therefore very common among Ras-related and non Ras-related proteins.

## 5.3.    Ras activity is related to processing events and localisation.

Another issue concerning the localisation of Ras is whether farnesylation is important for its interaction with effectors and regulators independently of its role in membrane localisation. A measure of the biological activity of various Ras proteins was obtained by performing MAP kinase assays on cytosolic fractions from transfected COS cells since the kinase cascade from

Ras through Raf to MAP kinase has been established. Cytosolic fractions are used because the major MEK kinase activity (80%) in the cytosol of serum starved COS cells expressing Ras proteins has been identified as p42 MAP kinase (Cadwallader *et al*, in press). The cytosolic fractions were incubated with kinase dead MEK immobilised on beads and [$^{32}$P]γATP. The phosphorylation of MEK can then be measured and used as a readout for Ras activity.

Expression of oncogenic H-ras increased MAP kinase activity seven fold over the activity observed in control transfections with empty vector. Cytosolic, farnesylated H-ras C181S, C184S was equipotent to plasma membrane localised H-ras in activating MAP kinase. By contrast, the other cytosolic H-ras mutants: H-ras C186S and myristoylated H-ras C181S,C184S,C186S did not elevate MAP kinase activity above control levels. These data indicate that farnesylation is required for cytosolic Ras to activate the MAP kinase pathway and that myristoylation cannot substitute the farnesylation function. Moreover, myristoylation significantly abrogated the activities of plasma membrane localised H-ras (with a wild type C-terminus) and farnesylated, non-palmitoylated H-ras (C181S,C184S). Interestingly, myristoylated, palmitoylated H-ras C186S, which localised normally to the plasma membrane was biologically active, suggesting that the requirement for farnesylation can be waived if Ras is correctly localised. The activities of the K-ras proteins showed a similar profile to the corresponding H-ras mutants. Wild type K-ras and farnesylated, cytosolic K6Q both had significant activity whereas cytosolic K-ras C186S did not activate the MAP kinase pathway. Myristoylation compromised the activities of both wild type K-ras and K6Q but efficiently rescued the activity of K-ras C186S.

As a second measure of biological activity, the myristoylated H-ras and K-ras mutants were assayed for focus forming ability in NIH 3T3 fibroblasts. Of the Ras proteins that mislocalised to the cytosol, only farnesylated Ras (H-ras C181S,C184S and K6Q) retained the ability to transform NIH 3T3 cells. The other cytosolic Ras mutants: K-ras C186S, H-ras C186S and myristoylated H-ras C181S,C184S,C186S all of which failed to activate MAP kinase, also failed to form foci. However, myristoylated palmitoylated H-ras C186S, and myristoylated K-ras C186S were both able to transform NIH 3T3 cells. In the transformation assay, as in the MAP kinase assay, myristoylation reduced the biological activity of plasma membrane localised H-ras and K-ras with wild type C-termini.

These data begin to address an interesting question, namely to what extent plasma membrane localisation and Ras biological activity are separable, i.e. is farnesylation important for the interaction of Ras with its regulators and

effectors independently of promoting membrane binding. In a completely *in vitro* system farnesylated Ras2 is a much more potent activator of adenyl cyclase than unmodified Ras2 (Kurodi *et al*, 1993). Moreover, geranylgeranylated H-ras Gly12 is growth inhibitory at low levels of expression (Cox *et al*, 1992) arguing that the specific isoprenoid is important to some aspect of Ras biology. I have used MAP kinase activation as an indirect measure of Raf activity in cells expressing Ras. Ras in the GTP form binds with high affinity to the N-terminal CR1 domain of Raf (Vojtek *et al*, 1993; Warne *et al*, 1993), but the role of this interaction in the activation of Raf is unclear. Raf is predominantly cytosolic in quiescent cells but activated Raf co-localises with Ras in Ras transformed HER313 cells, raising the possibility that Ras may be involved in translocating Raf to the plasma membrane (Traverse *et al*, 1993). My data show that farnesylation is absolutely required for Ras to activate the MAP kinase pathway when Ras is confined to the cytosol since unmodified Ras (H-ras C186S, and K-rasC186S) and H-ras modified by myristate only (H-ras C181S,C184S,C186S) were biologically inactive. However, plasma membrane localised Ras activates the MAP kinase cascade irrespective of whether the protein is farnesylated.

Myristoylation of farnesylated Ras proteins significantly reduces the extent to which these proteins activate MAP kinase. In the case of myristoylated H-ras C181S,C184S, one could postulate that this is because the protein is extensively mislocalised to intracellular membranes and may therefore also sequester Raf away from potential plasma membrane localised activators. This does not hold for myristoylated H-ras and myristoylated K-ras, which are both plasma membrane localised, but are, nevertheless, compromised in activity. Although at the plasma membrane, these proteins may be constrained by virtue of C-terminal and N-terminal lipidation in a manner that restricts access of Raf to the effector domain. This seems unlikely since the binding sites of Raf and GAP on Ras show considerable overlap (Zhang *et al*, 1993) and the interaction of GAP with myristoylated Ras was not compromised because Gly12 myristoylated mutants were not associated with GTP.

Alternatively, full biological activity may require that Ras interacts reversibly with the plasma membrane (**Figure 5.2**), a freedom denied Ras proteins with the two irreversible lipid modifications, farnesylation and myristoylation. Such a model predicts that Ras modified by myristoylation and reversible palmitoylation which has the potential to cycle on and off the membrane (Magee *et al*, 1987), should be more active than myristoylated H-ras.
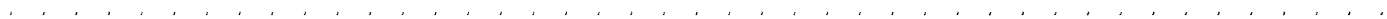
**Figure Legend 5.2.**

**A model for the cycling of Ras proteins.**

H-ras undergoes post-translational modifications at the CAAX motif becoming farnesylated, proteolysed and methylated. These processing events are irreversible and the protein is cytosolic. Membrane association of H-ras is achieved by palmitoylation. This is a reversible process and H-ras cycles on and off the membrane according to its palmitoylation state.

K-ras undergoes the same triplet of CAAX modifications but then becomes plasma membrane associated by electrostatic interaction between the polybasic domain and the negatively charged phospholipid head groups of the membrane. This interaction may be reversible by virtue of its weakness or phosphorylation may neutralise the positive charge and lead to membrane dissociation.

F = farnesyl

P = palmitate

+ = polybasic domain

Figure 5.2

A model for the cycling of Ras proteins



H-ras

K-ras(B)
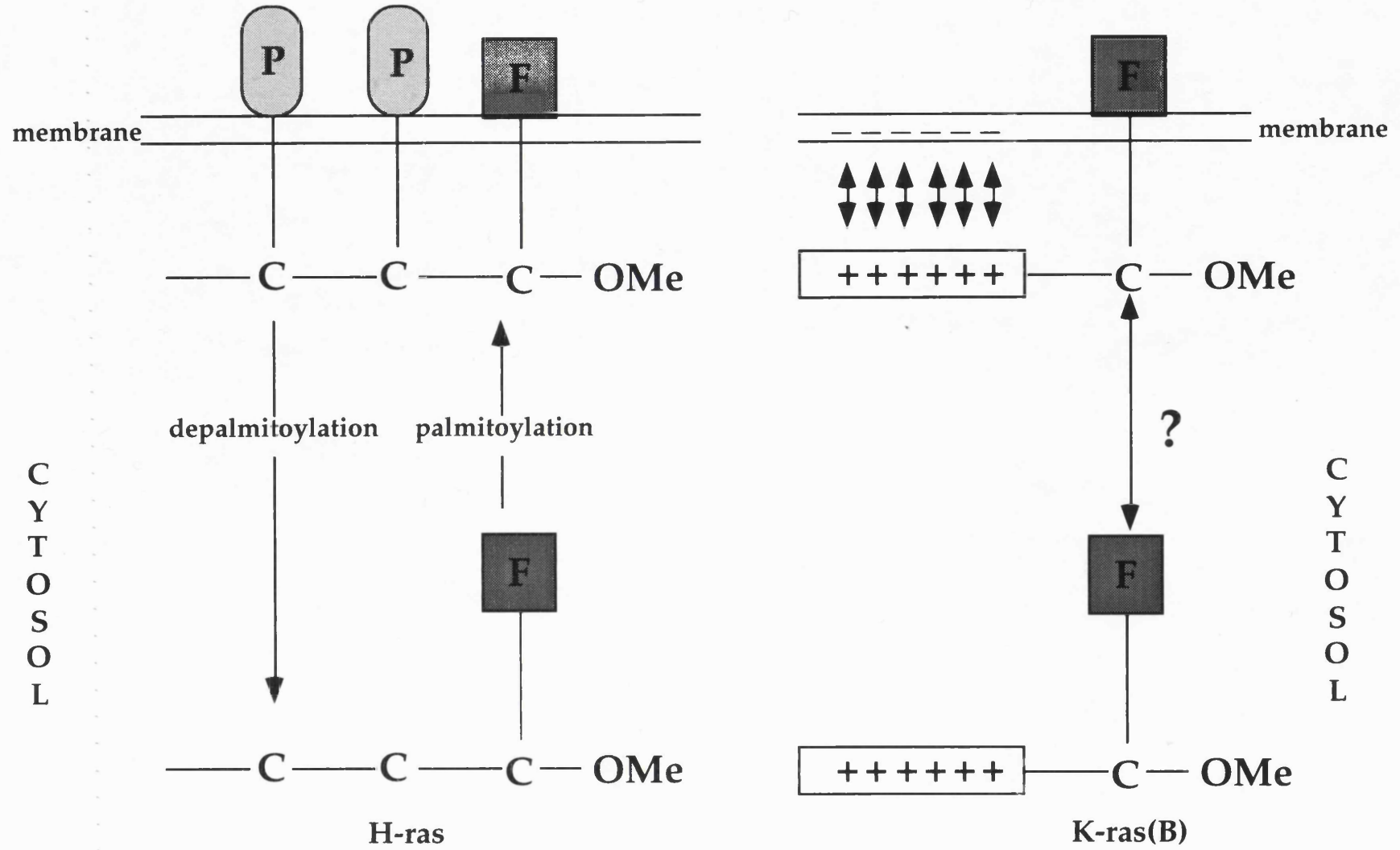
The results presented here support this model. Normally processed, plasma membrane localised H-ras and the myristoylated and palmitoylated mutant (MC186S) showed similar ability to activate MAP kinase. Both of these proteins (H-ras and MC186S) are modified by one irreversibly attached lipid, farnesyl or myristate, and one reversibly attached lipid, palmitate. These proteins therefore have the potential to associate with and dissociate from the plasma membrane. Myristoylated, farnesylated and palmitoylated H-ras showed significantly reduced ability to activate MAP kinase. This protein is modified by two irreversible lipid modifications, farnesylation and myristoylation, together with reversible palmitoylation. This protein would therefore be expected to remain membrane associated.

The geranylgeranylated modified H-ras mutant proteins were shown to have reduced MAP kinase activity compared to farnesylated H-ras. The mislocalised geranylgeranylated mutant (1/4CCIL) had MAP kinase activation levels similar to the negative control whilst the geranylgeranylated and palmitoylated mutant (HCCIL) showed reduced activation compared to farnesylated and palmitoylated H-ras. This reduction in MAP kinase activation may be due to the stronger membrane association of the geranylgeranylated proteins which even if palmitoylation is a reversible process, may impede the ability to associate with and dissociate from the plasma membrane.

Quantitatively K-ras was a more potent activator of MAP kinase than H-ras. Qualitatively the presence or absence of the polybasic domain had the same effect on the activities of the differently lipidated K-ras proteins as palmitoylation had on the activities of the correspondingly myristoylated/ farnesylated H-ras proteins. Myristoylated, farnesylated K-ras with a polybasic domain was less active in MAP kinase assays presumably due to its two irreversible lipid modifications. Mislocalised myristoylated K-ras also showed reduced activity. It is not known whether the interaction of the K-ras polybasic domain with the plasma membrane is reversible but these results suggest that it is.

There was a good correlation between the ability of the variously modified Ras proteins to activate MAP kinase and their ability to transform NIH3T3 fibroblasts, consistent with MAP kinase activation being a key event in Ras transformation. The only discrepancy between the two assays of Ras biological activity was the relative potencies of H-ras and K-ras. H-ras Gly12Val is a more potent transforming gene than K-ras Gly12Val in NIH3T3 fibroblasts (Hancock *et al*, 1989; Hancock *et al*, 1990;

Marshall and Hancock, unpublished data), but K-ras Gly12Val is the more potent activator of MAP kinase in COS cells. Whether the different mechanisms of membrane attachment of K-ras and H-ras in some way modulate their interactions with effector proteins and can therefore account for these data is an intriguing proposition. Cell transformation may require the activation of several pathways not just the MAP kinase cascade and may also involve Ras-independent signals. K-ras whilst being a potent stimulator of MAP kinase may be less able to activate these other pathways that are essential for cell transformation.

The model for the reversible interaction of prenylated proteins, such as Ras, with membranes is supported by research on other G-proteins. These proteins are involved in reactions that require fast response times and are repeatedly being stimulated. It has recently been reported that the N-terminus of the $\alpha$-subunit of the photoreceptor G protein transducin, T$\alpha$, is modified by heterogeneous fatty acids including laurate (C12:0), unsaturated C14:2 and C14:1 fatty acids, and a small amount of myristate (Kokame et al, 1992). Myristate is postulated to be the membrane anchor of Gi$\alpha$ or Go$\alpha$ which dissociate from the $\beta\gamma$ subunit after activation by GTP and receptor. Transducin $\alpha$, it is suggested, has a looser association with T$\beta\gamma$ than the other N-terminal myristoylated G-proteins due to the less hydrophobic nature of the C12:0, C14:2 and C14:1 fatty acids compared to myristate. This weak interaction would permit the rapid association and dissociation of T$\alpha$ that are catalysed by metarhodopsin II within a photoresponse time of vision (Vuong et al, 1984).

G-protein-coupled receptor kinases phosphorylate G-protein-coupled receptors when they are in their active or stimulated conformations (Palzewski and Benovic, 1991). The $\beta$-adrenergic receptor kinase ($\beta$ARK1) (Benovic et al, 1989) and rhodopsin kinase (Lorenz et al, 1991) are both members of this family. The ability of these kinases to rapidly phosphorylate G-protein coupled receptors following stimulation depends on prenylation.

Rhodopsin kinase is farnesylated at its C-terminus (Inglese et al, 1992) and translocates to rod outer segment membranes in a light dependent manner. The prenylated rhodopsin kinase then associates with the membrane-embedded receptor and phosphorylates it leading to quenching of the response. A geranylgeranylated rhodopsin kinase mutant is tightly associated with rod outer segment membranes even in the dark and

shows no light-stimulated translocation. This is presumably due to the greater hydrophobicity of geranylgeranyl compared to farnesyl.

The γ subunits of heterotrimeric G-proteins are geranylgeranylated apart from transducin γ which is farnesylated. The βγ subunits dissociate from the α subunit during agonist-mediated stimulation and activate βARK in vitro (Haga and Haga, 1990; Pitcher et al, 1992). βARK interacts with the prenylated βγ complex and this interaction appears to facilitate or target βARK to the lipid embedded β-adrenergic receptor which is then phosphorylated and the system becomes desensitised.

## 5.4. Functions of the hypervariable region

The hypervariable domain is a region (residues 165-180) that shows less than 20% homology between any two ras genes. This is in marked contrast to the rest of the protein which shows significant homology especially at the N-terminus (the first 80 amino acids are identical for the three human ras genes) and the extreme C-terminus (the CAAX motif). This region varies in sequence but has approximately the same length within in each of the three main Ras sub branches. The Rho group has the shortest region, 14 - 17 residues, Ras proteins have 18 - 30 amino acids, where in the Ypt/Rab group, the length varies between 27 - 47 amino acids (Valencia et al, 1991).

The conservation of the region between species throughout evolution suggests that it is necessary and performs a conserved function. It could account for different functions of the various Ras proteins by allowing interactions with specific proteins/effectors or direct the protein to specific sites within the cell such as different types of membrane. Particular C-terminal sequences may determine association with specific membranes possibly through interactions with membrane bound proteins.

Previously it has been reported that removal of amino acids 165-184 from H-ras (Lacal et al, 1986; Willumsen et al, 1985; 1986) or increasing the size of the H-ras hypervariable region (Willumsen et al, 1985) does not affect H-ras transforming ability. This suggested that the H-ras hypervariable region (HVR) merely acts as a junction between the N-terminus and the C-terminus and had no specific function.

The polybasic domain of Rap1A consists of two small polybasic regions separated by one amino acid. Rap1A is Golgi localised whilst K-ras is targeted to the plasma membrane. The polybasic domains of other Ras-related proteins also consist of positively charged regions separated by

several amino acids at various distances from the CAAX motif. It was wondered whether a plasma membrane targeting signal was generated by the spatial relationship between the polybasic domain and the CAAX motif and whether increasing the distance between these regions would alter the localisation pattern of K-ras. To test this theory insertions of various lengths were placed between the polybasic domain and the CAAX motif of K-ras. No effect was seen on plasma membrane localisation or biological activity when 12 extra amino acids were inserted into this region, however an 18 amino acid insertion produced a biologically inactive protein which showed little plasma membrane localisation. It is therefore possible to significantly increase the distance between the polybasic domain and the CAAX motif but beyond a certain limit (between 12 and 18 amino acids), plasma membrane targeting and focus formation is abolished.

Since the distance between the plasma membrane and the CAAX motif could be significantly increased without altering the localisation pattern of K-ras, it would appear that the plasma membrane targeting signal is contained within the polybasic domain itself and is not related to the distance between it and the CAAX motif.

The K-ras mutant with 18 amino acids inserted in between the polybasic domain and the CAAX motif is an interesting protein. It is presumably farnesylated and processed normally yet it is cytosolic. It would therefore be expected to be able to transform NIH 3T3 cells with an activity comparable to that of the polybasic domain mutant, K6Q, which is also farnesylated and cytosolic. However the K-ras insertion mutant is nontransforming whilst the K6Q mutant is capable of transformation. This would suggest that whilst the plasma membrane targeting signals of the hypervariable region are not required for transformation (i.e. the polybasic domain mutant K6Q is cytosolic but transforms), the length of the hypervariable region appears to affect the ability to cause transformation. An insertion of 18 amino acids into the hypervariable region may distort the conformation of the protein such that it can longer interact efficiently with its effectors to activate the signal transduction pathway that leads to cell transformation. This constraint on the length of hypervariable region could account for the conservation of the length of the region among the proteins of the Ras superfamily.

In order to see if the hypervariable region has a functional role, deletions of varying lengths were made within the hypervariable region of H-ras. None of the deletions involved the plasma membrane localisation

signals of the CAAX motif or the cysteine palmitoylation sites and consequently all of the deletion mutants were found to be plasma membrane localised and palmitoylated. In NIH 3T3 transformation assays the oncogenic Gly12Val hypervariable deletion mutants were all found to be severely compromised for focus formation. This was unexpected since previous work had reported that this region was not required for biological activity. The mutants used in those studies (Lacal *et al*, 1986; Willumsen *et al*, 1985; 1986) had two activating mutations and used a different expression vector to the data presented here but it is hard to see how these differences would account for these discrepancies.

The wild type Gly12 hypervariable deletion mutants were found to be 1-5% GTP loaded implying that they interact normally with p120GAP. p120GAP increases the intrinsic GTPase activity of the proteins leading to the release of GTP and binding to GDP. The oncogenic Gly12Val hypervariable deletion mutants were found to be 74-84% GTP loaded and would by implication bind p120GAP normally since the Gly12 mutants appear to. However their intrinsic GTPase activity is not increased by p120GAP and they remain predominantly in the GTP bound state.

Since the important signal transduction pathway elucidated for Ras involves a kinase cascade, the biological inactivity of the oncogenic Gly12Val hypervariable deletion mutants may be due to an inability to activate the MAP kinase cascade signal transduction pathway. The oncogenic (Val12) H-ras hypervariable deletion mutants were all found to be severely reduced for MAP kinase activation, which may account for their lack of transformation activity. It is also feasible that numerous pathways need to be activated in order to bring about cellular transformation and removal of the hypervariable region affects one or more of these yet to be discovered pathways. The lack of transformation activity and the reduced MAP kinase activation of the hypervariable region deletion mutants suggests that the hypervariable region is possibly involved in the interaction between Ras and its effector(s) *in vivo*. Alternatively, deletion of the hypervariable region may cause conformational changes in the Ras protein which distort the effector binding site such that the effector (e.g. Raf) can no longer interact with Ras or that the affinity between the effector and Ras is weakened. However the binding sites of p120GAP and Raf show considerable overlap (Zhang *et al*, 1993) and the interaction of p120GAP with the hypervariable deletion mutants was not compromised

because the wildtype (Gly12) hypervariable deletion mutants were not bound to GTP.

The Asp17 (N17) mutation of H-ras results in an increased affinity for GDP and an inability to release GEF and bind GTP (Feig and Cooper, 1988). Expression of this protein is therefore severely growth inhibitory. However, the Ser17Asn H-ras hypervariable deletion mutants were not growth inhibitory to NIH 3T3 cells suggesting that GEF interaction is affected by removal of the hypervariable region. The ability of the Ser17Asn H-ras hypervariable deletion mutants to rescue NIH proliferation suggests that they are no longer irreversibly bound to GEF and can therefore release GDP and bind GTP. The removal of the hypervariable region may affect GEF/Ras interaction by reducing the affinity of binding, by disrupting the GEF binding site due to conformational changes within the protein following hypervariable region removal, or by preventing GEF interaction due to the altered positioning of Ras in relation to the plasma membrane.

The currently known three dimensional structure of Ras indicates that it is a compact globular structure with amino acids 165-175 forming an α helix that protrudes from the main body of the protein. Amino acids 175-186 are also predicted to form an α helix so the assumed structure of Ras is a globular core with the hypervariable region projecting from it like a stalk (**Figure 5.3.**). I propose that removal of the hypervariable region leads to the main body of the protein being positioned much closer to the plasma membrane than usual. This new position may block the Sos binding site or lead to less avid binding of Sos due to the inability to access all of its binding sites.

The GEF binding site of H-ras is thought to involve amino acids 63-75 and 100-110 (Segal et al, 1993). These regions are highly conserved among Ras-related proteins implying that they have an important functional role. Residues 100-110 form part of the α helix 3 and loop 7 in the three dimensional structure of H-ras and are, according to the proposed model for the structure of Ras, positioned close to the C-terminus of the protein. Moreover, when the C-terminal lipid modifications of Ras have targeted the protein to the plasma membrane, the H-ras GEF 100-110 binding site would probably be on the plasma membrane facing surface of the protein.

The **insert** mutant, which had one part of the hypervariable region replaced with an unrelated sequence, was made to resolve the issue as to whether the specific amino acid sequence of the hypervariable region was

**Figure Legend 5.3.**

**The influence of the hypervariable domain on GEF interaction.**

Ras forms a globular structure with the hypervariable region and the CAAX motif protruding away from the main body of the protein. The hypervariable region allows sufficient space between the plasma membrane and the Ras protein for GEF interaction. Removal of the hypervariable region results in the movement of the protein closer to the plasma membrane which restricts GEF interaction.

P = palmitate

F = farnesyl

# Figure 5.3.
The influence of the HVR on GEF interaction



H-ras

H-ras hypervariable deletion mutant
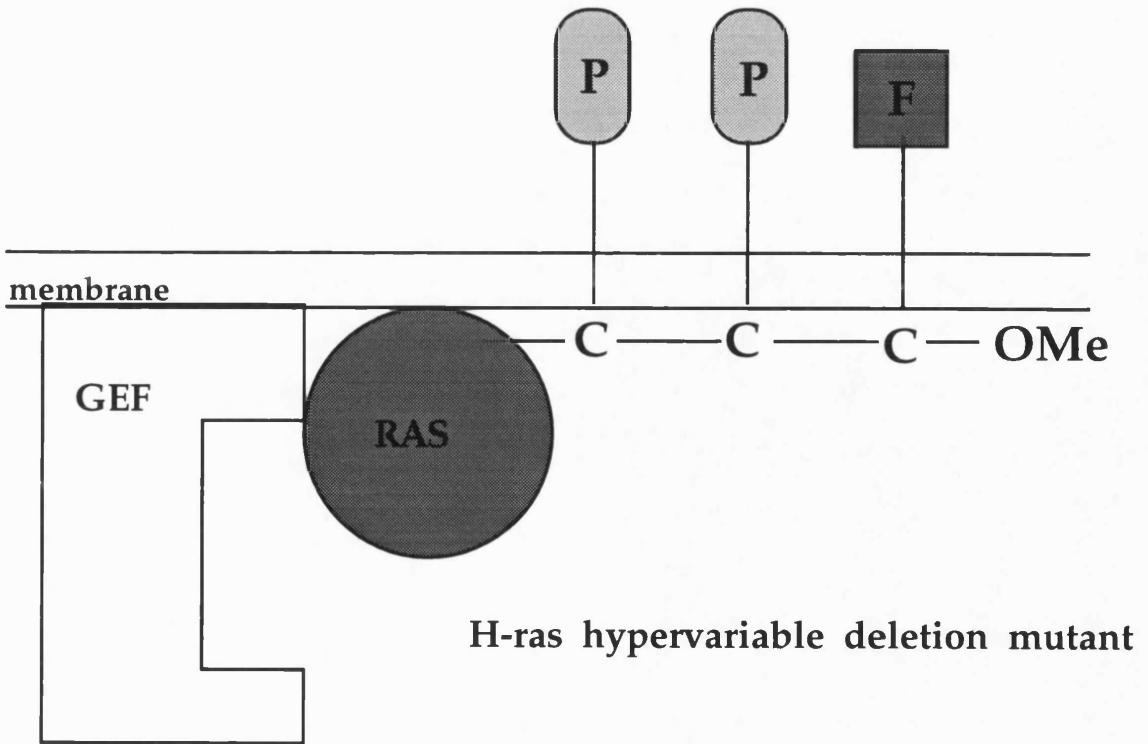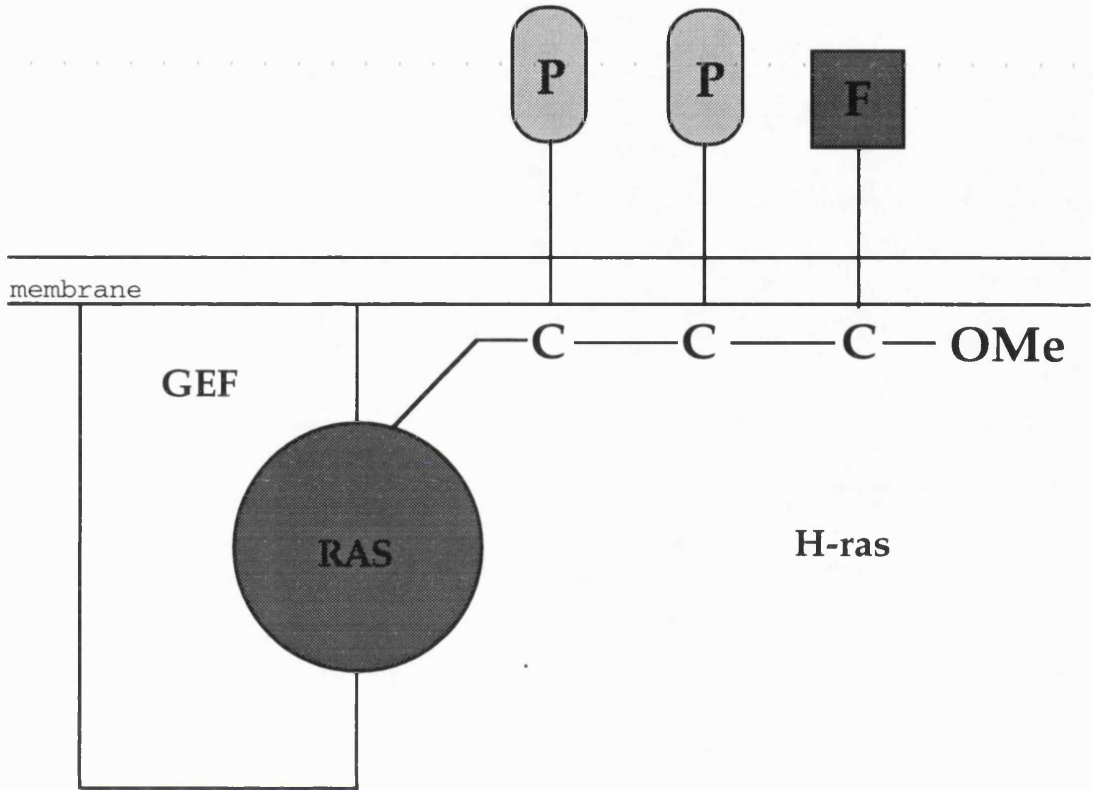
important. The oncogenic (Val12) **insert** protein was found to have a similar ability to transform NIH 3T3 cells as oncogenic (Val12) H-ras. It also had similar MAP kinase activation potency and was as toxic in NIH 3T3 co-transfection experiments as oncogenic Val12 H-ras. This showed that although the domain is absolutely required for biological activity, the actual sequence of the hypervariable region is not critical.

The hypervariable region therefore appears to be necessary for GEF interaction and it may also be important for the effector interaction of Ras *in vivo*. In order to examine the possible functions of the hypervariable region in more detail, further experiments will be performed. The role of the hypervariable domain in effector interaction will be studied by measuring the binding affinity between the deletion mutants and Raf, a known Ras effector. This will determine whether Ras/Raf interaction is affected by removal of the hypervariable region. Since oncogenic Gly12Val H-ras (at 5µg/plate) was found to be toxic in the co-transfection experiments performed, the oncogenic Gly12Val hypervariable deletion mutants will be tested in these assays. If effector interaction is dependent upon the hypervariable region, it is expected that these mutants will not be toxic in these assays.

The GEF role of the hypervariable region and the model that I have proposed will be tested further by examining GEF/Ras interaction *in vivo* and *in vitro*. This will be done by measuring GTP loading of the wildtype Gly12 hypervariable deletion mutants when they are co-transfected with full length Sos and with the catalytic domain alone of Sos. If the model I have proposed is correct, I may find that the smaller catalytic domain of Sos will still be able to interact with amino acids 100-110, a plasma membrane facing interactive site, despite its closer proximity to the plasma membrane. Full length Sos may promote less efficient GTP loading since it is too large to access this site in the context of the hypervariable deletion.

## 5.5. Conclusions

This thesis examines the role of the post-translational modifications of Ras and how these relate to biological activity. The ability to substitute different processing events for one another and their effect on protein localisation and biology was examined.

The plasma membrane targeting signals of Ras are confined to the C-terminus and consist of the CAAX motif together with either a polybasic domain (K-ras) or palmitoylation (H-ras). It was found that

geranylgeranylation or myristoylation could target Ras to the plasma membrane only in the presence of the second signals of palmitoylation or a polybasic domain. Myristoylation and geranylgeranylation could also replace the normal farnesylation of Ras to produce biologically active proteins.

A model is proposed where the ability of Ras to activate the MAP kinase cascade depends on the localisation of the protein to the plasma membrane and the potential reversibility of this interaction.

The work on the hypervariable region showed that the distance between the polybasic domain and the CAAX motif of K-ras could be significantly increased without affecting plasma membrane localisation or biological activity. The hypervariable region of H-ras was found to be essential for biological activity since deletions produced non-transforming proteins which although GTP loaded, had reduced ability to activate the MAP kinase cascade. Lack of focus formation may be a consequence of this and/or the inability to activate other essential pathways.

The ability of the Ser17Asn H-ras hypervariable deletion mutants to rescue proliferation led to the proposal of a model whereby deletion of the hypervariable region alters the ability of GEFs to interact with Ras by conformational change within the protein or by the positioning of the protein relative to the plasma membrane. If this model is correct, the hypervariable region is required to connect the N-terminus and the C-terminus in a way that allows the GEF access to Ras without interference from the plasma membrane.

Further study of the hypervariable region is planned and will involve a thorough analysis of the ability of the deletion mutants to interact with Sos and a study of effector interaction.

Note added in proof.

The ability of the predominantly cytosolic but farnesylated forms of Ras to transform NIH 3T3 cells is now believed to be a consequence of the over-expression of the protein in these cells and the resulting association of a small proportion of the protein with the plasma membrane. It is this small amount of plasma membrane localised protein that is responsible for the ability to transform NIH 3T3 cells.

# Chapter 6
# Materials and methods

## 6.1. DNA Techniques
## 6.1.1. Plasmid preparations
### 6.1.1.1. Large scale plasmid preparations

A single colony was picked from a selection plate and inoculated into 500ml of L-broth containing ampicillin. This culture was then grown at $37°C$ overnight. The cells were collected by centrifugation and resuspended in 10ml of buffer P1 (50mM Tris/HCl, 10mM EDTA, pH8.0 with RNAse), 10ml of buffer P2 (0.2M NaOH, 1% SDS) were then added, the tube was inverted several times and incubated at room temperature for 5min. 10ml of buffer P3 (3.0M KAc) were then added and after mixing a 20min incubation on ice was performed. The solution was centrifuged and the supernatant was applied to a Qiagen-tip 500 that been equilibrated with 10ml of buffer QBT (750mM NaCl, 50mM MOPS, 15% EtOH, pH7.0, 0.15% Triton X-100). The Qiagen-tip 500 was then washed with 2x30ml of buffer QC (1000mM NaCl, 50mM MOPS, 15% EtOH, pH7.0) before eluting the DNA from the column with 15ml of buffer QF (1250mM NaCl, 50mM Tris/HCl, 15% EtOH, pH8.5). The DNA was precipitated with 0.7vols of isopropanol, washed with 70% ethanol, dried and taken up in TE buffer. All buffers were supplied in a kit from Qiagen.

### 6.1.1.2. Small scale plasmid preparation

For rapid screening of recombinants small scale preparations of plasmid DNA from overnight or 6h 1.5ml cultures were used. The miniculture was microfuged for 5min and the supernatant discarded. The pellet was taken up in 100μl of cold lysis solution (50mM glucose, 10mM TrisCl pH8.0, 1mM EDTA, 10mg/ml lysozyme) and placed on ice for 5min. 200μl of 0.2M NaOH/1% SDS was added and after mixing gently 150μl of 3M NaAc pH4.2 was added followed by a 15min incubation on ice. The solution was vortexed and microfuged for 15min at $4°C$. The supernatant was transferred to a fresh tube and the DNA precipitated with 2volumes of 100% ethanol. The pellet was washed with 70% ethanol, dried and taken up in 50μl of TE buffer. RNAse was included in all restriction digests to remove contaminating RNA and most enzymes performed adequately on this DNA. Occasionally 10ml overnight cultures were grown and these were processed in exactly the same manner but all volumes were doubled.

## 6.1.2. Preparation of single stranded DNA

### 6.1.2.1.Large scale preparation of single stranded DNA

The highly purified single stranded (SS) DNA used as a template for oligonucleotide directed mutagenesis was produced using the following protocol. A single plaque was picked from a fresh transfection and inoculated into a 1.5ml culture of L-broth containing 15μl of a fresh overnight culture of JM109. The inoculum was grown for 4hr at $37^{\circ}C$ with agitation after which the cells were removed by centrifugation. The supernatant was transferred to a 100ml culture of JM109 in log phase growth $(OD_{550}=0.3)$ and grown for a further 4hr. The cells were removed by centrifugation and the phage precipitated by an hour long incubation at $4^{\circ}C$ with 0.2 volumes of 20% polyethylene glycol /2.5M NaCl (PEG/NaCl) solution. The phage pellet obtained after centrifugation was taken up in 0.5ml TE buffer, microfuged to remove any remaining cells and re-precipitated with 200ml PEG/NaCl. The pellet was taken up in 500μl TE buffer and extracted twice with equal volumes of neutralised phenol at $20^{\circ}C$ for 15min to remove the protein coat of the phage. Two chloroform extractions were performed before recovering the SS DNA by precipitation with NaAc and 100% ethanol. The pellet was washed with 70% ethanol, dried and taken up in 200μl TE buffer. The concentration was determined by spectrophotometry, adjusted to 1μg/μl and checked by agarose gel electrophoresis before being stored at $4^{\circ}C$.

### 6.1.2.2.Small scale SS DNA preparations

Rapid small scale preparations were used for screening new recombinants after subcloning and preparing SS DNA for sequencing. Single plaques from a fresh transfection were inoculated into 1.5ml of L-broth containing 20μl of fresh overnight culture of JM109. The inoculum was grown for 5hr at $37^{\circ}C$ with agitation and the cells were removed by centrifugation. The phage were precipitated with 0.2volumes PEG/NaCl solution. After recovery, the phage was taken up in 100μl TE buffer and extracted with neutralised phenol for 15min at $20^{\circ}C$. The phenol was removed and the phage extracted once with chloroform. SS DNA was recovered by centrifugation after precipitation with NaAc and 100% ethanol. The pellet was washed with 70% ethanol, dried and taken up in 30μl of TE buffer. The DNA was checked on agarose gels against control DNA to confirm the presence of a cloned insert and to determine the approximate concentration of the DNA before sequencing.

### 6.1.3. Bacterial transformations

Competent cells were prepared in advance for transformations. A bacterial colony was picked from a minimal agar plate and grown to saturation overnight. The culture was diluted 50 times into a volume of 200ml and grown to log phase ($OD_{550}$=0.3). The culture was incubated on ice for 5min before being centrifuged at 4,000rpm for 10min. The cells were taken up in 100ml ice cold 0.1M $CaCl_2$/ 15% glycerol; 200µl aliquots were snap frozen in liquid nitrogen and stored at -70°C.

For transformations a vial of competent cells was thawed on ice and a maximum of 100ng plasmid DNA added. Incubation continued on ice for a further 40min and the cells were heat shocked at 42°C for 1min, before being returned to ice for 5min. If antibiotic selection was required 0.8ml L-broth was added and the cells incubated at 37°C for 45min before plating out, in dilutions, on antibiotic agar plates. For phage, the transformed cells were aliquoted into 3ml melted top agar (at 42°C) containing 200µl fresh overnight culture of cells to provide a lawn and poured over prewarmed L-agar plates. 1mM IPTG and 0.02% X-gal were included in the top agar or on the antibiotic agar plates if blue/white selection of recombinants was required. All plates were incubated overnight at 37°C.

For later experiments competent cells were obtained from Gibco BRL. The strain used was DH5α and the transformation procedure was as follows. The ligation mixture was added to 50µl of competent cells on ice. After 30min the cells were heat-shocked for 20 seconds at 37°C. Following a 2min incubation on ice, 0.95ml of L-broth were added and this mixture was incubated at 37°C for 45min before being plated out onto antibiotic agar plates.

### 6.1.4. Oligonucleotide directed mutagenesis

Sense oligonucleotides were designed to introduce various point mutations into Ras cDNAs. Oligonucleotides were obtained from the Chester Beatty "in house" synthesis service and later by Chiron. Before the oligonucleotides could be used, they had to be removed from the column and purified. 1ml of concentrated $NH_4$ solution was drawn through the column over a time span of approximately 1 hour. The solution was collected and placed in a screw top eppindorf at 55°C overnight. The tube was placed on ice before adding NaAc and ethanol to precipitate the oligonucleotide. This precipitation was repeated and after washing, the pellet was resuspended in water. The oligonucleotides prepared by Chiron were already purified and

only required resuspending in water. The concentration of oligonucleotide was then determined by spectrophotometry. A kit from Amersham (oligonucleotide-directed *in vitro* mutagenesis system version 2.1 RPN 1523) which is based on the method of Fritz Eckstein and co-workers (Sayers *et al*, 1988; Taylor *et al*, 1985) was used.

Oligonucleotides were 5'-phosphorylated with T4 polynucleotide kinase. 10μg of SS DNA template was annealed with 4pM of phosphorylated oligonucleotide by heating to 70°C for 5min and then 37°C for 30min. Extension and ligation of the annealed oligonucleotide into closed circular DNA were performed overnight at 15°C with Klenow fragment and T4 DNA ligase. The nucleotide mix contained dCTPαS rather than dCTP. Any remaining SS non-mutant DNA was removed by filtration in 0.5M NaCl through nitrocellulose membranes. The DNA was then precipitated with NaAc and ethanol. The washed pellet was resuspended in the appropriate buffer and digested with Nci1 or Pvu1 at 37°C for 90min. The nicked template strand was then digested by exonuclease III. The digestion time must be sufficient to remove all the template DNA from the site of mutation but to leave some annealed to the mutagenised strand. Repolymerisation and ligation of the gapped DNA were performed to create a homoduplex containing the desired mutation(s). The DNA was then used to transform competent JM1O9 or TG1 cells. The mutant progeny were screened by DNA sequencing before subcloning.

A new mutagenesis kit was recently introduced by Amersham - the Sculptor™ *in vitro* mutagenesis system (RPN 1526). This new system enabled the whole mutagenic process to be performed in one day rather than two.

## 6.1.5. PCR Mutagenesis

On some occasions it was found that the efficiency of the *in vitro* mutagenesis system was very low and an alternative strategy was devised. Antisense oligonucleotides with the desired mutations were designed and purified as described previously. Polymerase chain reactions (PCRs) were set up using 100pM of each primer/oligonucleotide and 500ng of template DNA. A typical reaction would be - 94°C for 2.5min, 40 cycles of 94°C for 1min, 46°C for 45sec and 72°C for 3min, followed by 10min at 72°C before being cooled down to 15°C. After the reaction was completed, an aliquot was run out on an agarose gel alongside a control sample which contained no DNA to check that no contamination had occurred. The DNA was extracted with phenol/chloroform before being precipitated with NaAc and ethanol. The DNA was resuspended in TE buffer and the appropriate restriction enzyme buffer

and digested. The appropriate fragment was collected and then ligated with in most cases the plasmid pGEM 9fz- (Promega). The ligated DNA was then used to transfect competent JM109 cells which were plated out onto L-agar plates containing ampicillin and IPTG/X-gal. Recombinants were then screened by restriction analysis and any colonies showing the correct digestion pattern were analyzed by double stranded sequencing before subcloning.

## 6.1.6. DNA Sequencing

DNA sequencing of SS and double stranded DNA was performed using the dideoxy chain termination method (Sanger et al, 1977). Before sequencing double stranded DNA, the DNA was denatured by alkali (2M NaOH), neutralized with NH4Ac and precipitated with ethanol. The sequencing reactions were performed using the Sequenase version 2.0 kit which uses a modified T7 polymerase (United States Biochemical Corporation). The label used was [α $^{35}$S]dATP (Amersham SJ1304, >1000Ci/mmol). The reactions were resolved in 6% polyacrylamide wedge gels (upper thickness 0.4mm, 1.5mm at the bottom) containing 7M urea with 1xTBE as running buffer. The gels were run at a constant power of 80W, fixed for 1hr in 10% acetic acid and then dried under vacuum at 90°C before overnight autoradiography.

## 6.1.7. Subcloning and recombinant screening

Following restriction digests, DNA fragments for subcloning were resolved in agarose gels and extracted onto positively charged paper (DEAE-cellulose, NA45 Schleicher-Schuell) by electroelution. The paper slips were rinsed in TE buffer before being incubated at 70°C for 2hr in 0.4ml of 1M NaCl/20mg/ml Arginine free base solution to elute the DNA from the paper which was then discarded. The DNA was precipitated with 2vols of 100% ethanol and recovered by centrifugation. The fragments were quantified on an agarose gel before being ligated overnight using T4 ligase. A 1:3 molar ratio of backbone to insert was generally used. The ligations were plated out onto selection plates with competent JM109 cells and colonies were screened by restriction analysis of small scale preparations of plasmid DNA before proceeding to large scale preparations of the correct recombinant.

Later a different method for collecting DNA fragments was used. This method involved cutting out the gel slice that contained the desired fragment, cutting the slice into small pieces and adding 200µl of phenol. The sample was then frozen in a dry ice/ethanol bath for 5min, centrifuged for 15min and the top aqueous layer was collected into a fresh tube. 200µl of TE was

then added to the agarose/phenol remaining and a further aqueous layer collected after centrifugation. The aqueous layers were combined, extracted with phenol/chloroform and the DNA was then precipitated with ethanol.

## 6.2. *In vivo* assays

### 6.2.1. Electroporation of COS cells

COS cells were transfected using an electroporation method based on Chu *et al* (1987) and using the Bio-Rad gene pulser transfection apparatus. Cells were grown to confluence in DMEM containing 10% v/v donor calf serum, trypsinised from the culture flask and washed once with DMEM/10% v/v donor calf serum. The cells were then washed twice with HeBs (20mM Hepes pH7.05, 137mM NaCl, 5mM KCl, 0.7mM $Na_2HPO_4$, 6mM glucose). Cells were counted and the appropriate number were resuspended in 240$\mu$l HeBs, 10$\mu$l of salmon sperm DNA (10mg/ml) and 10ml of plasmid DNA (1$\mu$g/ml). This mixture was placed in a cuvette and pulsed in the gene pulser at 250V and a capacitance of 125$\mu$F. The time constant was generally between 4 and 6 mSec. The cells were then left at room temperature for 10min before being plated out onto dishes in DMEM containing 10%v/v donor calf serum. If several aliquots of the same transfection were required for differential metabolic labelling or for labelling and immunofluorescence, they were divided 12hr after transfection.

### 6.2.2. Metabolic labelling

COS cells were incubated in labelling medium 48hr after electroporation and were labelled overnight or for shorter periods of time depending on the assay. Methionine free medium was used with 50$\mu$Ci/ml of [$^{35}$S] methionine (Du Pont/NEN NEG-072 EXPRE$^{35}$S$^{35}$S protein labelling mix, 1000Ci/mmol). After labelling, cells were washed twice with PBS-A and once with lysis solution before being scraped into ice cold lysis solution (1% Triton X100, 0.5% SDS, 0.1M NaCl, 5mM $MgCl_2$, 20mM TrisCl pH7.4, 0.5% sodium deoxycholate). The cells were left on ice for 10min, vortexed and centrifuged for 15min at 13,000rpm. The supernatant was taken into a fresh tube and stored at -70$^\circ$C until required.

### 6.2.3. Triton X114 assays

COS cells were aliquoted on to 30mm dishes 12hr after electroporation; four dishes being set up for each experiment. 60hr later two dishes were incubated in methionine free DMEM for 1hr and then pulse labelled with

[$^{35}$S] methionine at 300μCi/ml for 10min. One dish was washed and returned to DMEM with 10% v/v donor calf serum for 4hr before harvesting. The other dish was washed twice with PBS-A and then lysed in ice cold 1% Triton X114. Other dishes were labelled with [$^3$H] palmitic acid (Du Pont/NEN NET-043, 52Ci/mmol) or [$^3$H] myristic acid (Du Pont/NEN NET-830, 30Ci/mmol) at 0.75mCi/ml for 4hr in DMEM. All cells were lysed on ice for 10min in 1% Triton X114 in Tris Buffered Saline (TBS, 10mM TrisCl pH7.5, 150mM NaCl) after being scraped off the plates into eppindorf tubes. After vortexing and centrifugation, the supernatant was transferred to a new tube and incubated at 37°C for 2min. The Triton solution separates at this temperature and the detergent and aqueous phases were then separated by centrifugation for 2min at room temperature (Bordier, 1981). The upper aqueous phase was placed in a fresh tube and each phase was restored to a Triton concentration of 1% by the addition of 0.1ml of 10% TX114 stock solution to the aqueous phase and 0.9ml of TBS to the detergent phase. The samples were stored on ice for 10min, vortexed and immunoprecipitated. [$^3$H] palmitic and myristic acid lysates were immunoprecipitated directly after removal of the insoluble cell debris with no partitioning being performed.

The TX114 used has to be taken through a series of partitions to remove contaminants of incorrect chain length. 3ml of TX114 were dissolved in 50ml of ice cold TBS and the solution incubated at 37°C for 5min, the phases were separated by centrifugation and the upper layer discarded. The volume was restored to 50ml with TBS and the whole process repeated 3 more times. The final aqueous phase was discarded and not replaced. The detergent stock solution containing 10-12% TX114 was diluted 1:10 in TBS to make the lysis solution used for these experiments.

### 6.2.4. Cell fractionation

COS cells were transfected onto 100mm dishes and processed 60hr after electroporation. Cells were washed twice in ice cold PBS-A and scraped into 1ml of ice cold hypotonic lysis solution (10mM TrisCl pH7.5, 5mM MgCl$_2$, 1mM DTT, 1mM PMSF) and left to swell on ice for 30min. All subsequent steps were carried out on ice or at 4°C. The cells were homogenised with 30 strokes in a Dounce homogeniser and then centrifuged for 5min at 1,500g. The post nuclear supernatant was removed and centrifuged at 60,000rpm for 30min. The s100 supernatant was removed and the p100 pellet was taken up in 200μl of membrane buffer (50mM TrisCl pH7.5, 50mM NaCl, 5mM MgCl$_2$, 1mM DTT, 1mM PMSF). Both fractions were stored at -70°C until required.

If cells had been labelled overnight, they were incubated in normal DMEM for 6hr before harvesting. The TCA precipitable radioactivity of the s100 portion was determined as described below and $2 \times 10^7$ cpm of the s100 and an equivalent percentage of the p100 were immunoprecipitated before running on SDS polyacrylamide gels.

To prevent proteolysis occurring, protease inhibitors were included in the lysis solutions used to harvest COS cells. These inhibitors included leupeptin (100μg/ml), aprotinin (10μg/ml) and soy bean trypsin inhibitor (10μg/ml) (Sigma).

### 6.2.5. Salt washing of COS cell membranes

50μl aliquots of the [$^{35}$S] methionine-labelled p100 fractions were taken up in 1ml of wash buffer containing 1M NaCl (1M NaCl, 1mM DTT, 10mM TrisCl pH8.0, 1mM PMSF) and incubated at room temperature for 30min. The samples were then centrifuged at 60,000rpm for 30min to reisolate the p100 fraction (p100'). This was taken up in lysis buffer and immunoprecipitated along with the new s100 fraction (s100').

### 6.3. Protein Analysis

### 6.3.1. Immunoprecipitations of Ras

Lysates of cells that had been labelled overnight had their TCA precipitable radioactivity determined by spotting 5μl of lysate onto a glass filter which was then dried, washed twice in 10% TCA, twice in 5% TCA and twice in ethanol. The filter was dried and counted in scintillation fluid. $2 \times 10^7$ cpm were usually immunoprecipitated, whilst the whole sample from a TX114 assay was used. Nonspecific proteins were removed by clearing the samples for 2hr with sepharose CL-4B beads (Sigma) which were prepared for use by swelling in PBS-A for 2hr. The beads were removed by centrifugation and the supernatant transferred to a fresh tube. The supernatant was incubated with Y13-259 monoclonal antibody at a final dilution of 1:1000 for 2hr. The protein/antibody complex was captured with 50μl anti-rat IgG coated protein A-sepharose beads in a 2hr incubation. The anti-rat beads were made by swelling the beads for 10min in PBS-A and then incubating them for 4hr with anti-rat IgG (whole molecule) before washing 3x with PBS-A. All incubations were performed at 4°C with mixing. The beads were washed three times in standard lysis buffer and once with PBS-A. The beads were then taken up in 50μl of SDS sample buffer and boiled for 4min. The samples were then

vortexed, centrifuged and the supernatant was loaded directly onto an SDS polyacrylamide gel.

### 6.3.2. Polyacrylamide gel electrophoresis and fluorography

All the experiments described use the standard Laemmli gel system. The resolving gel was 15% polyacrylamide, with 0.1% SDS and buffered to pH8.8 with TrisCl. The stacking gel was 3% polyacrylamide, 0.1% SDS and buffered to pH7.4 with TrisCl. The running buffer was 0.192M glycine, 0.1% SDS and 25mM Tris base. Samples were boiled in SDS sample buffer (0.1M DTT, 0.1% SDS, 0.01% bromophenol blue, 50% glycerol, TrisCl pH7.5) for 4min before being loaded onto the gels. Gels were stained for 20min in 0.15% Coomassie Brilliant Blue G (Sigma), 10% acetic acid, 50% methanol before being destained in 37% methanol, 37% acetic acid for 2-12hr. Gels were then fluorographed in Enlightening Autoradiography Enhancer (Du Pont/NEN) for 30min before being dried under vacuum at 60°C. Dried gels were autoradiographed at -70°C for between 4hr and 3 weeks as required.

Later experiments used pre-cast Tris - Glycine "Laemmli" PAGE gels of 14% from Novex. Samples were boiled in SDS sample buffer before being loaded and the gels were treated exactly as described above after running.

### 6.3.3. Quantification of incorporation of radioactivity into immunoprecipitated proteins

Labelled proteins were immunoprecipitated, resolved by SDS-PAGE, fluorographed and autoradiographed as described. The gel slice containing the band of interest was cut out using the autoradiogram as a guide. The slice was rehydrated for 5min in 1.5ml pronase buffer (10mM TrisCl pH7.8, 10mM EDTA, 0.5% SDS) and the Whatman paper adhering to the gel slice removed. Protease (Pronase E, Sigma) was added to a final concentration of 1mg/ml and the solution incubated at 37°C for 48hr. The gel slice and solution were taken up in scintillation fluid and counted. (The pronase treatment releases labelled amino acids from the gel slice and so reduces quenching).

Later experiments were quantified using an Ambis β–scanner. This scanned the gels directly for between 2 and 18hr. The cpm for each band could then be viewed directly on the screen and a printout of the image could also be made.

### 6.3.4. Western blotting

The protein content of COS cell s100 fractions was determined by the Bradford reaction (Bio-Rad). 3μg of a s100 fraction and an equal proportion of the p100 fraction were resolved by SDS-PAGE in 15% gels and the proteins transferred to nitrocellulose using a semi-dry blotting system (Pharmacia). The filter was blocked overnight in PBS-T (phosphate-buffered saline with 0.1% Tween 20) containing 5% (w/v) dried milk, washed briefly in PBS-T and incubated with an anti-protein A antibody (Sigma) diluted 1:1500 in PBS-T/5% milk. After washing, the filter was incubated for 1hr with an anti-rabbit horseradish peroxidase conjugate (Amersham) diluted 1:1000 in PBS-T/5% milk and developed after washing in PBS-T using an ECL detection kit (Amersham) according to the manufacturer's instructions. Exposure times varied from 10sec to 10min.

For Ras detection, the monoclonal antibody Y13-259 was used at a dilution of 1:1,000 whilst the second step goat anti rat HRPO was used at 1:5,000.

### 6.4. GDP / GTP loading of Ras

The GDP/GTP loading of Ras proteins was determined using the method of Downward $et$ $al$ (1990). COS cells were transfected by electroporation and grown on 6cm dishes. 72hr after transfection, they were incubated in phosphate free DMEM for 1hr before being labelled with $^{32}$P-orthophosphate (ICN, 64014 300μCi/ml) at 100μCi/ml for 4hr. Cells were lysed in 1.5ml of cold lysis buffer (50mM Hepes pH7.4 containing 1% Triton X-100, 100mM NaCl, 5mM $MgCl_2$, 1mg of BSA/ml, 10mM benzamidine, 10μg of leupeptin/ml, 10μg of aprotinin/ml and 10μg of soybean trypsin inhibitor/ml). Centrifugation at 15,000g for 2min removed the nuclei and 1ml of the resulting supernatant was added to 50μl of RaR - PAS bead slurry followed by the addition of 0.5M NaCl (100μl), 0.5% deoxycholate and 0.05% sodium dodecyl sulphate (100μl). Samples were rotated at 4°C for 15min, the beads were pelleted and the supernatant was taken into fresh tubes together with 100μl of rabbit-anti-rat protein A sepharose (RaR - PAS) Y13 - 259 bead slurry. These immunoprecipitates were rotated for 40min before being washed 8 times with 1ml of 50mM HEPES pH7.4, 500mM NaCl, 5mM $MgCl_2$, 0.1% Triton X-100 and 0.005% SDS. After the final wash as much liquid was removed as possible before eluting the bound nucleotide in 35μl of 2mM EDTA, 2mM DTT, 0.2% SDS, 0.5mM GTP and 0.5mM GDP by heating at 68°C for 20min. Thin layer chromatographic (TLC) separation of the eluted

nucleotides (3 x 5µl loadings of each sample) was performed on PEI - cellulose plates run in 1M LiCl. The separated nucleotides were quantitated by direct scanning for beta radiation using an Ambis β-scanner and visualised by autoradiography.

## 6.5. MAP kinase assays

Kinase dead MEK (MEKB) (Macdonald *et al*, 1993) was transfected into Sf9 cells to generate a recombinant baculovirus. Frozen Sf9 cell pellets that had been infected with the MEKB baculovirus were lysed in 5 volumes of hypotonic buffer [20mM Tris-HCl pH 8.2], 1mM ethyleneglycol-bis-(β-aminoethyl ether0-$N,N,N'$-tetraacetic acid) containing 10µg of leupeptin/ml. Centrifugation at 12,000g at 4°C for 10min removed any insoluble material. NaCl and n-octylglucoside were added to the supernatant to 80mM and 0.1% respectively. After a 10min incubation at room temperature, the solution was again centrifuged for 10min at 12,000g (4°C). The MEKB in the resulting supernatant was adsorbed for 1 hour at 4°C to glu-glu monoclonal antibody (Grussenmeyer *et al*, 1985), covalently bound to protein G sepharose. The beads were sequentially washed with 10 volumes of hypotonic buffer, hypotonic buffer containing 0.5% NP-40, hypotonic buffer containing 100mM NaCl and hypotonic buffer. The beads were then washed twice with 5 volumes each of the following buffer (50mM Tris, 75mM NaCl, 25mM NaF, 5mM $MgCl_2$, 5mM EGTA, 100µM $NaVO_4$) and finally resuspended in 4ml of the same buffer.

COS cells were electroporated as previously described. Cells were serum starved for 18 hours prior to harvesting. The cells were washed twice in PBS-A and scraped on ice into 0.5ml of buffer A (10mM Tris pH7.5, 25mM NaF, 5mM $MgCl_2$, 1mM EGTA, 1mM DTT, 100µM $NaVO_4$). After 10 minutes on ice, the cells were homogenised with 30 strokes in a Dounce homogeniser. Following a 5 minute spin at 1500g, the membranes were pelleted at 100,000g. The supernatant (s100) was removed whilst the p100 fraction was rinsed briefly with buffer A before being resuspended in 0.5ml of buffer B (50mM Tris, 75mM NaCl, 25mM NaF, 5mM $MgCl_2$, 5mM EGTA, 100µM $NaVO_4$, 1% NP40). The protein content of the s100 was measured using the Bradford reaction. Typically 40 - 50µg of the s100 fraction was used for the kinase assay.

The lysate aliquot was adjusted to 75µl with buffer C (50mM Tris, 75mM NaCl, 25mM NaF, 5mM $MgCl_2$, 5mM EGTA, 100µM $NaVO_4$). 25µl of a 1:5 suspension of MEKB beads, 1.5µl of 100mM $MgCl_2$, 0.5µl of cold ATP and 2µl of $\gamma[^{32}P]$ ATP (NEN, NEG002H 300µCi/mmol) were added to each

sample. The reactions were vortexed at $30^{\circ}$C for 30 minutes and then placed on ice. 60μl of CL4B beads were added to each sample and the beads were then washed with 1ml of buffer B and 1ml of buffer C before being resuspended in 40μl of 2x Laemmli sample buffer. After separation by SDS PAGE (10%), the amount of radioactivity incorporated into MEKB was measured by scanning the gels using an Ambis β-scanner.

## 6.6. Immunofluorescence / MDCK microinjection

Immunofluorescence analysis was kindly performed by H. Paterson, Institute of Cancer Research, Chester Beatty Laboratories, London, using the following protocol. MDCK cells growing on glass coverslips were microinjected intracytoplasmically using a Zeiss/Eppendorf semi-automatic microinjecting device. Approximately $2 \times 10^{-11}$ ml plasmid DNA at 0.2mg/ml was injected. After 18-20hr at $37^{\circ}$C the cells were fixed in 3% paraformaldehyde/50mM ammonium chloride/0.2% Triton X100 for 10min, washed and then incubated in monoclonal antibody 7F7 (Schultz et al, 1988) at 1:1000 dilution in PBS-A for 1hr followed by a 1:400 dilution of goat anti-mouse immunoglobulin coupled to FITC (Pierce). Stable NIH 3T3 cell lines transfected K-ras were isolated from NIH 3T3 co-transfections with pSV2neo (described below) and screening colonies for Ras expression. Cells were cultured overnight on glass coverslips and then treated as described above for the microinjected MDCK cells.

For permeablisation experiments, cells were treated with a solution of 0.5% saponin-0.2% bovine serum albumin in PBS for 3min and fixed in 4% formaldehyde in PBS for 20min. The coverslips containing the fixed cells were washed for 30min in a solution of 0.05% saponin-0.2% BSA in PBS. All further washes and incubations were performed in this solution. Permeablisation removes all soluble proteins from cells leaving only membrane associated proteins.

Cells were examined using a MRC 500 confocal imagining system in conjunction with a Nikon Optiphot fluorescent microscope with a x60 planapo objective lens.

## 6.7. NIH 3T3 biological assays

All the DNA used for NIH 3T3 work was prepared from a 150ml overnight culture that was treated with Qiagen reagents and passed through a Qiagen column. After precipitation, the plasmid DNA was extracted once with phenol and twice with phenol/chloroform before being reprecipitated with NH4Ac and ethanol.

### 6.7.1. Transfections

Cells were thawed from a sample frozen in nitrogen and grown in 10cm dishes in 10% DMEM. Cells were only used up to passage 16. After trypsinisation cells were counted and seeded at $1.3 \times 10^5$ cells per 10cm plate in 10% DMEM. The following day a calcium chloride precipitate of the test DNA was added to each plate. A DNA / CaCl$_2$ solution together with human placental DNA as carrier was added dropwise to a solution of 2X HBS (280mM NaCl, 50mM Hepes, 1.5mM Na$_2$HPO$_4$.12H$_2$O) through which air was bubbled. After 30min the DNA / CaCl$_2$ precipitate was added to a plate of cells and left on for 18 - 24hr. Generally three identical plates were set up for each DNA under test. Plates were washed twice with Tris saline (4mls of 1M Hepes pH7.1 and 10mls of donor calf serum per 200ml of Tris saline) to remove the DNA precipitate and then incubated in 5% DMEM. Medium was changed twice a week and foci were generally seen 10 - 14 days after transfection. Cells were fixed in formal saline (10%) and the number of foci were counted. After counting cells were then stained in 1% crystal violet.

### 6.7.2. Co-transfections

NIH 3T3 cells were seeded at $2 \times 10^5$ per 6cm dish in 10% DMEM. A DNA / CaCl$_2$ precipitate containing the test plasmid and pSV2neo was added to each plate and left for 18 - 24hr before being washed away with Tris saline. The following day cells were trypsinised and divided between 3 x 10cm plates in 10% DMEM containing 1mg/ml G418 (Geneticin, Sigma). The medium was left for one week and then changed every three to four days. Foci were seen 10 -14 days after transfection and were counted after fixing with formal saline.

### 6.8. *In vitro* translations and isoprenoid analysis

R-[5-$^3$H] mevalonic acid 50μCi (NEN, NET-716) was dried under vacuum at -60$^o$C and taken up in 50μl nuclease-treated rabbit reticulocyte lysate (Promega) containing all 20 amino acids. Uncapped RNA (2μg) was added and translation performed at 30$^{\circ}$C for 90min. The whole lysate was partitioned in Triton X114 and the detergent partitioning fraction precipitated with 10% TCA. After incubation on ice for 1hr the precipitated proteins were collected by centrifugation, washed three times with 1ml cold acetone, dried and taken up in SDS sample buffer. Following SDS-PAGE, the gel was soaked in Enlightening (NEN) and autoradiographed. The labelled bands were digested with Pronase from gel slices excised using the autoradiogram as a

guide. $3 \times 10^5$ cpm of labelled peptide were cleaved using the method of Casey *et al* (1989) and analyzed by HPLC. The HPLC analysis was kindly performed by C.J. Marshall (Institute of Cancer Research, Chester Beatty Laboratories, London) and A.I. Magee (National Institute for Medical Research, London). Labelling of *in vitro* translates with S-adenosyl [$^3$H] methyl-methionine was carried out in the presence of canine pancreatic microsomal membranes (Promega) (Hancock *et al*, 1991a).

# Abbreviations

| | |
|---|---|
| a | aqueous |
| AAC | ADP/ATP carrier |
| ADP | adenosine diphosphate |
| ARF | ADP ribosylation factor |
| ATP | adenosine triphosphate |
| BSA | bovine serum albumin |
| BZA | 3-amino-1-carboxymethyl-5-phenyl-benzodiazepin |
| $C_{15}$ | farnesyl |
| $C_{20}$ | geranylgeranyl |
| $CaCl_2$ | calcium chloride |
| cAMP | adenosine 3',5'-cyclic-monophosphate |
| CHM | choroideremia |
| CHML | choroideremia-like |
| CR | conserved region |
| d | detergent |
| dATP | deoxyadenosine triphosphate |
| dCTP | deoxycytidine triphosphate |
| DEAE | diethylaminoethyl |
| DMBA | 7,12-dimethylbenz[a]anthracene |
| DMEM | Dulbecco's modified Eagle's medium |
| DNA | deoxyribonuleic acid |
| DTT | dithiothreitol |
| ECL | enhanced chemiluminescent |
| EDTA | ethylenediaminetetraacetic acid |
| EGF | epidermal growth factor |
| EGTA | ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N',-tetraacetic acid |
| ERK | extracellular signal related kinase |
| EtOH | ethanol |
| FITC | fluorescein isothiocyanate |
| FMLP | N-formyl-methionyl-leucyl-phenylalanine |
| FPP | farnesyl pyrophosphate |
| FTase | farnesyltransferase |
| G418 | geneticin |
| GAP | GTPase activating protein |
| GDI | guanine nucleotide dissociation inhibitor |
| GDP | guanosine diphosphate |

| | |
|---|---|
| GDS | guanine nucleotide dissociation stimulator |
| GEF | guanine nucleotide exchange factor |
| GGPP | geranylgeranyl pyrophosphate |
| GGTase | geranylgeranyltransferase |
| GNRP | guanine nucleotide releasing protein |
| GRF | guanine nucleotide releasing factor |
| GTP | guanosine triphosphate |
| HeBs | Hepes buffered saline |
| HMG CoA | 3-hydroxy-3-methylglutaryl-CoA |
| HPLC | high performance liquid chromatography |
| HVR | hypervariable region |
| IPP | isopentyl pyrophosphate |
| IPTG | isopropyl-1-thio-β-D-galactoside |
| KAc | potassium acetate |
| LiCl | lithium chloride |
| MAP | mitogen activated protein |
| MgCl$_2$ | magnesium chloride |
| MOPS | 3-(N-morpholino) propane sulphonic acid |
| MTA | methylthioadenosine |
| NH$_4$ | ammonium |
| NaAc | sodium acetate |
| NaCl | sodium chloride |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NaF | sodium fluoride |
| Na$_2$HPO$_4$ | disodium hydrogen phosphate |
| NaOH | sodium hydroxide |
| NaVO$_4$ | sodium vanadate |
| NGF | nerve growth factor |
| NMT | myristoyl CoA : protein N-myristoyl transferase |
| NMV | nitroso - methylurea |
| NH$_4$Ac | ammonium acetate |
| NP40 | Nonidet P40 |
| PBS-A | Dulbecco's phosphate buffered saline (calcium and magnesium free) |
| PBS-T | phosphate-buffered saline with 0.1% Tween 20 |
| PCR | polymerase chain reaction |
| PEG | polyethyleneglycol |
| PH | pleckstrin homology |

| | |
|---|---|
| pI | isoelectric point |
| PMSF | phenylmethylsulphonyl fluoride |
| P100 | membrane fraction |
| Ran | Ras-related nuclear protein |
| REP | Rab escort protein |
| RNA | ribonucleic acid |
| SAM | S-adenosyl-[$^3$H]-methyl-methionine |
| SCR | structurally conserved regions |
| SDS | sodium dodecylsulphate |
| SDS-PAGE | sodium dodecylsulphate polyacrylamide gel electrophoresis |
| SH2 | Src-homology-2 |
| SH3 | Src-homology-3 |
| S100 | cytosolic fraction |
| TBS | Tris buffered saline |
| TCA | trichloroacetic acid |
| TE | Tris/EDTA (buffer) |
| TLC | thin-layer chromatography |
| TrisHCl | Tris(hydroxymethyl)aminoethane |
| Tween 20 | polyoxyethylene sorbitan monolaurate |
| TX100 | t-octylphenoxypolyethoxyethanol |
| TX114 | octylphenoxypolyethoxyethanol |
| X-gal | 5-bromo-4-chlor-3-indoyl-β-D-galactoside |

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# A CAAX or a CAAL motif and a second signal are sufficient for plasma membrane targeting of ras proteins

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Mutational analysis of p21[ras] has shown that plasma membrane targeting requires the combination of a CAAX motif with a polybasic domain of six lysine residues or a nearby palmitoylation site. However, it is not known from these studies whether these signals alone target p21[ras] to the plasma membrane. We now show that these C-terminal sequences are sufficient to target a heterologous cytosolic protein to the plasma membrane. Interestingly, the key feature of the p21[K-ras(B)] polybasic domain appears to be a positive charge, since a poly-arginine domain can function as a plasma membrane targeting motif in conjunction with the CAAX box and p21[K-ras(B)] with the polylysine domain replaced by arginines is biologically active. Since some ras-related proteins are modified by geranylgeranyl rather than farnesyl we have investigated whether modification of p21[ras] with geranylgeranyl affects its subcellular local-ization. Geranylgeranyl can substitute for farnesyl in combining with a polybasic domain to target p21[K-ras(B)] to the plasma membrane, but such geranylgeranylated proteins are more tightly bound to the membrane. This increased avidity of binding is presumably due to the extra length of the geranylgeranyl alkyl chain.
*Key words:* CAAX/plasma membrane/prenylation/ras/ targeting

## Introduction

The p21[ras] proteins are localized to the inner surface of the plasma membrane (Willingham *et al.*, 1980). Mutational analysis has shown that one element of plasma membrane localization is an intact CAAX motif at the C terminus of the protein (Willumsen *et al.*, 1984; Hancock *et al.*, 1989). A CAAX motif (C = cysteine, A = aliphatic, X = any amino acid) is found at the C terminus of all ras proteins and many other cellular proteins. The motif undergoes a triplet of closely coupled post-translational modifications. Firstly, a prenoid derivative is linked as a thioether to the cysteine residue (Hancock *et al.*, 1989; Casey *et al.*, 1989); second, the -AAX amino acids are removed by proteolysis (Gutierrez *et al.*, 1989) and third, the $\alpha$-carboxyl group of the now C-terminal cysteine residue is methyl-esterified (Clarke *et al.*, 1988; Gutierrez *et al.*, 1989). We have shown recently that all three of these post-translational processing events at the CAAX motif are required for efficient membrane binding of p21[K-ras(B)] (Hancock *et al.*, 1991).

The p21[ras] proteins (Casey *et al.*, 1989), nuclear lamin B (Farnsworth *et al.*, 1989) and the $\gamma$-subunit of transducin (Fukada *et al.*, 1990; Lai *et al.*, 1990) are all prenylated with $C_{15}$ farnesyl. Certain other CAAX containing proteins, including the ras-related proteins K*rev*1/*rap*1A (Kawata *et al.*, 1990; Buss *et al.*, 1991) and G25K (Maltese and Sheridan, 1990), and the $\gamma$-subunits of brain G-proteins (Yamane *et al.*, 1990; Mumby *et al.*, 1990) have been shown to be geranylgeranylated. The CAAX motifs of these $C_{20}$ modified proteins all terminate with a leucine residue indicating that the X amino acid determines whether a CAAX motif is a substrate for a farnesyl or geranylgeranyl transferase (Seabra *et al.*, 1991; Finegold *et al.*, 1991). It is not known why some proteins are farnesylated and others are geranylgeranylated nor whether the alkyl chain length affects subcellular localization or avidity of membrane association.

The modifications of the CAAX motif provide only one part of the signal for the subcellular localization of proteins to cellular membranes. The CAAX motif of p21[ras] combines with a second signal contained within the C-terminal hypervariable domain of ras proteins to target plasma membrane localization. This second signal comprises either a cysteine palmitoylation site in the case of p21[H-ras], p21[N-ras] and p21[K-ras(A)], or a polybasic domain comprising six consecutive lysine residues (amino acids 175−180) in the case of p21[K-ras(B)] (Hancock *et al.*, 1990). Similarly, the nuclear lamins A and B require both a CAAX motif and a nuclear localization signal for correct targeting to the nuclear membrane (Holtz *et al.*, 1989).

While mutational analysis of p21[ras] demonstrates that both the CAAX motif and either palmitoylation or a polybasic domain are necessary for plasma membrane localization of p21[ras] it is not clear whether the combination of these signals is sufficient for plasma membrane localization. It is possible that there are other domains which are also required. To address this question we have investigated whether a heterologous cytosolic protein, protein A, can be targeted to the inner surface of the plasma membrane by a CAAX motif in combination with a polybasic domain or a palmitoylation signal. We have also studied whether other positively charged amino acid sequences can substitute for the wild type polylysine tract of p21[K-ras(B)] as subcellular targeting motifs and determined whether CAAX motifs which direct geranylgeranylation can target p21[ras] and protein A to the inner surface of the plasma membrane.

## Results

### Plasma membrane targeting of protein A

In the light of our previous studies using a mutational analysis (Hancock *et al.*, 1989, 1990) it was of interest to determine whether the polybasic domain of p21[K-ras(B)] together with a CAAX motif could function as a plasma membrane targeting sequence for an heterologous protein. Using polymerase

chain reaction (PCR) the coding sequence for the C-terminal 17 amino acids of p21$^{K-ras(B)}$ was amplified from a K-ras(B) cDNA and cloned onto the C terminus of protein A using a restriction site introduced into the K-ras(B) coding sequence during amplification. Figure 1 shows that the chimeric protein expressed in COS cells localized entirely to the P100 fraction on subcellular fractionation, whereas protein A without p21$^{K-ras(B)}$ C-terminal sequences localized predominantly (80%) to the S100 fraction (Figure 1). The addition of the 17 C-terminal amino acids of K6Q, a mutant p21$^{K-ras(B)}$ protein with the polylysine domain replaced with six glutamine residues (Hancock et al., 1990), did not shift protein A into the P100 fraction (Figure 1).

The subcellular localization of the protein A chimeras was further analysed by immunofluorescent studies in MDCK
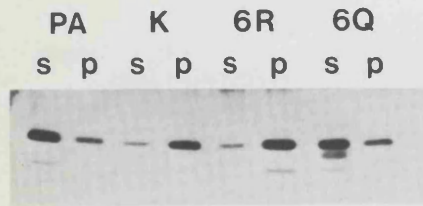


Fig. 1. COS cells expressing protein A (PA), or protein A chimeras with the C-terminal 17 amino acids from K-ras(B) (K), K6Q (6Q) and K6R (6R) were fractionated into P100 (p) and S100 (s) fractions. The polylysine domain of wild type K-ras(B) is replaced with polyglutamine in the K6Q mutant protein and with polyarginine in the K6R mutant K-ras(B) protein. Equal proportions of the S100 and P100 fractions were resolved by SDS−PAGE and Western blotted using an anti-protein A primary antibody.

cells transiently expressing the proteins following micro-injection of plasmid DNA. Figure 2 shows that protein A with wild type p21$^{K-ras(B)}$ C-terminal sequences localized to the plasma membrane and protein A with K6Q C-terminal sequences localized to the cytosol. The only observable effect of a CAAX motif in the absence of a polybasic domain was to exclude protein A from the nucleus (Figure 2). Together the data from the cell fractionation experiments and the immunofluorescence localization shows that a polylysine domain can combine with a CAAX motif to target a heterologous protein to the plasma membrane but a farnesylated CAAX motif in isolation does not lead to plasma membrane (or any other membrane) association. Similarly, when the C-terminal 10 amino acids of p21$^{H-ras}$ comprising two cysteine palmitoylation sites plus a CAAX motif were cloned into the C terminus of protein A, the chimeric protein localized to the plasma membrane (Figure 3). However, if both of the cysteine residues required for palmitoylation are replaced with serines then the protein is cytosolic just like the protein A−K6Q chimera (Figure 2).

We next analysed the ability of polybasic domains with fewer than six Lys → Gln substitutions to function as plasma membrane targeting motifs. Figure 4 shows that as the number of lysine residues within the polybasic domain was progressively reduced, the protein A chimeras were increasingly localized to the cytosol. No plasma membrane localization occurs when there are fewer than two lysines in the polybasic domain. Thus the polybasic domain functions for a heterologous protein exactly as for p21$^{K-ras(B)}$ (Hancock et al., 1990).
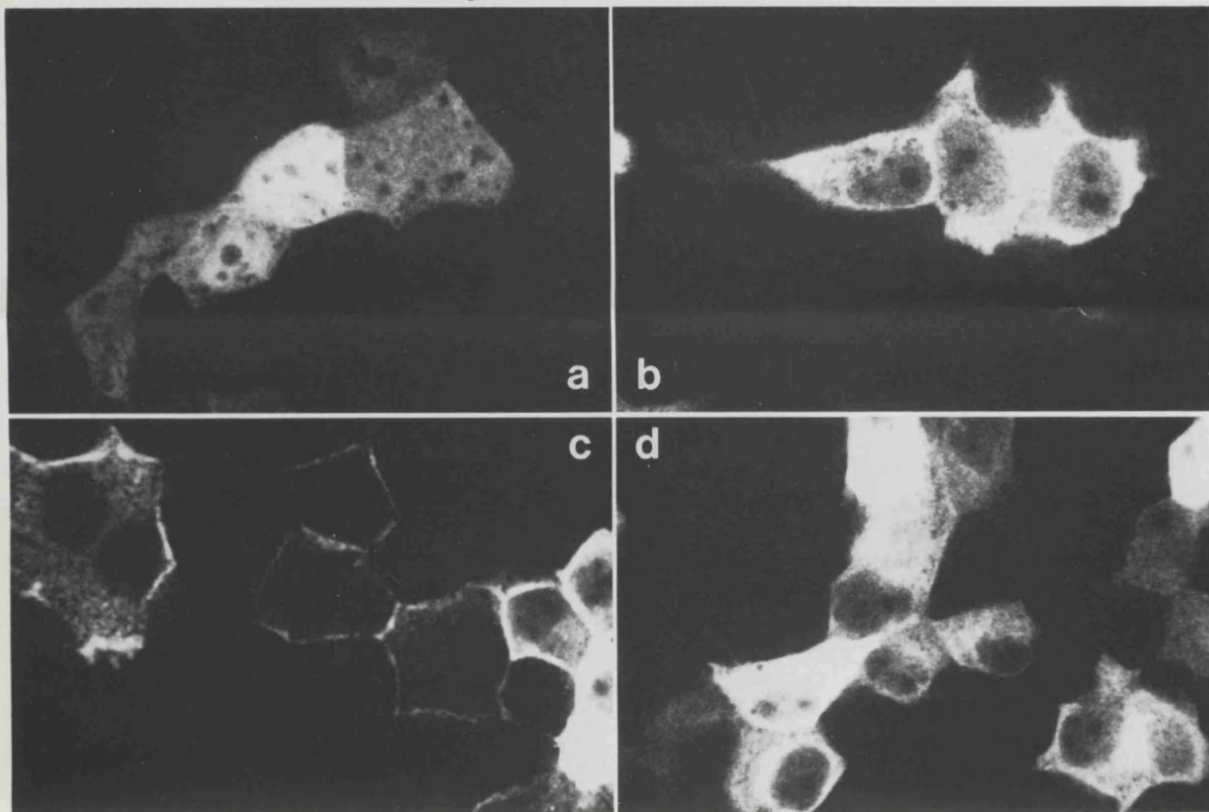


Fig. 2. Immunofluorescent analysis of MDCK cells transiently expressing protein A or protein A chimeric proteins following microinjection of plasmid DNA. (a) Protein A: cytoplasmic and nuclear staining. (b) Protein A with a CVLS C-terminal motif alone (from p21$^{H-ras}$): cytoplasmic staining only. (c) Protein A with 17 C-terminal amino acids from wild type p21$^{K-ras(B)}$: plasma membrane staining. (d) Protein A with 17 C-terminal amino acids from the K-ras(B) mutant, K6Q, which has the polylysine domain replaced with polyglutamine: cytoplasmic staining only.

### Polyarginine can substitute for polylysine as a plasma membrane targeting motif

It is possible that the polylysine domain could function as part of a plasma membrane targeting signal in two ways. First, since the domain is positively charged at physiological pH, an electrostatic interaction with negatively charged phospholipid head groups may be important. Second, there may be a specific docking protein for $p21^{K-ras(B)}$ in the plasma membrane with which the polylysine domain interacts. If the second model is true it is probable that substituting other positively charged amino acids for the lysine residues would comprise the function of the domain. To address these possibilities we constructed a mutant $p21^{K-ras(B)}$ protein, K6R, which has six Lys → Arg substitutions at amino acids 175−180.

The biological activity of oncogenic mutant K6R (Gly12 → Val) was tested in focus assays on NIH3T3 cells, and found to be similar to $p21^{K-ras(B)}$ (Gly12 → Val) (Table I). Immunofluorescence studies on NIH cell lines transformed by the K6R mutant showed that the K6R protein localized to the plasma membrane (data not shown) and subcellular fractionation of COS cells expressing the protein showed >90% localization to the P100 fraction. The 17 C-terminal amino acids of K6R were then cloned onto the C terminus of protein A and the chimeric protein expressed in COS and MDCK cells. Subcellular fractionation demonstrated that the chimeric protein was >90% P100 associated (Figure 1) and the immunofluorescence studies presented in Figure 4 show
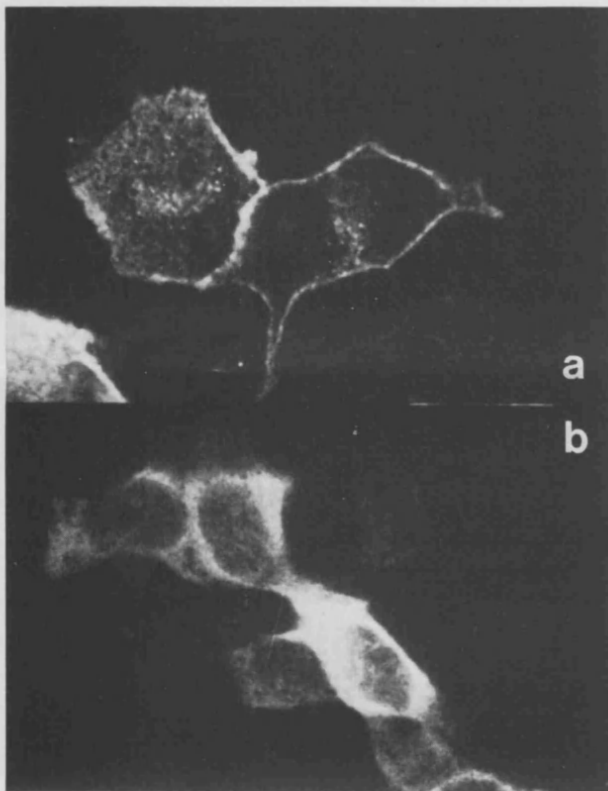


**Fig. 3.** Immunofluorescent analysis of MDCK cells transiently expressing protein A or protein A chimeric proteins following microinjection of plasmid DNA. (a) Protein A with the C-terminal 10 amino acids from $p21^{H-ras}$: plasma membrane staining. (b) Protein A with the C-terminal 10 amino acids from $p21^{H-ras}$ Cys181, Cys184 → Ser, this protein A chimera is therefore not palmitoylated: cytoplasmic staining only (compare with Figure 2d).

that the protein A−K6R chimera localized to the plasma membrane of MDCK cells.

### Analysis of the subcellular localization of geranylgeranylated $p21^{K-ras(B)}$

So far the only ras-related protein with a $C_{20}$ geranylgeranyl modification that has had its intracellular localization determined is rap1. Both rap1A and rap1B have C-terminal polybasic domains like $p21^{K-ras(B)}$ and Beranger et al. (1991) have shown that antibodies against rap1 stain the Golgi. It was therefore of interest to determine whether a $C_{20}$ modification of $p21^{K-ras(B)}$ would alter its subcellular localization. Two K-ras(B) constructs were made which changed the wild type CVIM sequence to CAIL, the CAAX motif of a brain G-protein γ-subunit and CCIL, the CAAX motif of ral. The same CAAX box mutations were also made in the K6Q K-ras(B) mutant.

To confirm that the $p21^{K-ras(B)}$ CAIL and CCIL proteins were modified by geranylgeranylation, they were translated in vitro in a rabbit reticulocyte lysate labelled with [$^3$H]mevalonic acid (Hancock et al., 1991). Following SDS−PAGE and fluorographic detection the ras proteins were excised from the polyacrylamide gel, digested out of the gel slices and subjected to methyliodide cleavage (Casey et al., 1989). The products of the cleavage were analysed by HPLC. For both proteins the counts retained on the column co-eluted with the $C_{20}$ geranylgeraniol standard and no label was detected in the position of farensol. In addition, both the CAIL and CCIL proteins incorporated label from S-adenosyl-[$^3$H]methyl-methionine when translated in vitro in the presence of microsomal membranes (data not shown). Thus the CAIL and CCIL mutant $p21^{K-ras(B)}$ proteins are geranylgeranylated and methylesterified. A recent study of the post-translational processing of full length ral (Kinsella et al., 1991) also found that the CCIL motif was geranylgeranylated. These data are therefore consistent with the hypothesis that CAA(X = L) motifs direct geranylgeranylation rather than farnesylation. Such motifs are also methylated and by implication they must be -AA(X = L) proteolysed.

We next investigated whether the presence of a $C_{20}$ alkyl chain affected the subcellular distribution of the $p21^{K-ras(B)}$ protein. Fractionation of COS cells expressing the CAIL and CCIL proteins showed that they were localized to the P100 fraction (Figure 5) and immunofluorescence studies of NIH3T3 cells expressing the proteins showed strong plasma membrane staining (Figure 6). These results demonstrate that the presence of a $C_{20}$ rather than a $C_{15}$ alkyl group has little effect on the localization of K-ras(B) proteins with an intact polybasic domain. However, Figure 5 shows that a $C_{20}$ alkyl group profoundly altered the subcellular distribution of K-ras(B) proteins in which the polybasic domain has been changed to six uncharged glutamine residues. Subcellular fractionation of COS cells expressing the geranylgeranylated K6QCAIL and K6QCCIL proteins (with the mutations Lys175−180 → Gln) revealed that they were >90% localized to the P100 fraction whereas the farnesylated K6Q protein is >90% localized to the S100 fraction (Figure 5 and Hancock et al., 1990). Interestingly, examination by immunofluorescence of NIH3T3 cells expressing K6QCAIL and K6QCCIL revealed that the proteins were not localized to the plasma membrane (Figure 6). Thus while the presence
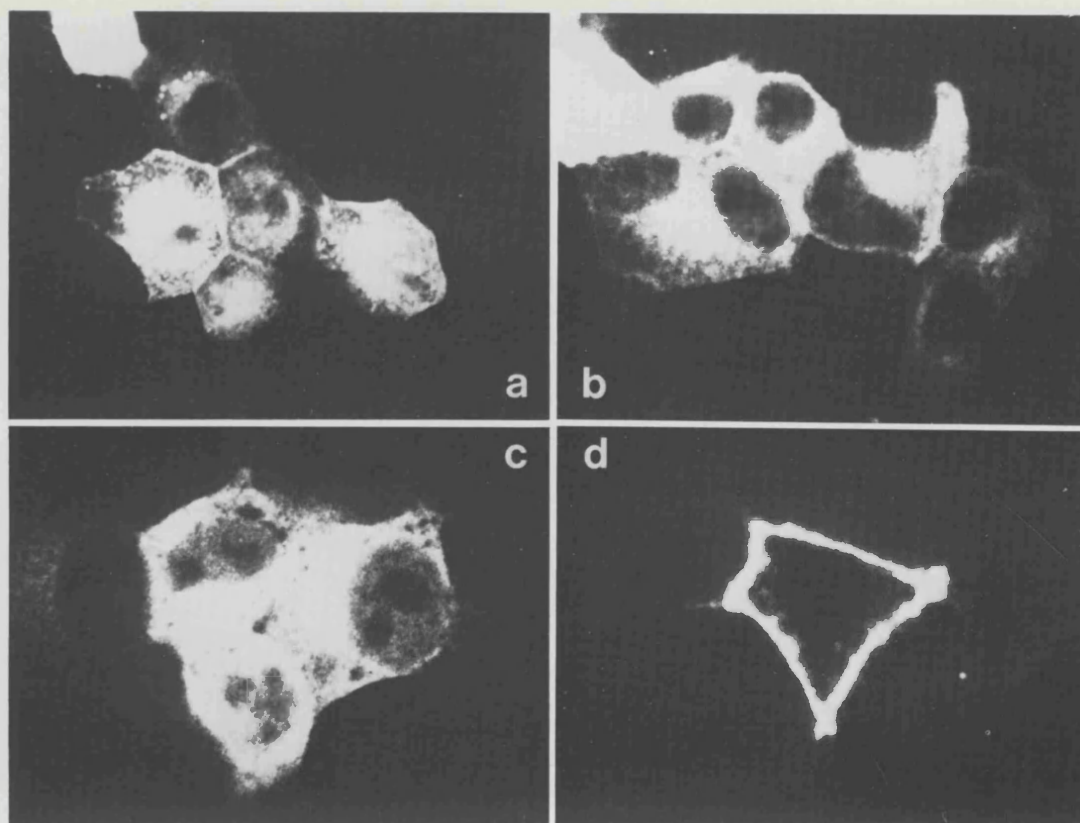
**Fig. 4.** Immunofluorescent analysis of MDCK cells transiently expressing protein A or protein A chimeric proteins following microinjection of plasmid DNA. (**a**) Protein A with the 17 C-terminal amino acids from a K-ras(B) protein with three glutamine substitutions in the polybasic domain: combination of plasma membrane and cytosolic staining. (**b**) Protein A with the 17 C-terminal amino acids from a K-ras(B) protein with four glutamine substitutions in the polybasic domain: predominantly cytosolic staining with weak plasma membrane staining. (**c**) Protein A with the 17 C-terminal amino acids from a K-ras(B) protein with five glutamine substitutions in the polybasic domain: cytosolic staining only. (**d**) Protein A with the 17 C-terminal amino acids from a K-ras(B) protein with the polylysine domain replaced with polyarginine: plasma membrane staining.

of a $C_{20}$ chain leads to the association of the K6QCCIL and K6QCAIL proteins with P100 membranes, it does not restore plasma membrane association. This is in agreement with our previous observation that both a polybasic domain and a CAAX motif are required for plasma membrane localization (Hancock *et al.*, 1990). The presence of a farnesyl or geranylgeranyl chain appeared to have little effect on the biological activity of oncogenic K-ras(B) proteins, as measured in NIH3T3 transformation assays (Table I), either in the context of an intact polybasic domain or in K-ras(B) proteins with the K6Q substitutions. This result, therefore, demonstrates that for transforming activity prenylation with a $C_{15}$ or a $C_{20}$ alkyl chain is effective.

The $C_{20}$ modified K-ras(B) proteins are avidly associated with the membrane pellet. Table II shows that a 1 M salt wash removes 78% of farnesylated $p21^{K-ras(B)}$ from the P100 fraction whereas $\leq 15\%$ of geranylgeranylated $p21^{K-ras(B)}$ is removed under the same conditions. The tighter membrane association of the geranylgeranylated proteins is independent of the presence of a polybasic domain. However, 0.5% Triton X-100 solubilizes >90% of all the geranylgeranylated and farnesylated K-ras(B) proteins from the P100 fraction (Table II), thus suggesting that the K6QCAIL and K6QCCIL proteins are associated with an intracellular membrane rather than a high molecular weight cytoplasmic protein complex. We are currently investigating to which intracellular membrane compartment these geranylgeranylated, polybasic mutant proteins are being targeted.

Finally, Figure 7 shows that the subcellular distribution

**Table I.** NIH3T3 focus assays

| ras construct | Relative activity |
|---|---|
| K-ras | 1.0 |
| K6R | 1.0 |
| K-ras CCIL | 0.88 |
| K-ras CAIL | 0.64 |
| K6Q | 0.38 |
| K6Q CCIL | 0.64 |
| K6Q CAIL | 0.2 |

Relative transforming efficiencies of K-*ras(B)* (Val12) cDNAs with altered C termini. 20 ng of each EXV plasmid was transfected with 20 $\mu$g normal human DNA as carrier onto $1.3 \times 10^5$ NIH3T3 cells. Foci were scored 14−16 days following transfection. K-ras(B) (Val12) with a wild type C terminus gave 1−1.9 foci/ng and results are expressed relative to this value.
CCIL = C-terminal CCIL motif substituted for wild type CVIM.
CAIL = C-terminal CAIL motif substituted for wild type CVIM.
K6Q = $p21^{K-ras(B)}$ with the mutations Lys175−180 → Gln.

of protein A chimeras with the 17 C-terminal amino acids from the CCIL and K6QCCIL K-ras(B) constructs is exactly the same as the parent K-ras(B) proteins. Moreover, immunofluorescence studies of MDCK cells expressing these proteins show that the protein A chimeras have the same localization as the K-ras(B) CCIL and K6QCCIL proteins in NIH3T3 cells (data not shown). Thus the membrane targeting phenotypes associated with the $C_{20}$ modified CAA(X = L) motif are fully defined by these primary amino acid sequences.
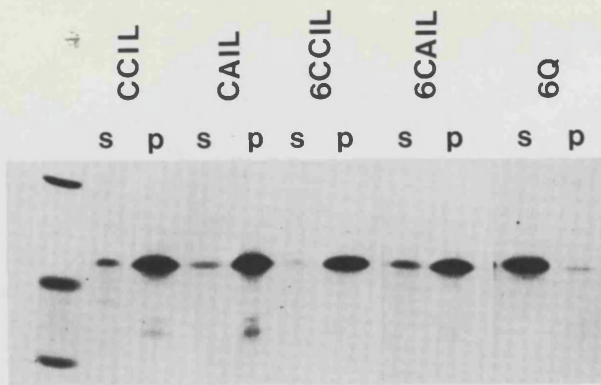
**Fig. 5.** COS cells expressing K-ras(B) proteins with metabolically labelled with [$^{35}$S]methionine for 18 h and fractionated into P100 (p) and S100 (s) fractions. Equal proportions of each fraction were immunoprecipitated, resolved by SDS−PAGE and autoradiographed. CCIL = p21$^{K-ras(B)}$ with a CCIL C-terminal motif; CAIL = p21$^{K-ras(B)}$ with a CAIL C-terminal motif; 6CCIL = p21$^{K-ras(B)}$ Lys175−180 → Gln with a CCIL C-terminal motif. 6CAIL = p21$^{K-ras(B)}$ Lys175−180 → Gln with a CAIL C-terminal motif; 6Q = p21$^{K-ras(B)}$ Lys175−180 → Gln (wild type CAAX motif).

## Discussion

We have shown previously that a C-terminal polybasic domain, comprising six lysine residues (amino acids 175−180) of p21$^{K-ras(B)}$ and the palmitoylation sites (Cys181, Cys184) of p21$^{H-ras}$, are required in addition to the CAAX motif for plasma membrane localization (Hancock *et al.*, 1990). These experiments, however, do not address whether other protein sequences might also be required for the subcellular targeting of p21$^{ras}$. The data presented here exclude this possibility. The C-terminal 17 amino acids from p21$^{K-ras(B)}$ are sufficient to target the heterologous protein, protein A, to the plasma membrane. Similarly the C-terminal 10 amino acids from p21$^{H-ras}$, comprising the palmitoylation sites and the CAAX motif contain all the necessary information for the plasma membrane localization of protein A. In related experiments we have shown that the same C-terminal sequences can be used to target the cytosolic GAP protein to the plasma membrane (Huang,D., Marshall,C.J. and Hancock,J.F., in preparation). A CAAX motif in isolation however is insufficient to target proteins to the plasma membrane or any other membrane.

The sequential replacement of lysine residues 175−180 with glutamine results in a progressive loss of the targeting function of the polybasic domain of p21$^{K-ras(B)}$ (Hancock *et al.*, 1990). Increasing cytosolic localization is also seen with the protein A−K-ras chimeras as the number of glutamine substitutions within the polybasic domain increases. The simplest interpretation of these data is that the polybasic sequence operates as a membrane targeting motif via the positive charge on the side chains of the constituent lysine residues. This model is supported by the observation that a polybasic domain comprising six arginine residues and hence with the same net positive charge as six lysines can fully substitute for the wild type polybasic domain.

We have also shown here that a C$_{20}$ geranylgeranyl moiety can substitute for C$_{15}$ farnesyl and a target p21$^{K-ras(B)}$ to the plasma membrane implying that the chain length of the alkyl group is not important if the polybasic domain is intact. It is interesting that the geranylgeranylated p21$^{K-ras(B)}$

**Table II.** Salt and Triton wash of COS cell membranes

| ras protein | Percentage P100 washout | |
| --- | --- | --- |
| | 1 M NaCl | 0.5% Triton |
| K-ras | 78 | 92 |
| K-ras CCIL | 11 | 90 |
| K-ras CAIL | 13 | 94 |
| K6Q CCIL | 12 | 92 |
| K6Q CAIL | 15 | 95 |

[$^{35}$S]Methionine-labelled P100 fractions were prepared from COS cells expressing K-ras(B) proteins. Aliquots of these fractions were washed with 1 M NaCl for 30 min at 20°C or 0.5% Triton X-100 for 10 min on ice and the membranes repelleted at 120 000 *g*. K-ras(B) protein was immunoprecipitated from the S100 and P100 fractions, resolved by SDS−PAGE and cut out of the gel following autoradiography. The amount of K-ras(B) protein remaining in the membrane pellet and the amount washed out into the S100 fraction was then quantified by scintillation counting of pronase digests of the gel slices. Results (mean of two experiments) are presented as percentage P100 washout, calculated as (c.p.m. in ras in the S100 wash fraction) × 100%/(total c.p.m. in ras). The mutant K-ras(B) proteins are described in the legend to Table I.

was not targeted to the Golgi like the rap1 proteins (Beranger *et al.*, 1991) which are geranylgeranylated and have a polybasic domain upstream of their CAA(X = L) motifs. One interpretation of these results is that there are other domains within the rap1 proteins which determine Golgi localization. Such domains may override the apparent plasma membrane localization signals located at the C terminus of the rap1 proteins. Alternatively the different spacing of the polybasic domains and the CAA(X = L) motifs of the rap1 proteins compared with these motifs in the CCIL and CAIL mutant K-ras(B) proteins may be of relevance to their different subcellular localizations.

A significant difference between geranylgeranylated and farnesylated proteins relates to their relative avidity of membrane binding. We have shown here that the presence of a 20 carbon C-terminal isoprenoid chain results in an avid membrane binding which is resistant to 1 M salt extraction. In contrast, a 15 carbon isoprenoid chain together with a polybasic domain, as in p21$^{K-ras(B)}$, leads to a weaker membrane association. The presence of an additional lipid, palmitic acid, near the C terminus, results in farnesylated p21$^{H-ras}$ binding to membranes with an avidity comparable to that of a geranylgeranylated protein (Hancock *et al.*, 1990). However, the avidity of membrane binding cannot be the sole determinant of plasma membrane association since wild type K-ras(B) protein is plasma membrane associated but bound less avidly to membranes than the K6QCCIL and K6QCAIL proteins which are not associated with plasma membrane. These data also show that there are differences in the strength of membrane association between certain ras and ras-related proteins. It is possible that these differences in membrane association reflect different functional requirements of the proteins.

The data presented here also show that replacement of the farnesyl group with geranylgeranyl results in the polybasic mutant (Lys175−180 → Gln) protein being targeted to an intracellular membrane. We have yet to establish whether the K6QCCIL and K6QCAIL mutant K-ras(B) proteins are being targeted to a specific membrane compartment; however, the immunofluorescence analyses and preliminary sucrose gradient fractionations we have performed are not consistent with a Golgi localization. It is possible, however,
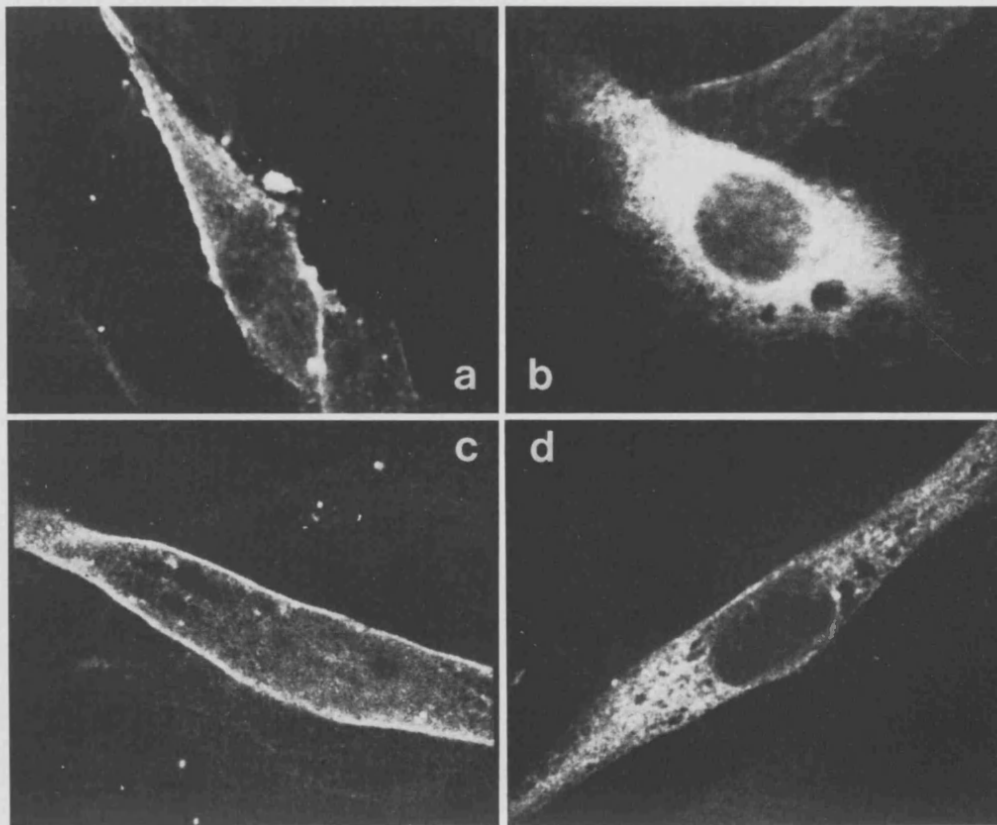
**Fig. 6.** NIH3T3 cell lines derived from the focus assays described in Table I were examined for immunofluorescence after incubation with the monoclonal antibody Y13-238 followed by an anti-rat FITC conjugate. This methodology allows specific staining of transfected K-ras(B) proteins [see Hancock *et al.* (1990) for a detailed description and discussion]. (**a**) p21$^{K\text{-ras(B)}}$ with a CCIL C-terminal motif: plasma membrane staining. (**b**) p21$^{K\text{-ras(B)}}$ Lys175−180 → Gln with a CCIL C-terminal motif: no plasma membrane staining, the P100 associated protein is diffusely localized through the cell. (**c**) p21$^{K\text{-ras(B)}}$ with a CAIL C-terminal motif: plasma membrane staining. (**d**) p21$^{K\text{-ras(B)}}$ Lys175−180 → Gln with a CAIL C-terminal motif: no plasma membrane staining, the P100 associated protein is diffusely localized through the cell.

given the avidity with which geranylgeranylated proteins bind to cell membranes that, in the absence of a second signal or signals directing plasma membrane (polybasic domain) or Golgi (rap1 specific domains) localization, the K6QCCIL and K6QCAIL mutant K-ras(B) proteins bind nonspecifically to all accessible intracellular membranes.

## Materials and methods

### Plasmids and mutagenesis
The K6R mutation was created using oligonucleotide directed mutagenesis. The tails for the protein A chimeric proteins were synthesized by PCR using the oligonucleotides 5'-ACAGAATTCAAAGATGAGCAAAGATG and 5'-AATTCTAGAGTACTAGATATGCCTTAAG to amplify a 109 bp fragment comprising the final 54 bp of the K-ras(B) coding sequence together with 37 bp of 3' untranslated sequence plus new restriction sites (18 bp). Wild type K-ras(B) cDNA and the mutant K-ras(B) cDNAs K3Q, K4Q, K5Q, K6Q (Hancock *et al.*, 1990) and K6R were used as PCR templates. The PCR reaction was denatured for 2 min at 94°C followed by 25 cycles of 94°C for 30 s, 54°C for 45 s and 72°C for 2 min before being held at 72°C for 10 min.

The PCR product was digested with *Eco*RI and *Xba*I cloned into pGEM-9Zf(−) (Promega) for sequencing, and subsequently cloned in frame into the C-terminal polylinker of protein A in a eukaryotic expression vector (described in Hancock *et al.*, 1989).

### COS cell expression
COS cells were electroporated using a method based on that described by Chu *et al.* (1987). Cells were grown to 75% confluence, harvested by trypsinization, washed twice in HeBS (20 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na$_2$HPO$_4$, 6 mM dextrose) and counted. 3 × 10$^6$ cells were resuspended in 240 μl HeBS and 10 μg plasmid DNA
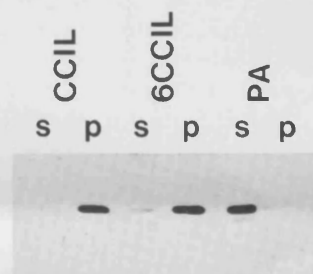


**Fig. 7.** COS cells expressing protein A (PA) or protein A chimeras with the 17 C-terminal amino acids from K-ras(B) mutant proteins with CCIL C-terminal motifs were fractionated into S100 (s) and P100 (p) fractions. Equal proportions of each fraction were resolved by SDS−PAGE and Western blotted with an anti-protein A primary antibody. The protein A−CCIL chimera (CCIL) has a wild type polylysine domain, whereas the protein A−6QCCIL chimera (6CCIL) has had this domain replaced with polyglutamine.

plus 100 μg sonicated salmon sperm DNA added in a total volume of 20 μl water. The suspension was dispensed into a 0.4 cm Bio-Rad electroporation cuvette and pulsed at 250 V/125 μF (giving a time constant of ~6 ms). Cells were allowed to rest at room temperature for 10 min before seeding to 100 mm tissue culture dishes.

COS cells were harvested 72 h after transfection and fractionated as described by Cales *et al.* (1988) except that membranes were pelleted at 120 000 *g* for 30 min. Where required, cells were metabolically labelled for 18 h prior to harvesting with 100 μCi/ml Expre[$^{35}$S][$^{35}$S] labelling mix (NEN, NEG072) in methionine-free DMEM. Immunoprecipitations of fractionated COS cells, quantification of immunoprecipitated p21$^{ras}$ and salt washing of P100 fractions were performed as described previously (Hancock *et al.*, 1989, 1990).

## Focus assays

NIH3T3 focus assays were performed as described by Marshall *et al.* (1981) using 20 μg normal human placental DNA as carrier and 20 ng plasmid DNA. Foci were counted 14 days after transfection.

## Western blotting

The protein content of COS cell S100 fractions was determined by the Bradford reaction. 3 μg of a S100 fraction and an equal proportion of the P100 fraction were resolved by SDS−PAGE in 15% gels and the proteins transferred to nitrocellulose using a semi-dry blotting system (Pharmacia). The filter was blocked overnight in PBS-T (phosphate-buffered saline with 0.1% Tween 20) containing 7.5% (w/v) dried milk, washed briefly in PBS-T and incubated for 1 h with an anti-protein A antibody (Sigma, P3775) diluted 1:1500 in PBS-T. After washing the filter was incubated for 1 h with an anti-rabbit horseradish peroxidase conjugate (Amersham, NA9340) diluted 1:1000 in PBS-T and developed using an ECL detection kit (Amersham) according to the manufacturer's instructions. Exposure times on Hyperfilm-ECL (Amersham) ranged from 10 s to 10 min.

## In vitro translations and isoprenoid analysis

R-[5-$^3$H]mevalonic acid 50 μCi (NEN, NET716) was dried under vacuum at −60°C and taken up in 50 μl nuclease-treated rabbit reticulocyte lysate (Promega) containing all 20 amino acids. Uncapped RNA (2 μg) was added and translation performed at 30°C for 90 min. The whole lysate was partitioned in Triton X-114 and the detergent partitioning fraction precipitated with 10% TCA. After incubation on ice for 1 h the precipitated proteins were collected by centrifugation, washed three times with 1 ml cold acetone, dried and taken up in Laemmli sample buffer. Following SDS−PAGE the gel was soaked in Enlightening (NEN) and autoradiographed. The labelled bands were digested with pronase from gel slices excised using the autoradiogram as a guide. 3 × 10$^5$ c.p.m. of labelled peptide were cleaved with methyl iodide using the method of Casey *et al.* (1989) and analysed by HPLC as previously described (E.Fawell, J.F.Hancock, T.Giannakouros, C.Newman, J.Armstrong and A.I.Magee, submitted).

Labelling of *in vitro* translates with S-adenosyl[$^3$H]methyl-methionine was carried out in the presence of canine microsomal membranes (Promega) (Hancock *et al.*, 1991).

## Immunofluorescence/MDCK microinjection

MDCK cells were microinjected intracytoplasmically using a Zeiss/Eppendorf semi-automatic microinjecting device. Approximately 2 × 10$^{-11}$ ml plasmid DNA at 0.2 mg/ml were injected. After 18−20 h at 37°C the cells were fixed in 3% paraformaldehyde/50 mM ammonium chloride/0.2% Triton X-100 for 10 min, washed and then incubated in monoclonal antibody 7F7 (Schultz *et al.*, 1988) at 1:1000 dilution in PBS-A for 1 h, followed by a 1:400 dilution of goat anti-mouse immunoglobulin coupled to FITC (Pierce). Preparation of NIH3T3 cell lines expressing transfected K-ras(B) proteins has been described previously (Hancock *et al.*, 1990). Cells were examined using an MRC 500 confocal imaging system in conjunction with a Nikon Optiphot fluorescent microscope with a ×60 planapo objective lens.

## Acknowledgements

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# Methylation and proteolysis are essential for efficient membrane binding of prenylated p21$^{K-ras(B)}$

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Plasma membrane targeting of p21$^{K-ras(B)}$ requires a CAAX motif and a polybasic domain. The CAAX box directs a triplet of post-translational modifications: farnesylation, proteolysis of the AAX amino acids and methylesterification. These modifications are closely coupled *in vivo*. However, *in vitro* translation of mRNA in rabbit reticulocyte lysates produces p21$^{K-ras(B)}$ proteins which are arrested in processing after farnesylation. Intracellular membranes are then required both for proteolytic removal of the AAX amino acids and methylesterification of farnesylated p21$^{K-ras(B)}$. Binding of p21$^{K-ras(B)}$ to plasma membranes *in vitro* can then be shown to depend critically on AAX proteolysis and methylesterification since p21$^{K-ras(B)}$ which is farnesylated, but not methylated, binds inefficiently to membranes.
*Key words:* p21$^{K-ras(B)}$/*in vitro*/processing

## Introduction

A number of proteins including fungal mating factors, nuclear lamins, the transducin $\gamma$-subunit and p21$^{ras}$ all terminate in a C-terminal CAAX motif (C = cysteine, A = aliphatic, X = non aliphatic amino acid) and have been shown to undergo a triplet of post-translational modifications. This set of modifications comprises prenylation of the cysteine residue with a C$_{15}$ farnesyl moiety, proteolysis to remove the -AAX amino acids and carboxymethylation (Wolda and Glomset, 1988; Clarke *et al.*, 1988; Gutierrez *et al.*, 1989; Hancock *et al.*, 1989; Casey *et al.*, 1989; Lai *et al.*, 1990; Fukada *et al.*, 1990). For the *Saccharomyces cerevisiae* a-type mating factor and the transducin $\gamma$-subunit, structural determination has shown that following post-translational processing: cysteine is the C-terminal amino acid, farnesyl is linked to the sulphur by a thio ether bond and the $\alpha$-carboxyl group of the cysteine is methylesterified (Anderreg *et al.*, 1988; Lai *et al.*, 1990; Fukada *et al.*, 1990). Although the site of carboxylmethylation has not been determined in p21$^{ras}$ it is generally assumed to be on the C-terminal cysteine because, in all other features, the post-translational modifications of p21$^{ras}$ resemble the other proteins.

In the case of the nuclear lamins and p21$^{ras}$, the post-translational modifications of the CAAX motif combine with another signal to target localization to specific membranes (Holtz *et al.*, 1989; Hancock *et al.*, 1990). For the localization of p21$^{ras}$ to the inner surface of the plasma membrane, the second signal is contained within the C-terminal hypervariable domain and comprises a cysteine palmitoylation site (p21$^{H-ras}$, p21$^{N-ras}$) or a polybasic sequence [p21$^{K-ras(B)}$] (Hancock *et al.*, 1990). The net result of the CAAX modifications is to significantly increase the hydrophobicity of the processed protein (Gutierrez *et al.*, 1989). However, it has not been possible to determine whether all three post-translational modifications are required for membrane binding. In order to investigate the individual contributions of the CAAX modifications to membrane association we have made use of *in vitro* translated proteins.

Previous studies have shown that CAAX containing peptides are farnesylated *in vitro* in soluble cellular extracts (Reiss *et al.*, 1990; Schafer *et al.*, 1990) and that nuclear lamins A and B can be prenylated in a rabbit reticulocyte lysate (Vorburger *et al.*, 1989). In addition an enzyme has been identified in rat liver membranes which can methylesterify synthetic farnesyl:cysteine containing peptides (Stephenson and Clarke, 1990). These peptides mimic the C-terminal structure of farnesylated, -AAX proteolysed ras proteins, and hence the enzyme is an excellent candidate for the p21$^{ras}$ methyltransferase. Genetic studies in yeast suggest that the methyltransferase activity is encoded by, or dependent on, the STE14 gene product (Hrycyna and Clarke, 1990).

We now show that p21$^{K-ras(B)}$ is prenylated in reticulocyte lysates but that neither proteolysis nor methylation takes place. However, addition of pancreatic microsomes results in proteolysis and methylation to produce a fully processed p21$^{K-ras(B)}$ protein. Use of an inhibitor of the methylation step, in the presence of microsomes, allows prenylation and proteolysis but not methylation to occur. Thus, the two potential intermediates in processing, and the fully processed form of p21$^{K-ras(B)}$, can be assayed for binding to membranes.

## Results

### Post-translational processing of p21$^{K-ras(B)}$ in an in vitro system

Three K-*ras* cDNAs encoding wild-type p21$^{K-ras(B)}$, a K-ras protein with a Cys186 → Ser mutation (K186) and a K-ras protein with the polybasic domain replaced with uncharged glutamine residues (K6Q) (Hancock *et al.*, 1990) were cloned into pGEM plasmids. The genes were transcribed *in vitro* and the mRNA produced used to direct *in vitro* translation in a nuclease treated (message dependent) rabbit reticulocyte lysate. The extent of the post-translational processing of the translated proteins was then determined.

Post-translational modifications which occur at the C-terminal CAAX motif increase the hydrophobicity of p21$^{ras}$ (Gutierrez *et al.*, 1989; Hancock *et al.*, 1989). This is readily assayed by the partitioning of radiolabelled ras proteins after Triton X-114 phase separation, followed by

immunoprecipitation and gel electrophoresis (Bordier, 1981; Gutierrez et al., 1989; Hancock et al., 1989). Aliquots of reticulocyte lysates containing [$^{35}$S]methionine labelled p21$^{K\text{-}ras(B)}$ proteins were therefore assayed by Triton X-114 partitioning. Figure 1 shows that the K186S control protein, which is blocked for step I processing (Hancock et al., 1989), is found exclusively in the aqueous phase while 20% of wild-type K-ras protein partitions into detergent. This result indicates that only a small proportion of the translated protein is being processed in the unmodified lysate.

To investigate whether the low level of processing was due to an inadequate supply of mevalonic acid (MVA, the precursor of farnesyl pyrophosphate) the lysates were supplemented with MVA during the translation incubation. Under these conditions 70% of the translated wild-type protein partitioned into the detergent phase of Triton X-114. MVA supplementation had no effect on the processing of the K186 protein (Figure 1). The K6Q protein was processed to the same extent as wild-type p21$^{K\text{-}ras(B)}$ in the MVA supplemented lysate (Figure 1). We have previously shown that mutations in the polybasic domain of p21$^{K\text{-}ras(B)}$ have no detrimental effect on processing of the CAAX motif in vivo (Hancock et al., 1990). To confirm that p21$^{K\text{-}ras(B)}$ was being prenylated in vitro, translations were performed in lysates supplemented with [$^{14}$C]mevalonolactone. Figure 2 shows that label was incorporated into both wild-type p21$^{K\text{-}ras(B)}$ and the K6Q mutant but not into the K186 protein. A recent study has shown that rabbit reticulocyte lysate farnesylates recombinant, Escherichia coli expressed p21$^{ras}$ protein (Schaber et al., 1990), suggesting strongly that the prenyl group attached to p21$^{K\text{-}ras(B)}$, translated in vitro, is C$_{15}$ farnesyl.

Following farnesylation, p21$^{ras}$ undergoes proteolysis to remove the -AAX amino acids (Gutierrez et al., 1989) and methylesterification (Clarke et al., 1989; Gutierrez et al., 1989). By analogy with other CAAX containing proteins (Anderegg et al., 1988) the methylesterification is assumed

to occur on the newly exposed C-terminal cysteine residue. To investigate whether methylesterification was occurring in the lysate, translations of p21$^{K\text{-}ras(B)}$ were performed in the presence of S-adenosyl-[$^{3}$H]methionine (SAM) as a methyl donor. Figure 3A shows that no incorporation of label into the p21$^{K\text{-}ras(B)}$ protein was evident. However, since the p21$^{ras}$ methyltransferase has been localized to intracellular membranes (Stephenson and Clarke, 1990; Hrycyna and Clarke, 1990), the experiment was repeated in the presence of canine pancreatic microsomal membranes. Under these conditions methylation occurred on p21$^{K\text{-}ras(B)}$ and K6Q but not on the control K186 protein (Figure 3). We next investigated whether methylation could be inhibited by methylthioadenosine (MTA), which has been shown to significantly slow the methylesterification of nuclear lamins in vivo (Chelsky et al., 1989). Translations of p21$^{K\text{-}ras(B)}$ were therefore performed with added microsomes in the presence of 3 mM MTA, which is close to the limit of solubility of the compound (Chelsky et al., 1989). Control translations labelled with [$^{35}$S]methionine indicated that 3 mM MTA in DMSO (0.6% final concentration) inhibited translation by 29%. However, labelling of p21$^{K\text{-}ras(B)}$ by

**Fig. 2.** Prenylation of K-ras(B) proteins in vitro. Translations of p21$^{K\text{-}ras(B)}$ proteins were labelled with [$^{14}$C]mevalonic acid lactone (in the absence of added cold MVA). 25 μl of the lysate was then partitioned in Triton X-114 and the aqueous (a) and detergent (d) phases immunoprecipitated separately. K = wild-type p21$^{K\text{-}ras(B)}$, 186 = p21$^{K\text{-}ras(B)}$ (Cys186 → Ser), K6Q = p21$^{K\text{-}ras(B)}$ [(Lys175−180) → Gln].
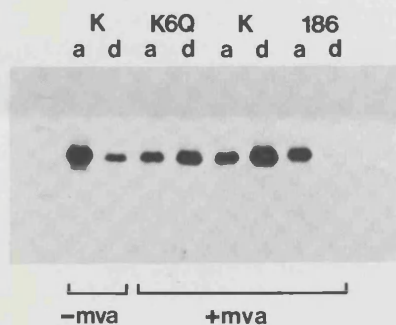
**Fig. 1.** Triton X-114 partitioning of in vitro translated K-ras proteins. K-ras proteins were translated in a reticulocyte lysate without added MVA (−mva) or with MVA at a final concentration of 5 mM (+mva). 2 μl aliquots of the [$^{35}$S]methionine-labelled translation reactions were partitioned in Triton X-114 and the aqueous (a) and detergent (d) phases immunoprecipitated and resolved by SDS−PAGE. To estimate the extent of processing the radioactivity incorporated into the aqueous and detergent partitioning forms of p21$^{K\text{-}ras(B)}$ was determined as described (Materials and methods). In the unmodified lysate, 20% of the translated wild-type p21$^{K\text{-}ras(B)}$ (K) is processed (i.e. partitions into detergent), whereas if the lysate is supplemented with MVA, the processed fraction increased to 70%. The K6Q mutant protein which has had the polybasic domain replaced with six glutamine residues is processed to the same extent as wild-type p21$^{K\text{-}ras(B)}$. The Cys186 → Ser mutant (186) is blocked for step I processing and partitions exclusively into the aqueous phase.
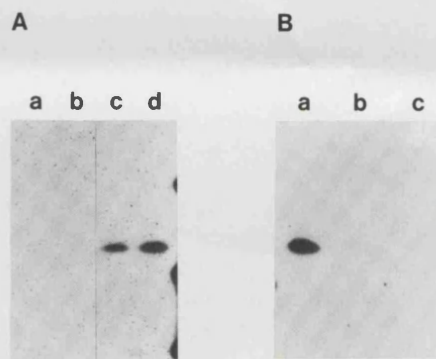
**Fig. 3.** Methylation of K-ras protein in vitro. **A.** Translations of p21$^{K\text{-}ras(B)}$ proteins were carried out in the presence and absence of canine microsomal membranes in lysates supplemented with 5 mM MVA and labelled with SAM. 25 μl lysate was immunoprecipitated, resolved by SDS−PAGE and fluorographed. a = K6Q minus microsomes, b = p21$^{K\text{-}ras(B)}$ minus microsomes, c = K6Q plus microsomes, d = p21$^{K\text{-}ras(B)}$ plus microsomes. **B.** SAM labelling of in vitro translated p21$^{K\text{-}ras(B)}$ (lanes a and b) and p21$^{K\text{-}ras(B)}$ Ser186 (lane c) proteins was performed in the presence of microsomes as described above, and the proteins immunoprecipitated. MTA (final concentration 3 mM) was included in the translation of p21$^{K\text{-}ras(B)}$ in lane b.

SAM was reduced to ~5% of control in the presence of 3 mM MTA (Figure 3B).

Since the methyltransferase responsible for methylating p21$^{ras}$ is membrane bound, we wished to determine whether the protease responsible for removing the -AAX amino acids was present in the cytosol or was associated with the microsomal membranes. To address this question, tryptophan substitutions were made in the p21$^{K-ras(B)}$ protein at amino acid 28, N-terminal of the CAAX motif and amino acid 189 at the extreme C-terminus. Since there are no tryptophan residues in wild-type p21$^{K-ras(B)}$, tryptophan substitutions allow specific labelling of individual residues within the protein to determine whether a particular residue is still present in processed forms of p21$^{ras}$ (Gutierrez *et al.*, 1989). The 28W and 189W substituted proteins were translated in [$^3$H]tryptophan supplemented lysates, with and without added microsomal membranes. Figure 4 shows that both the aqueous and detergent partitioning forms of the control 28W protein are labelled by [$^3$H]tryptophan irrespective of the presence of microsomes. However, with the 189W protein [$^3$H]tryptophan label is only found in the detergent phase in the absence of added microsomes (Figure 4B). The control $^{35}$S-labelled experiment in Figure 4A shows that the tryptophan substitution at amino acid 189 does not prevent processing to a detergent partitioning form, although processing is slowed by ~60%. Therefore, only if microsomes are present in the lysate is amino acid 189 removed from the processed K-ras protein, indicating that the -AAX proteolytic activity is, like the methyltransferase, associated with the microsomal membranes. The complete loss of all the label at amino acid 189 suggests that the protease present in the added microsomal membranes is sufficient to fully process all of the prenylated protein. A repeat of this experiment in the presence of 3 mM MTA
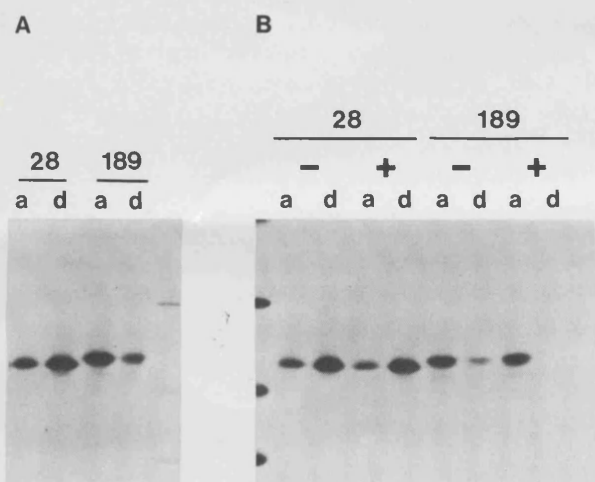
showed that the methyltransferase inhibitor did not block the proteolytic removal of amino acid 189W (data not shown).

### Prenylation alone is insufficient for efficient membrane binding

Given that *in vitro* translation, depending on the presence of microsomes, could be used to produce both fully and partially processed forms of p21$^{K-ras(B)}$ it was of interest to assess the relative importance to membrane binding of the various CAAX modifications. The proportion of processed p21$^{K-ras(B)}$ present in the lysate was first determined by Triton X-114 partitioning, immunoprecipitation and SDS−PAGE. This estimation is necessary since only processed p21$^{ras}$ binds to membranes *in vivo* (Hancock *et al.*, 1989). Varying amounts of reticulocyte lysate were incubated with P100 membrane fractions prepared from COS cells. The P100 fractions were then reisolated by
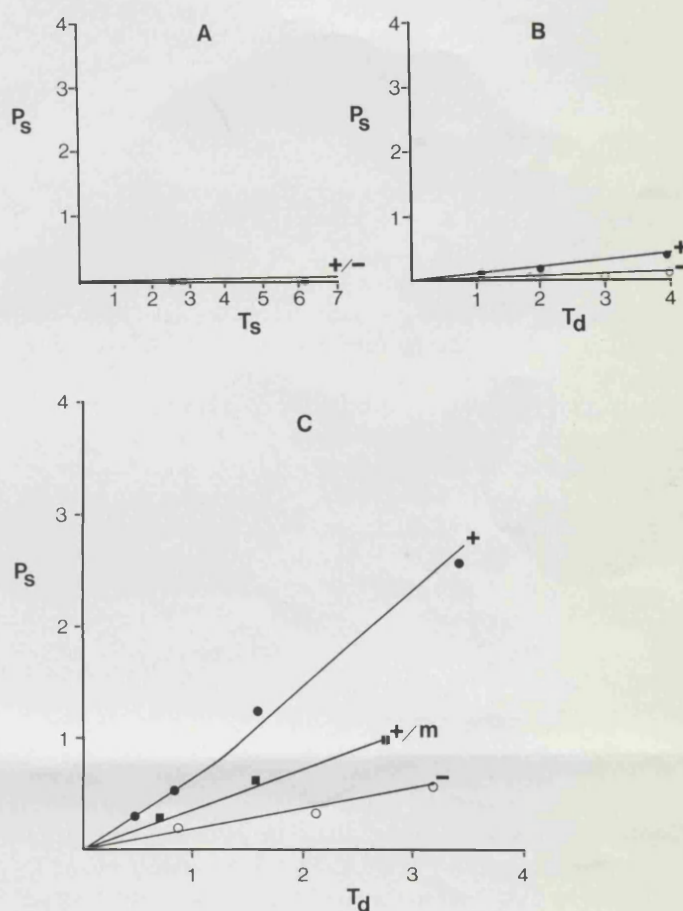


Fig. 5. Membrane binding of *in vitro* processed K-ras(B) proteins. Translations of p21$^{K-ras(B)}$ proteins were performed in the presence (+) and absence (−) of pancreatic microsomes. Labelling was with [$^{35}$S]methionine in MVA supplemented lysates. For wild-type p21$^{K-ras(B)}$ translations were also performed with microsomes in the presence of 3 mM MTA (+/m). Varying amounts of the lysates were incubated with P100 fractions prepared from COS cells and the amount of p21$^{K-ras(B)}$ bound to the acceptor membranes determined as described (Materials and methods). The amount of p21$^{K-ras(B)}$ binding specifically to the P100 acceptor membranes (P$_S$) is plotted as c.p.m. × 10$^{-5}$ along the y axis. The amount of p21$^{K-ras(B)}$ incubated with the membranes is given as T$_D$ (=detergent partitioning soluble ras protein) or, for the K186 protein, T$_S$ (=total soluble ras protein). T$_D$ and T$_S$ are plotted on the x axis as c.p.m. × 10$^{-5}$. The results of an individual experiment are shown on the graphs, but each of the plotted lines summarizes data pooled from 4−8 independent experiments. **A** p21$^{K-ras(B)}$ Ser 186, **B** p21$^{K-ras(B)}$ [(Lys175−180) → Gln] = K6Q, **C** p21$^{K-ras(B)}$.



Fig. 4. The -AAX protease is membrane associated. **A**. Translations of 28W and 189W substituted p21$^{K-ras(B)}$ proteins were labelled with [$^{35}$S]methionine. The lysates were supplemented with MVA but the translations were performed without microsomes. Aliquots of the reactions were partitioned in Triton X-114 and the aqueous (a) and detergent (d) phases immunoprecipitated. The presence of a tryptophan residue at amino acid 189 reduces the detergent partitioning fraction from 70% (wild-type control) to 30%. **B**. Translations of 28W and 189W substituted p21$^{K-ras(B)}$ proteins, labelled with [$^3$H]tryptophan, were carried out in the absence (−) or presence (+) of microsomal membranes in lysates supplemented with MVA. Aliquots of the reactions were partitioned in Triton X-114 and the aqueous (a) and detergent (d) phases immunoprecipitated.

**Table I.** Summary of C-terminal processing in a rabbit reticulocyte lysate

| Construct | Micro | MTA | C-terminus | $F_D$ | Membrane binding |
|---|---|---|---|---|---|
| K186S | − | − | −−S V I M(−) | 0% | 0% |
| K wt | − | − | −−C V I M(−) $\vert$ $C_{15}$ | 70% | 20% |
| K wt | + | + | −−C(−) $\vert$ $C_{15}$ | 70% | 40% |
| K wt | + | − | −−C−Me $\vert$ $C_{15}$ | 70% | 60−80% |

The table gives C-terminal structures of K-ras proteins translated in reticulocyte lysates, supplemented with MVA, depending on the presence of microsomes and/or MTA. $F_D$ is the proportion of the protein partitioning into the detergent phase of Triton X-114 under the specified reaction conditions and the final column gives the extent of membrane association of the processed protein. K wt = wild-type p21[K-ras(B)], K186S = p21[K-ras(B)] Cys186 → Ser, (−) = free C-terminal α-carboxyl group, −Me = methylesterified α-carboxyl group, $C_{15}$ = farnesyl.

centrifugation at 100 000 $g$ and the amount of p21[K-ras(B)] bound determined (legend to Figure 5). Following translation, Triton X-100 was added to the lysates to a final concentration of 0.05% in order to maximize the proportion of soluble protein. In subsequent incubations of lysate with acceptor membranes the final concentration of Triton was adjusted to 0.02%. In similar experiments with pp60[src], Resh (1989) has shown that 0.02% Triton has no detrimental effect on the acceptor membranes. Control centrifugations performed in the absence of added P100 fraction showed that ~5% of p21[K-ras(B)] protein, translated in the absence of microsomes, was insoluble under these reaction conditions. This fraction increased to ~15% in translations performed in the presence of microsomes. There was, however, no evidence to suggest that p21[K-ras(B)] was binding to the microsomes since the pellet contained aqueous and detergent partitioning forms of p21[K-ras(B)] in similar proportions to those found in the soluble fraction. Thus the pellet was not significantly enriched for processed p21[K-ras(B)].

Figure 5 shows the membrane binding of p21[K-ras(B)] proteins that have been processed in the presence and absence of microsomes. None of the K186 control protein bound to the P100 membranes in the conditions of this assay (Figure 5A) and <10% of processed K6Q protein bound to the acceptor membranes whether or not the protein was proteolysed and methylated (Figure 5B). This result is consistent with the observed distribution of the K6Q mutant protein in vivo (Hancock et al., 1990).

Farnesylated, non-proteolysed, non-methylated wild-type p21[K-ras(B)] did bind to the P100 membranes but only inefficiently; Figure 5C shows that <20% of the farnesylated protein bound to the acceptor membranes over a wide range of p21[K-ras(B)] concentrations. However, p21[K-ras(B)] protein translated in the presence of microsomal membranes bound much more extensively to the P100 membranes. Figure 5C shows that 60−80% of the fully processed p21[K-ras(B)] present in the lysate bound to the acceptor membranes over the range of p21[K-ras(B)] concentrations investigated. This figure compares favourably with the proportion of

p21[K-ras(B)] found associated with the P100 fraction in vivo (Hancock et al., 1990). Approximately 40% of the p21[K-ras(B)] processed in the presence of microsomes and MTA bound to the acceptor membranes (Figure 5C). Thus farnesylated, -AAX proteolysed, predominantly non-methylated p21[K-ras(B)] binds less extensively to membranes than the fully methylated protein.

Finally, to exclude the possibility that the endogenous SAM concentration was limiting for full methylation of the protein, p21[K-ras(B)] was translated with added microsomes in lysates supplemented with both MVA and cold SAM. Adding SAM to a final concentration of 50 $\mu$M produced no increment in the P100 binding of p21[K-ras(B)] protein translated and processed in such modified lysates. However, SAM concentrations of 100 $\mu$M or above significantly inhibited translation.

It is also of interest to note that ~32 $\mu$g of microsomal membranes are added to 50 $\mu$l of reticulocyte lysate in this protocol. Therefore binding of p21[K-ras(B)] to 4 $\mu$g of acceptor plasma membrane is carried out in the presence of up to an 8-fold excess of intracellular membranes; a striking demonstration of the specificity of p21[K-ras(B)] binding to plasma membrane.

## Discussion

The plasma membrane targeting of p21[ras] requires the C-terminal CAAX motif and a second signal of either palmitoylation or a polybasic motif. The requirement for the second signal in directing plasma membrane targeting has been addressed in vivo using mutant ras proteins (Hancock et al., 1990). Dissecting the individual roles of farnesylation, AAX proteolysis and methylesterification in plasma membrane targeting is difficult in vivo since these modifications appear to be very closely coupled and there are no specific inhibitors for proteolysis and methylation. Furthermore mutations introduced into the CAAX motif block all processing by preventing farnesylation, which is the first post-translational modification of p21[ras] (Hancock et al., 1989).

We have shown here that farnesylation, proteolysis and methylation of p21[K-ras(B)] are possible in a reticulocyte lysate supplemented with MVA and microsomal membranes. Farnesylpyrophosphate transferase is present in the soluble fraction of the lysate, as it is in the soluble fraction of other cell extracts (Vorburger et al., 1990; Reiss et al., 1990). By implication from the results presented here, the synthetic enzymes of the isoprene pathway are also present in the soluble fraction of the lysate. It has been shown previously that the methyltransferase activity is membrane associated (Stephenson and Clarke, 1990); our results show that the -AAX protease is also found on intracellular membranes. This is consistent with recent work on the processing of C-terminal peptides of p21[ras] in soluble in vitro systems. The peptides were found to be modified by the addition, predominantly of $C_{15}$ farnesol, but were not proteolysed or methylesterified (Schafer et al., 1990).

It is interesting to note that the processing of p21[K-ras(B)] in this in vitro system is very rapid and clearly exhausts the available supply of MVA unless extra is provided. We have noted previously that the processing of p21[K-ras(B)] in vivo is much more rapid than that of p21[H-ras] and p21[N-ras] (Hancock et al., 1989). We have also observed that the

processing of p21$^{H\text{-ras}}$ is much slower than that of p21$^{K\text{-ras(B)}}$ in the reticulocyte lysate to the extent that the endogenous supply of MVA is not limiting over the course of the incubation (data not shown). The recent observation that the p21$^{K\text{-ras(B)}}$ CAAX peptide is a much better substrate for farnesyltransferase than the p21$^{H\text{-ras}}$ CAAX peptide (Reiss et al., 1990) rationalizes these data. Similarly the presence of a tryptophan residue at amino acid 189 slows processing presumably by making the modified CAAX sequence a poorer substrate for the farnesyltransferase.

The data presented here, summarized in Table I, show that farnesylation alone increases the hydrophobicity of p21$^{K\text{-ras(B)}}$ sufficiently to cause partitioning into the detergent phase of Triton X-114. However, farnesylated, non-proteolysed, non-methylated p21$^{K\text{-ras(B)}}$ associates inefficiently with cell membranes. Removal of the -AAX amino acids produces a 2-fold increment in the extent of membrane binding in vitro, and subsequent methylation results in a further 2-fold increment in membrane binding (Table I). These experiments also show that, as in the intact cell, the combination of a processed CAAX motif and a polybasic domain is essential for the in vitro membrane binding of p21$^{K\text{-ras(B)}}$.

The interesting question arising from these data is why 'AAXing' and methylation should significantly increase the extent of membrane binding of farnesylated p21$^{K\text{-ras(B)}}$. Removal of the last three amino acids may be required because they sterically hinder insertion of the farnesyl group into the lipid bilayer or a receptor protein. The requirement for methylation is presumably to neutralize the negative charge on the ionized carboxyl group which could cause repulsion from the negatively charged head groups of membrane phospholipids. If p21$^{K\text{-ras(B)}}$ does bind to a receptor protein in the plasma membrane, an analogy with another CAAX protein, the yeast mating factor-a, is apparent. A chemically demethylated form of the mature factor has been shown to be biologically inactive (Anderegg et al., 1989), which may reflect an inability of the demethylated factor to bind efficiently to its receptor on the target cell.

## Materials and methods

### Plasmids and focus assays
All mutations in K-ras(B) cDNA were constructed using oligonucleotide directed mutagenesis and the cDNA fully sequenced before subcloning into the expression vector pGEM-9Z(f-) (Promega). RNA was translated in vitro using T7 DNA dependent RNA polymerase and linearized plasmid as template.

### Translation reactions
Nuclease treated reticulocyte lysate was obtained from Promega, UK, and translations performed according to the manufacturer's instructions. Typically reactions were performed at 30°C for 90 min using 2 µg uncapped RNA in a reaction volume of 50 µl. Mevalonic acid lactone (Sigma) was converted to MVA by alkalkine hydrolysis as described (Kita et al., 1980), stored as an aqueous 100 mM stock solution at −20°C and 2.5−5 µl added to the translation reactions where indicated. Canine pancreatic microsomal membranes (Promega) were stored at −70°C and thawed on ice immediately before use. 7.2 EQ of membranes (3.6 µl) were used in a 50 µl translation reaction. Following translation, 2.5 µl of 1% Triton X-100 in NT buffer (50 mM Tris−Cl, pH 7.5, 50 mM NaCl, 1 mM MgCl$_2$) was added to adjust the final Triton concentration to 0.05%.

Methionine free amino acid mix (Promega) was used for the labelling of translations with [$^{35}$S]methionine (Amersham, SJ204; at 1.2 mCi/ml$^{-1}$ final concentration) in 50 µl reactions. Appropriate 1 mM L-amino acid (Sigma) mixes were prepared for labelling translation reactions with MVA and S-adenosyl-methionine (all 20 amino acids) or tryptophan (19 amino

acids minus tryptophan). These reactions were labelled with 50 µCi R-[2-$^{14}$C]mevalonic acid lactone (Amersham, CFA660) after removing the benzene solvent under a stream of nitrogen at 37°C, or 100 µCi SAM (ICN, 24051H) or 25 µCi [5-$^3$H]tryptophan (NEN, NET782) after removing the ethanol/H$_2$SO$_4$ and water/ethanol solvents respectively under a vacuum at −60°C. The dried label was taken up directly in reticulocyte lysate in each case and the translations performed in a final volume of 25 µl. MTA (Sigma) was kept as a 0.5 M stock in DMSO at −20°C and thawed immediately before use.

### Triton X-114 partitioning and immunoprecipitations
2 µl of a [$^{35}$S]methionine-labelled reaction, or 25 µl of a [$^3$H]tryptophan or [$^{14}$C]MVA labelled translation reaction were taken up in 1 ml of ice cold 1% Triton X-114, warmed to 37°C for 2 min and the aqueous and detergent phases separated by a 2 min microfuge spin (Gutierrez et al., 1989; Hancock et al., 1989). Immunoprecipitations with Y13-259 and resolution of the proteins by SDS−PAGE were performed as described (Gutierrez et al., 1989; Hancock et al., 1988). Gels were soaked in Enlightening (NEN) before being autoradiographed at −70°C. $^{35}$S-Labelled gels were autoradiographed for 4−12 h and $^3$H- and $^{14}$C-labelled gels were autoradiographed for 7−28 days.

To measure the radioactivity in immunoprecipitated p21$^{ras}$, autoradiograms were used as guides to cut out gel slices containing the ras protein. These were re-hydrated and digested with proteinase K (100 µg/ml) in Tris buffered saline at 37°C for 48 h, the gel slice and proteinase K solution were taken up in scintillation fluid and counted. The proportion of processed p21$^{ras}$ in an aliquot of lysate (= F$_D$) is then calculated as (c.p.m. in p21$^{ras}$ partitioning into detergent)/(total c.p.m. in p21$^{ras}$).

### Membrane binding assays
P100 fractions were prepared from COS cells as described (Cales et al., 1988), adjusted to a protein concentration of 0.5 µg/µl and stored in NT buffer in aliquots at −70°C until required. Reticulocyte lysate (5−60 µl) was incubated with 8 µl (4 µg) P100 fraction at 20°C for 30 min. The volume of the reaction was 50 µl (for 20 µl lysate or less) or the minimum volume, required for a final Triton X-100 concentration of 0.02%. In reactions using >20 µl lysate, Triton X-100 concentrations were adjusted so that the final concentration was always 0.02%. After the 30 min incubation the P100 fraction was reisolated by centrifugation at 100 000 g at 4°C for 30 min. The S100 fraction was removed and 5× concentrated SDS sample buffer added. The P100 pellet was taken up into 1 × SDS sample buffer. Both fractions were boiled and 20−50% of the sample resolved by SDS−PAGE. The amount of radioactivity in p21$^{ras}$ in each sample was then determined as described above. The amount of insoluble p21$^{ras}$ present was determined by carrying out a control incubation in the absence of added P100 fraction. The c.p.m. present in insoluble p21$^{ras}$ were then subtracted from the c.p.m. isolated in the presence of P100 (in proportion to the amount of p21$^{ras}$ present in the reaction) to determine the amount of p21$^{ras}$ binding specifically to the acceptor membranes (P$_S$).

The processed p21$^{ras}$ available in an incubation to bind to the acceptor membranes (T$_D$ = total detergent partitioning c.p.m.) was calculated as (total c.p.m. in S100 + total c.p.m. in P100 − insoluble c.p.m.) × F$_D$.

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## Note added in proof

We have now demonstrated by methyliodide cleavage (Casey *et al.*, 1989) and HPLC analysis that the isoprenoid covalently bound to p21[K-ras (B)] following *in vitro* translation is $C_{15}$ farnesyl