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University of Alberta

Variable Lipidation of Ras Isoforms Directs their Differential Membrane Association

by

Christine Mattar 

A thesis submitted to the Faculty of Graduate Studies and Research in Partial fulfillment of the requirements for the degree of Master of Science

Department of Biochemistry

Edmonton, Alberta
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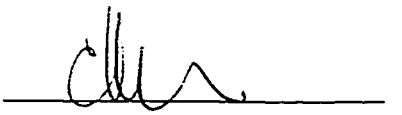

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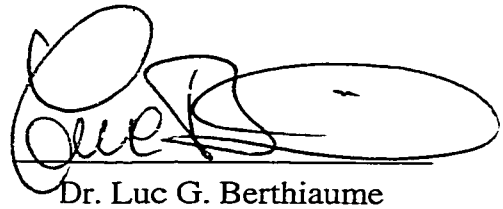
ABSTRACT

The three major Ras isoforms, N-Ras, H-Ras and K-Ras4B, are highly homologous except for their C-terminal regions, known as "hypervariable domains". Plasma membrane association of these isoforms is essential for their signalling activity, and is mediated by variable lipid modifications found within their respective hypervariable domains. All three isoforms possess a C-terminal CaaX motif directing protein prenylation, and are consequently farnesylated on the CaaX cysteine. Farnesylation is combined with a second membrane binding signal to enable stable plasma membrane binding. The "second signal" is found upstream of the prenylcysteine: N-Ras and H-Ras respectively utilize mono- and di-palmitate second signals, while K-Ras4B utilizes a polybasic second signal. We sought to determine whether the variably lipidated hypervariable domains could direct differential membrane association of these isoforms. To investigate this, we appended wild-type and lipidation-mutant forms of the C-terminal 14 amino acids from each isoform to the C-terminus of green fluorescent protein (GFP). Using a variety of techniques, including confocal microscopy, we demonstrated that the variably lipidated GFP-Ras chimeras differentially associated with vesicular structures, endoplasmic reticulum and Golgi membranes in a lipidation-dependent fashion. These results confirm and augment the emerging model of Ras trafficking currently described in the literature.

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Variable Lipidation of Ras Isoforms Directs their Differential Membrane Association** by **Christine Mattar** in partial fulfillment of the requirements for the degree of Master of Science.



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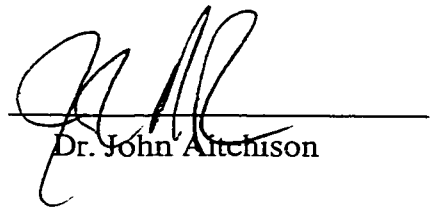

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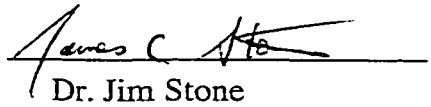

Dr. Jim Stone

Date: 2 August 2000

For my parents, still the smartest people I know
For Suzie, Evie, Eddie and Rich, who brighten the world
And for Bryan, my answered prayer

Acknowledgments

At last...

I am thankful for the friends who sustained me day to day, and occasionally, moment to moment: Carrie Soltys, Zhao Yang, Helen Everett, Val Templeman, Dean Schieve and James McCabe. I am indebted to my committee, Dr. Tom Hobman, Dr. John Aitchison and Dr. Jim Stone, for their patient advice. I am also indebted to Dr. Bob Ryan for his mentorship. Et toi, Luc! Thank you to my supervisor Dr. Luc Berthiaume for the insightful years I spent in his lab. And thank God for making me stubborn enough to persevere.

'For I know the plans that I have for you' declares the Lord 'plans for welfare and not for calamity to give you a future and a hope'

Jeremiah 29:11

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
°C	degrees Celsius
Ci	Curie
CLB	cold lysis buffer
CMV	cytomegalovirus
COS-7	CV-1 origin, SV40-transformed African baby green monkey kidney cell line
dATP	deoxyadenosine 5′-triphosphate
dCTP	deoxycytidine 5′-triphosphate
DEAE	diethylaminoethyl
dGTP	deoxyguanosine 5′-triphosphate
diI-LDL	1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethylindocarbocyanine perchlorate-low density lipoprotein
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide 5′-triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine 5′-triphosphate
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced GFP
ER	endoplasmic reticulum
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FTase	farnesyl protein transferase
x*g*	acceleration due to gravity

GFP	green fluorescent protein
GGTase	geranylgeranyl protein transferase
Gln	glutamine
Gly	glycine
hGH	human growth hormone
HLB	hypotonic lysis buffer
HMG-CoA	3-Hydroxy-3-Methylglutaryl-Coenzyme A
HPLC	high performance liquid chromatography
IC16	[^{125}I] iodopalmitate palmitate analogue
IP	immunoprecipitation
kDa	kiloDalton
L	liter
LB	Luria-Bertani Medium
Leu	leucine
MCS	multiple cloning site
Met	methionine
mg	milligram
ml	milliliter
mm	millimeter
mM	millimolar
MVA	mevalonic acid lactone or mevalonolactone
MW	molecular weight
NA	numerical aperture
NaOH	sodium hydroxide
ng	nanogram
nm	nanometers; unit of light wavelength measurement

NP-40	Nonidet P-40
OD_{260}	optical density at 260 nanometers
PAGE	polyacrylamide gel electrophoresis
PAS	protein A sepharose
PAT	protein S-acyltransferase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Phe	phenylalanine
pmol	picomole
PMSF	phenylmethylsulfonylfluoride
P100	particulate fraction resulting from 100 000xg centrifugation
PVDF	polyvinylidenelfluoride
rpm	revolutions per minute
RSGFP	red-shifted GFP
SDS	sodium dodecyl sulfate
Ser	serine
SOB	super optimal broth
SOE	splicing by overlap extension
S100	soluble fraction resulting from 100 000xg centrifugation
SV40	Simian virus 40
T	total fraction derived from cell homogenization before centrifugation
TE	tris-EDTA
Thr	threonine
TR	Texas Red
Tris	tris(hydroxymethyl)aminomethane
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride

U	units
µCi	microcurie
µl	microliter
V	volt
v/v	volume per unit volume
WT	wild-type
w/v	weight per unit volume

1.0 INTRODUCTION

1.1 Covalent Lipid Modification of Proteins - Overview

The earliest report describing the phenomenon of protein lipidation was Folch and Lees' description of brain myelin lipidation in 1951 (Folch and Lees, 1951). The introduction of protein lipidation as a modern field of study began some thirty years later with the discovery of viral glycoprotein lipidation in eukaryotic cells (Schmidt and Schlesinger, 1979; Schmidt *et al.*, 1979). In the past two decades, the study of protein lipidation has identified several hundred diverse lipid-modified proteins which vary in function and subcellular localization. These proteins are covalently modified by a variety of lipids and lipid combinations. The lipid modifications function to stabilize the tertiary protein structure, enhance or inhibit protein-protein intractions, regulate enzymatic activity, or impart hydrophobicity, which facilitates their association with various cellular membranes. In turn, these membrane associations enable specific protein-protein interactions which are essential for a number of distinct cellular processes.

The covalent lipid modification of proteins is traditionally divided into three categories: acylation, which encompasses *N*-myristoylation and palmitoylation, prenylation, which encompasses farnesylation and geranylgeranylation, and glypiation, which is the modification of cell surface proteins with the glycosyl phosphatidylinositol glycolipid (Table 1.1). An emerging category may be described as "novel lipidation" and includes covalent modification of specific proteins with retinoic acid (Takahashi and Breitman, 1989; Breitman and Takahashi, 1996 and Myhre *et al.*, 1996), cholesterol (Porter *et al.*, 1996) and *n*-

octanoyl (Kojima *et al.*, 1999). Only acylation and prenylation will be considered in this context, as these are the two modifications relevant to the study of Ras lipidation.

1.2 Protein Acylation

Protein acylation most commonly refers to the modification of proteins by myristate (C14:0), a saturated 14-carbon fatty acid, and/or palmitate (C16:0), a saturated 16-carbon fatty acid (Table 1.1). Proteins may also be modified by laurate (C12:0), myristoylate (C14:1), stearate (C18:0), oleate (C18:1(9)) and arachidonate (C20:4 (5,8,11,14)) (Resh, 1999; Dunphy and Linder, 1998). In this context, only *N*-myristoylation and palmitoylation will be considered.

1.2.1 *N*-Myristoylation

Protein myristoylation was first desribed for calcineurin B and the catalytic subunit of protein kinase A after the identification of an N-terminal blocking group as myristate (Carr *et al.*, 1982; Aitken and Cohen, 1984). The enzymology of *N*-myristoylation has since been extensively characterized (Rudnick *et al.*, 1992; Johnson *et al.*, 1994). *N*-myristoylation is a cotranslational process which occurs at the N-terminus of proteins beginning with the consensus sequence:

Met-Gly-X-X-X-Ser/Thr

where X represents almost any amino acid (Table 1.1). However, *E*-myristoylation of the ε-amino group on internal lysine residues has also been reported for the interleukin 1α propiece (Stevenson *et al.*, 1993). During *N*-myristoylation, the initiating methionine residue is cotranslationally cleaved from the appropriate protein by methionine amino peptidase, leaving glycine as

the terminal amino acid. N-myristoyl transferase (NMT; Towler *et al.*, 1987; Duronio *et al.*, 1992) catalyzes transfer of myristate from myristoyl-CoA to the terminal glycine residue in the nascent peptide while it is still bound to the ribosome (Deichaite *et al.*, 1988; Wilcox *et al.*, 1987 and Rudnick *et al.*, 1990). The glycine residue is critical; substitution of glycine with any other amino acid abrogates myristoylation (Resh, 1993). In addition, NMT is highly selective for myristoyl-CoA, and shorter or longer fatty acyl CoAs are not transferred (Devadas *et al.*, 1992; Kishore *et al.*, 1993). Myristate is covalently bound to the protein by an amide bond, and the modification is irreversible, with a half-life equivalent to the half-life of the modified protein (Figure 1.1) (Wolven *et al.*, 1997).

Greater than one hundred myristoylated proteins have been identified, which vary in function and subcellular localization (reviewed in Resh, 1996 and Resh, 1999). Myristoylated proteins include the Src family of tyrosine kinases, Gα subunits of heterotrimeric G-proteins, retroviral proteins such as HIV-1 Gag and Nef, and those involved in a variety of cellular processes, such as myristoylated alanine rich C Kinase substrate (MARCKS), recoverin and nitric oxide synthase (Resh, 1999). Proteins modified by myristate may be membrane-associated, localizing to the plasma membrane (Moffett *et al.*, 2000), endoplasmic reticulum (Hecker *et al.*, 1994), Golgi (Haun *et al.*, 1993) and mitochondrial membranes (Borgese *et al.*, 1996), may associate with the cytoskeleton, or may exist in the cytoplasm as soluble proteins. Myristoylated proteins, such as recoverin and ADP ribosylation factor (Arf), become soluble by sequestering the myristate moiety within hydrophobic pockets formed by the three-dimensional protein structure (Ames *et al.*, 1994; Tanaka *et al.*, 1995 and Haun *et al.*, 1993).

Myristoylation of proteins serves several functions; it may stabilize tertiary protein structure, as with the catalytic subunit of protein kinase A (Zheng *et al.*, 1993), or may facilitate membrane association, as with the Src family tyrosine kinases (Cross *et al.*, 1984; Kamps *et al.*, 1985) or retroviral Gag proteins (Weaver and Panganiban, 1990; Schultz and Rein, 1989; Gottlinger *et al.*, 1989; Bryant and Ratner, 1990). Active site fatty acylation may act as an enzyme regulation mechanism, as suggested for the mitochondrial matrix enzyme methylmalonate semialdehyde dehydrogenase (Deichaite *et al.*, 1993; Berthiaume *et al.*, 1994). Myristoylation may also enhance protein-protein interactions; for example, myristoylation of Gα subunits has been shown to increase their affinity for the βγ heterodimer (Linder *et al.*, 1991).

The modification of protein by myristate alone does not provide sufficient binding energy to stably anchor that protein within a cellular membrane (Peitzsch and McLaughlin, 1993). A second membrane-binding signal is required for stable membrane association (Cadwallader *et al.*, 1994). This second signal is found downstream of the myristoylglycine and is defined as one or more palmitoylated cysteine residues or proximal basic amino acids which form a polybasic domain (Resh, 1999). Electrostatic interactions between basic amino acids in the polybasic region and acidic membrane phospholipids (phosphatidylserine and phosphatidylinositol) greatly enhance the membrane binding of myristoylated proteins such as Src tyrosine kinase (Sigal *et al.*, 1994; Buser *et al.*, 1994), MARCKS (McLaughlin and Aderem, 1995) and HIV-1 Gag (Zhou *et al.*, 1994). Palmitoylation of a myristoylated protein serves to increase membrane association by increasing the hydrophobic character of that protein (Shahinian and Silvius, 1995). Proteins which utilize a myristate/palmitate

combination for membrane binding include several Src family tyrosine kinases and Gα subunits (Resh, 1999).

1.2.2 Palmitoylation

Protein palmitoylation represents the post-translational modification of the cysteine thiol group by a long chain fatty acid, most commonly palmitate (Resh, 1999 and Dunphy and Linder, 1998). The palmitate moiety is bound to cysteine by a reversible thioester bond, which may enable the dynamic association of certain signalling proteins with the plasma membrane (Figure 1.1) (Milligan *et al.*, 1995; Mumby, 1997). The half-life of palmitate varies and has been estimated at as little as 20 minutes for N-Ras (Magee *et al.*, 1987) and as great as 12 hours for the transferrin receptor (Omary and Trowbridge, 1981). Depalmitoylation would require a thioesterase activity, and a putative acyl protein thioesterase (APT1) was recently purified which depalmitoylates Ras and Gα subunits *in-vitro* (Duncan and Gilman, 1998).

Palmitoylated cysteine residues exist in various sequence contexts and no consensus for protein palmitoylation has been defined (Table 1.1). The nature of the palmitoylation reaction remains controversial; there is evidence in support of both enyzmatic and non-enzymatic mechanisms. This is due in part to the difficulty in purifying the elusive palmitoyl acyl transferase (PAT) enzyme(s) responsible for palmitoylating proteins. In addition, the non-enzymatic palmitoylation of Gα proteins and Yes tyrosine kinase peptides have been reported (Duncan and Gilman, 1996; Bano *et al.*, 1998). However, several groups have reported the partial purification of PAT activities which palmitoylate Gα subunits (Dunphy *et al.*, 1996), Src family tyrosine kinase Fyn (Berthiaume

and Resh, 1995), H-Ras protein and Ras peptide (Ueno and Suzuki, 1997; Liu *et al.*, 1996), and red blood cell spectrin (Das *et al.*, 1997). While the protein substrate specificities of these PAT activities are not known, the preferred acyl-CoA substrate appears to be palmitoyl-CoA (Berthiaume and Resh, 1995; Dunphy *et al.*, 1996), but other long chain fatty acyl CoAs may also be incorporated onto palmitoylated proteins by PAT. Several palmitoylated proteins, including the transferrin receptor (Nadler *et al.*, 1994), Gα subunits (Hallak *et al.*, 1994), myelin (Bizzozero *et al.*, 1987) and P-selectin (Fujimoto *et al.*, 1993) have been shown to heterogeneously incorporate myristate, stearate or arachadonate by a thioester linkage. Consequently, the term "S-acylation" has been suggested to more accurately describe the palmitate modification (Casey, 1995).

Palmitoylated proteins may be categorized into four types (Resh, 1996). Type I proteins are comprised of transmembrane or integral membrane proteins such as seven transmembrane receptors. These proteins are palmitoylated on cysteine residues adjacent to or just within the transmembrane sequence. Type II proteins include palmitoylated proteins that incorporate an isoprenoid within their C-termini; prior prenylation of these proteins is required for palmitoylation to occur. N-Ras, H-Ras and K-Ras4A are all examples of type II proteins. Type III proteins are modified by one or more palmitate moieties within their N-termini; type III proteins are typified by the Gα subunits Gαs, Gαq, Gα12, Gα13 and Gα16. The fourth type of palmitoylated protein is N-terminally modified by both palmitate and myristate. Myristoylation occurs first and is a prerequisite for palmitoylation. Type IV proteins include the Src family tyrosine kinases and the Gαi1, Gαo and Gαz subunits.

While palmitoylated proteins are primarily associated with the plasma membrane, they have also been localized to membranes of the Golgi apparatus, mitochondrion (Rebollo *et al.*, 1999) and the extracellular milieu (Sessa *et al.*, 1995; Pepinsky *et al.*, 1998). Protein palmitoylation has a variety of functions. Palmitoylation enhances membrane association of myristoylated, prenylated or intrinsically hydrophilic proteins (Cadwallader *et al.*, 1994), and may target proteins specifically to the plasma membrane, as is the case for Gα subunits (Dunphy *et al.*, 1996). Palmitoylation may also retain proteins within caveolae, which are highly ordered subdomains of the plasma membrane (Robbins *et al.*, 1995; Melkonian *et al.*, 1999; Shenoy-Scaria *et al.*, 1994). Receptor endocytosis (Alvarez *et al.*, 1990; Bouvier *et al.*, 1995), enzymatic activity (Berthiaume *et al.*, 1994), viral budding (Ivanova and Schlesinger, 1993), protein sorting (Breuer and Braulke, 1998; Yang *et al.*, 2000) and protein-protein interactions (Ponimaskin and Schmidt, 1998; Nakamura *et al.*, 1998; Sudo *et al.*, 1992) may all be regulated by protein palmitoylation. Palmitoylation may also act as a "second signal" on prenylated proteins, stably anchoring them within cellular membranes (Hancock, *et al.*, 1990).

1.3 Protein Prenylation

The study of protein prenylation began with the study of fungal mating peptides, when Kamiya and coworkers identified the presence of an S-farnesyl cysteine in the fungal mating peptide rhodotorucine A (Kamiya *et al.*, 1979a; Kamiya *et al.*, 1979b). In the following years, peptidyl sex hormones of genus *Tremella* fungi were also found to be S-isoprenylated and methyl esterified on C-terminal cysteine residues (Sakagami *et al.*, 1979; Sakagami *et al.*, 1981; Ishibashi *et al.*, 1984). The isoprenoid modification of fungal peptides was not immediately

connected with mammalian protein prenylation when it was first suggested in 1984. That year, Schmidt and coworkers had described the first post-translational incorporation of a radiolabelled mevalonate derivative into 3T3 fibroblast proteins (Schmidt *et al.*, 1984). The following year, the inhibition of DNA replication in mevalonate-starved mammalian cells was correlated with defects in the synthesis of prenylated proteins (Sinesky and Logel, 1985). This suggested a functional role for mammalian protein prenylation.

The isolation of the *Saccharomyces cerevisiae* RAM gene ultimately created the link between the post-translational processing of the prenylated fungal mating peptides and mammalian protein prenylation (Powers *et al.*, 1986). The RAM gene product was found to be required for post-translational processing of *S.cerevisiae* RAS2 and a-mating pheromone (Powers *et al.*, 1986). Mutations in the RAM gene inhibited both RAS2 processing or maturation (which normally resulted in increased mobility in SDS-PAGE), and membrane association. At this time, maturation of mammalian Ras was believed to involve palmitoylation of a cysteine residue found within the C-terminal CaaX motif (where C=cysteine, a=aliphatic amino acids, X=any amino acid) (Buss and Sefton, 1986). Because *S.cerevisiae* RAS2 possessed a similar C-terminal CaaX motif, it was assumed that RAM protein was the acyltransferase which palmitoylated RAS2. However, another study involving novel *S.cerevisiae* mutants defective in RAS2 processing suggested that maturation involved proteolytic cleavage at the C-terminus, which would account for the difference in SDS-PAGE mobility observed for processed versus unprocessed RAS2 (Fujiyama *et al.*, 1987). Other studies demonstrating carboxymethylation of rat embryo H-Ras (Clarke *et al.*, 1988) and farnesylation/methylesterification of *S.cerevisiae* a-mating pheromone

(Anderegg *et al.*, 1988) finally correlated these modifications with those found on the *Tremella* mating peptides.

The common CaaX motif found in H-Ras, a-mating pheromone and RAS2 now suggested that RAS2 processing involved farnesylation, proteolysis and carboxymethylation. Proteolysis and carboxymethylation of RAS2 was formally demonstrated in 1990 (Fujiyama and Tamanoi, 1990). The elucidation of CaaX motif-dependent prenylation and processing of all mammalian Ras isoforms soon followed (Hancock *et al.*, 1989; Casey *et al.*, 1989; Gutierrez *et al.*, 1989). In the intervening years, a growing number of prenylated proteins have been identified in a variety of organisms. These proteins mediate diverse cellular processes such as cellular growth, proliferation and transformation (Ras) (Barbacid, 1987), cytoskeletal organization (Rac and Rho) (Ridley and Hall, 1992; Ridley *et al.*, 1992) and vesicular transport (Rab).

1.3.1 General Features of Protein Prenylation and Processing

The process of protein prenylation encompasses three distinct reactions: (1) prenylation of a specific C-terminal cysteine residue by a farnesyl (C15) or geranylgeranyl (C20) isoprenoid, (2) proteolytic removal of amino acids downstream of the prenylcysteine to expose its α carboxyl and (3) carboxymethylation of the exposed prenylcysteine α carboxyl (Zhang and Casey, 1996).

The enzymology of prenylation is well characterized. Prenylation begins in the cytoplasm with the transfer of farnesyl or one or more geranylgeranyl moieties to proteins bearing the appropriate consensus sequence (Table 1.1). This is

catalyzed by the soluble enzymes farnesyl protein transferase (FTase) or geranylgeranyl protein transferase Type I or II (GGTase I or II) (Zhang and Casey, 1996; Sinesky, 2000). Most prenylated mammalian proteins are geranylgeranylated (Rilling *et al.*, 1990; Farnsworth *et al.*, 1990). Following prenylation, proteins associate with endoplasmic reticulum membranes where the last two processing reactions, C-terminal proteolysis and carboxymethylation, take place. Some prenylated proteins may then acquire a "second signal" required for stable membrane association. The second signal is found upstream of the prenylcysteine and is defined as either one or more palmitoylated cysteine residues or a stretch of proximal basic residues which form a polybasic domain (Schafer and Rine, 1992; Zhang and Casey, 1996). The fully processed and modified proteins associate with various endomembranes where they mediate a number of different cellular processes.

1.3.2 Farnesylation and Geranylgeranylation

Farnesylation and geranylgeranylation occur on the cysteine residue found within the C-terminal Ca_1a_2X motif, the general consensus sequence directing protein prenylation (Table 1.1). In this motif, the C=cysteine, a=aliphatic amino acids and X=specific amino acids whose identity determines the nature of the prenyl transferred. If X is aspartate, glutamine, methionine, serine or cysteine, the protein is a substrate for FTase. If X is leucine or phenylalanine, the protein is a substrate for GGTase I; the geranylgeranylation consensus sequence is often called a CaaL box for this reason (Yokoyama *et al.*, 1991; Moores *et al.*, 1991; Casey *et al.*, 1991; Reiss *et al.*, 1990). In addition, the a_1 position has a more relaxed amino acid specificity, while the a_2 position is more restrictive (Moores *et al.*, 1991; Reiss *et al.*, 1991a). The minimum sequence requirement for

prenylation is a CaaX tetrapeptide, and the conserved CaaX cysteine is essential for prenylation, as mutation of this residue to serine abolishes prenylation (Clarke, 1992; Glomset and Farnsworth, 1994). However, sequences upstream of the CaaX motif may influence the specificity of prenylation. This has been demonstrated for G protein γ subunits (Kalman *et al.*, 1995) and for RhoB, which is farnesylated or geranylgeranylated depending on the presence or absence of upstream cysteines (Adamson *et al.*, 1992). The prenyl moiety is covalently bound to the appropriate cysteine residue by a stable thioether bond, which is considered irreversible under physiological conditions (Casey *et al.*, 1989) (Figure 1.1).

The soluble zinc metalloenzymes FTase and GGTase I catalyze the transfer of farnesyl or geranylgeranyl from the cholesterol biosynthesis intermediates farnesyl pyrophosphate or geranylgeranyl pyrophosphate (Figure 3.1) to the CaaX protein (Reiss *et al.*, 1990; Moores *et al.*, 1991; Moomaw and Casey, 1992; Yokoyama *et al.*, 1993). The mammalian enzymes are heterodimers, consisting of an α and β subunit. The α subunits of these enzymes are identical (Zhang *et al.*, 1994) but the β subunits, which bind both the protein and prenyl substrate, demonstrate limited identity (30%)(Reiss *et al.*, 1991b; Ying *et al.*, 1994). While FTase and GGTase I are selective for their respective substrates *in-vitro*, some cross-specificity has been reported for K-Ras4B (James *et al.*, 1995), N-Ras (Whyte *et al.*, 1997) and RhoB (Armstrong *et al.*, 1995).

Geranylgeranylation may also occur on both cysteine residues within C-terminal CC or CxC motifs found exclusively in the Rab family of G-proteins (Table 1.1) (Moores *et al.*, 1991; Horiuchi *et al.*, 1991; Seabra *et al.*, 1992a).

Digeranylgeranylation has also been reported to occur on Rab proteins ending in CCXX or CCXXX motifs (Glomset and Farnsworth, 1994). The GGTase II enzyme catalyzes the transfer of geranylgeranyl to both cysteines of the CC and CxC motifs in a single cycle of the reaction (Farnsworth *et al.*, 1991; Farnsworth *et al.*, 1994). These reactions are mechanistically distinct from those prenylating CaaX proteins (Horiuchi *et al.*, 1991; Seabra *et al.*, 1992a). However, only those proteins terminating in the CxC motif are carboxymethylated, while those terminating in CC are not (Smeland *et al.*, 1994). Recognition of substrate proteins by GGTase II requires prior binding of the CC or CxC protein to an escort protein termed Rep1 (Rab escort protein) (Seabra *et al.*, 1992a; Seabra *et al.*, 1992b). Rep1 binds and presents the unprenylated protein to the GGTase II dimer (Andres *et al.*, 1993). Rep1 recognizes and binds sequences upstream of the CC or CxC motif within the three-dimensional context of the substrate protein, therefore Rep1 will not bind short CC or CxC peptides (Seabra *et al.*, 1992a; Beranger *et al.*, 1994). Consequently, GGTase II will not prenylate any short peptides or truncated chimeric proteins containing these prenylation motifs (Kinsella *et al.*, 1992; Khosravi-Far *et al.*, 1992).

1.3.3 Endoproteolysis of the –aaX Tripeptide

Following prenylation, the –aaX tripeptide downstream of the prenylcysteine is cleaved. Endoproteolysis is believed to occur at the cytoplasmic surface of the endoplasmic reticulum. The reaction requires a prenylated substrate and results in exposure of the prenylcysteine α carboxyl group and liberation of the –aaX tripeptide (Zhang and Casey, 1996; Ashby, 1998). Endoproteolysis may ultimately facilitate closer association between the prenyl group and cellular membranes (Ashby, 1998).

Endoprotease activity was initially isolated from canine, bovine and rat microsomal membranes using short prenylated peptides and *in-vitro* expressed Ras as substrates (Hancock *et al.*, 1991b; Ashby *et al.*, 1992; Ma and Rando, 1992; Jang *et al.*, 1993). Endoproteolysis required that the substrate possessed an unesterified C-terminus, amino acids in the L-configuration and a minimum prenyl cysteine dipeptide sequence to enable proteolysis (Ma and Rando, 1992; Ma *et al.*, 1992). The first CaaX endopeptidases isolated were integral membrane proteins from *S.cerevisiae*. Afc1p , or Ste24p (a-factor converting enzyme) and Rce1p (Ras and a-factor converting enzyme) were CaaX prenyl proteases which localized to the endoplasmic reticulum but shared no homology and demonstrated differences in substrate specificities (Schmidt *et al.*, 1998; Boyartchuk *et al.*, 1997; Fujimura-Kamada *et al.*, 1997). Human homologs of both Afc1p and Rce1p have also been isolated (Otto *et al.*, 1999; Kumagai *et al.*, 1999). The human Rce1p homolog hRce1 proteolyzes both farnesylated and geranylgeranylated substrates (Otto *et al.*, 1999), but the human Afc1p homolog HsSte24p remains relatively uncharacterized.

1.3.4 Carboxymethylation of the Prenylcysteine α Carboxyl

The proteolytic exposure of the prenylcysteine α carboxyl group enables the last step in processing, carboxymethylation. A single carboxymethyltransferase enzyme appears to catalyze carboxymethylation of CaaX prenyl proteins. The CaaX carboxymethyltransferase enzyme has been isolated from *S.cerevisiae* (Ste14p) (Sapperstein *et al.*, 1994), *Schizosaccharomyces pombe* and *Xenopus laevis* (Imai *et al.*, 1997), and human cells (pcCMT; prenylcysteine carboxyl methyltransferase) (Dai *et al.*, 1998). In contrast, the CxC carboxymethyltransferase reaction has not been extensively studied. Using

enzyme assays, subcellular fractionation and immunofluorescence and confocal microscopy, Ste 14p and pcCMT were shown to localize to the endoplasmic reticulum (Stephenson and Clarke, 1990; Romano *et al.*, 1998; Dai *et al.*, 1998). The carboxymethylation reaction is therefore believed to occur on the cytoplasmic face of the endoplasmic reticulum. However, mammalian pcCMT was additionally localized to the Golgi apparatus and nuclear envelope by confocal microscopy (Dai *et al.*, 1998).

The carboxymethylation reaction utilizes S-adenosyl-L-methionine (SAM) as the methyl donor, and even short peptides may serve as substrates. Carboxymethylation requires prior farnesylation of the protein or peptide substrate (Stephenson and Clarke, 1990; Hrycyna *et al.*, 1991), and both farnesyl and geranylgeranyl prenyl protein substrates are recognized equally well (Tan *et al.*, 1991). While the methylester bond formed by prenylcysteine methylation is stable, reports of reversible lamin B methylation (Chelsky *et al.*, 1987) and detection of methylesterase activity in cellular membranes (Tan and Rando, 1992) suggest this modification is potentially reversible.

Carboxymethylation contributes to the overall hydrophobicity of prenyl proteins primarily by neutralizing the ionized C-terminal carboxylate (Silvius and L'Heureux, 1994; Shahinian and Silvius, 1995). Methylation of the prenylcysteine is estimated to increase hydrophobicity by two log units (Black, 1992). Consequently, membrane association is also enhanced. The effect of carboxymethylation on prenyl peptide membrane binding is more pronounced with farnesylated peptides as compared to geranylgeranylated peptides (Silvius and L'Heureux, 1 994; Shahinian and Silvius, 1995). This is due to the greater

hydrophobicity imparted by the geranylgeranyl isoprenoid as compared to the farnesyl isoprenoid (Shahinian and Silvius, 1995; Epand, 1997).

In conclusion, each processing step in protein prenylation contributes substantially to the hydrophobicity and membrane association of a prenyl protein (Figure 1.2) (Gutierrez *et al.*, 1989; Silvius and L'Heureux, 1994; Epand *et al.*, 1993). However, other protein modifications such as ADP-ribosylation (Kuribara *et al.*, 1995), phosphorylation (Bailly *et al.*, 1991) and acylation may antagonize or enhance the membrane-binding function of prenylation (Giannakouros and Magee, 1992). In particular, palmitoylation of a number of prenyl proteins acts as a complementary "second signal" required for stable membrane association.

1.4 Role of the Second Signal in Prenyl Protein Membrane Association

Because of their branched and unsaturated nature, farnesyl and geranylgeranyl isoprenoids respectively impart hydrophobicities equivalent to 11- or 14-carbon saturated fatty acyl chains (Shahinian and Silvius, 1995; Epand, 1997). As a result, protein modification by a single isoprenoid would not be predicted to enable stable membrane association (Silvius and L'Heureux, 1994). It has long been established that stable membrane association of prenyl proteins requires a second membrane binding signal upstream of the prenylcysteine (Hancock *et al.*, 1990). As with myristoylated proteins (Section 1.2.1), this "second signal" is defined as either one or more palmitoylated cysteine residues, or a stretch of proximal basic amino acids forming a polybasic domain (Cadwallader *et al.*, 1994; Hancock *et al.*, 1990). In theory, palmitoylation alone is sufficient to enable sustained membrane binding (Shahinian and Silvius, 1995). Therefore,

palmitoylation effectively complements prenylation in anchoring prenyl proteins within cellular membranes.

While CaaX-directed prenylation and processing occurs in the cytoplasm and on the endoplasmic reticulum surface, palmitoylation of prenyl proteins has been suggested to occur at the plasma membrane (Schroeder *et al.*, 1996; Schroeder *et al.*, 1997; Dunphy *et al.*, 1996) within Golgi membranes (Solimena *et al.*, 1994), or on endoplasmic reticulum (ER) or ER-Golgi intermediate membranes (Bonatti *et al.*, 1989; Kasinathan *et al.*, 1990; Veit and Schmidt, 1993; Apolloni *et al.*, 2000). A "kinetic membrane trapping" model of protein palmitoylation has been proposed to explain how prenylated or myristoylated proteins acquire the palmitate second signal (Shahinian and Silvius, 1995). In this model, a prenylated or myristoylated protein may diffuse through the cytoplasm and "sample" various endomembranes until it encounters an appropriate "membrane-targeting receptor". This receptor may be PAT, the enzyme believed responsible for protein palmitoylation (Section 1.2.2). Once palmitoylated, the protein is stably anchored in the membrane where the palmitate second signal was acquired, potentially the plasma membrane. Alternatively, if palmitoylation occurred within Golgi membranes, the palmitate second signal might direct the vectorial, vesicular transport of the protein to the plasma membrane (Choy *et al.*, 1999; Apolloni *et al.*, 2000). The palmitate moiety is bound to cysteine by a thioester bond, which may reversible *in-vivo*, enabling the dynamic association of palmitoylated prenyl proteins with the plasma membrane (Milligan *et al.*, 1995; Mumby, 1997). Prenylated proteins incorporating a palmitate second signal include H-Ras, N-Ras and K-Ras4A (Hancock *et al.*, 1990), paralemmin (Kutzleb *et al.*, 1998), Rap2 (Beranger *et al.*, 1991a) and RhoB (Adamson *et al.*,

1992).

The presence of a polybasic domain upstream of the prenylcysteine also functions as an effective second signal (Hancock *et al.*, 1990). Myristoylated proteins may also utilize a polybasic second signal for stable membrane binding (Section 1.2.1). This type of second signal is intrinsic to the myristoylated or prenylated protein and is therefore present before lipidation occurs. The polybasic domain enhances membrane binding through electrostatic interaction with the negative head groups of membrane phospholipids (Murray *et al.*, 1997; Silvius and L'Heureux, 1994; Black, 1992). This synergistic combination of hydrophobic and electrostatic forces is sufficient to enable sustained membrane association (Epand, 1997). For example, the six basic amino acids comprising the Src tyrosine kinase second signal enhance binding to acidic-phospholipid-containing membranes 3000-fold (Buser *et al.*, 1994; Sigal *et al.*, 1994). Other studies involving the binding of K-Ras4B C-terminal peptides to lipid vesicles have demonstrated that electrostatic forces actually contributed more to K-Ras4B membrane binding than hydrophobic forces (Leventis and Silvius, 1998). Plasma membrane binding of K-Ras4B may not be mediated by a kinetic trapping mechanism. Instead, the polybasic domain has been suggested to function as a membrane surface-potential sensor (Leventis and Silvius, 1998). It remains to be determined whether the surface-potential sensor model applies to all lipidated proteins utilizing a polybasic second signal.

A novel myristate second signal may potentially be found in the farnesylated protein CLN3 (ceroid lipofuscinosis) (Pullarkat and Morris, 1999; Kaczmarski *et al.*, 1999). A 1.02 kb deletion within the gene encoding this 48 kDa protein

results in Batten disease, a primarily jeuvenile disease characterized by the lysosomal accumulation of lipopigments, which results in neurodegenerative disorders. CLN3 is predicted to have 5-10 transmembrane domains, as well as N-terminal myristoylation and C-terminal prenylation consensus sequences (Kaczmarski *et al.*, 1999). Although farnesylation of this protein was shown to occur *in-vitro* (Pullarkat and Morris, 1999), myristoylation has yet to be demonstrated. While the precise subcellular distribution of this protein has not been clearly elucidated, it appears to traffic through the secretory system to the plasma membrane (Haskell *et al.*, 1999). While the function of CLN3 lipidation is unclear, it remains an interesting and novel example of a prenylated, potentially myristoylated protein.

1.5 Functions of Prenylated Proteins

Prenylation and subsequent modifications of proteins facilitates their association with various endomembranes such as the plasma membrane and Golgi apparatus (Muntz *et al.*, 1992; Beranger *et al.*, 1991b), endosomes (Pizon *et al.*, 1994), peroxisomes (James *et al.*, 1994) and mitochondria (Rebollo *et al.*, 1999). This membrane association is necessary for the ultimate function of prenyl proteins, which mediate a number of diverse processes including signal transduction (Ras) (Barbacid, 1987), cytoskeletal organization and cellular morphology (Rac, Rho and paralemmin) (Ridley and Hall, 1992; Ridley *et al.*, 1992; Kutzleb *et al.*, 1998), and nuclear envelope structure (prelamin A and lamin B) (Lutz *et al.*, 1992; Holtz *et al.*, 1989; Farnsworth *et al.*, 1990). Prenylated proteins also function in vesicular transport (Rab) (Rodman and Wandinger-Ness, 2000), protein folding (Caplan *et al.*, 1992) and viral replication (Glenn *et al.*, 1992). Prenylation is also required for certain protein-protein interactions. Examples of

this include the interaction of Rab protein with guanine nucleotide dissociation inhibitor (Musha *et al.*, 1992), of *S.cerevisiae* a-factor mating pheromone with the STE3 receptor (Marcus *et al.*, 1991) and of K-Ras4B with tubulin *in-vitro* (Thissen *et al.*, 1997).

A large proportion of isoprenoid-modified proteins belong to the Ras superfamily of GTP-binding proteins (Figure 1.2). Table 1.2 lists a number of these and other prenylated proteins and their functions. Among the most well-characterized of the lipidated Ras superfamily proteins are the N-Ras, H-Ras, and K-Ras4B members of the Ras subfamily (Figure 1.2).

1.6 p21Ras GTP-binding Proteins

The three major isoforms of the 21 kDa GTP-binding p21Ras protein, designated N-Ras, H-Ras, and K-Ras4B, are expressed in virtually all tissues (Furth *et al.*, 1987; Lowy and Willumsen, 1993). The major isoforms are highly homologous proteins which are encoded by genes highly conserved throughout evolution, and may function in heterologous systems (Shilo and Weinberg, 1981; Defeo-Jones *et al.*, 1985; Kataoka *et al.*, 1985). The *ras* cellular proto-oncogenes were originally identified as the wild-type homologues of mutant transforming retroviral *ras* oncogenes v-H-*ras* and v-K-*ras* (Coffin *et al.*, 1981; Ellis *et al.*, 1981). These oncogenes were named for the Harvey and Kirsten murine sarcoma viruses they were initially isolated from, but no viral homologue has been shown to exist for cellular N-Ras. The *ras* genes include four coding exons; the encoded N-Ras and H-Ras proteins are 189 amino acids in length, while the K-Ras4B isoform is 188 amino acids because the fourth exon has one less codon than the N-Ras and H-Ras fourth codons (Lowy and Willumsen,

1993). The K-*ras* gene has two alternative fourth coding exons, A and B (Shimizu *et al.*, 1983). Alternative splicing of the fourth exon results in expression of K-Ras4A or K-Ras4B, which differ only in their C-terminal sequences. K-Ras4A is the form expressed in the viral oncogene (v-K-*ras*), and K-Ras4B is the proto-oncogenic form ubiquitously expressed in mammalian tissues (Capon *et al.*, 1983). The N-terminal sequences of the N-Ras, H-Ras and K-Ras4B isoforms are nearly identical, but their C-terminal 25 amino acids are highly divergent and are known as the "hypervariable domain" (Lowy and Willumsen, 1993). The major isoforms are variably lipidated within the hypervariable domain, which facilitates their differential interaction with cellular membranes (Hancock *et al.*, 1989; Hancock *et al.*, 1990; Hancock *et al.*, 1991a; Choy *et al.*, 1999; Apolloni *et al.*, 2000).

The p21Ras proteins mediate the transduction of a variety of extracellular signals by coupling phosphorylated transmembrane receptors, which receive the signal, to various intracellular effectors (Figure 1.3) (Pronk and Bos, 1994; Denhardt, 1996). As a result, p21 Ras mediates multiple signalling pathways. Upon receptor activation and autophosphorylation, recruitment of adaptor and activator proteins to the plasma membrane activates Ras by stimulating Ras GTP binding (Figure 1.3). In response to a given signal, Ras-GTP recruits specific effector proteins to the plasma membrane. Genetic and biochemical evidence has identified Raf, phosphatidylinositol-3 kinase (PI-3K) and RalGEF (guanine nucleotide exchange factor) as genuine Ras effectors (Katz and McCormick, 1997). These effectors propagate the signal by initiating signalling cascades, ultimately generating specific cellular responses (Figure 1.3). Ras signalling is downregulated upon hydrolysis of bound GTP to GDP by intrinsic Ras GTPase

activity, which is stimulated by the GTPase-activating proteins p120 GAP or neurofibromin 1 (Figure 1.3) (Pronk and Bos, 1994).

An important function of wild-type Ras proteins is the mediation of cellular growth and proliferation. Single amino acid substitutions, particularly at residues 12, 13 or 61, result in defective GTPase activity and constitutive Ras activation (Barbacid, 1987). The physiological consequence is cellular transformation, characterized by uncontrolled cell growth and, potentially, tumor formation. In order to mediate cellular growth and other signalling pathways, the Ras isoforms must associate with the plasma membrane, where the transmembrane receptors reside. Plasma membrane association is mediated by differential post-translational lipid modification of the major isoforms within their C-terminal hypervariable domains (Table 2.1).

The major Ras isoforms were initially thought to be largely redundant in function as a result of the high degree of homology within their N-terminal sequences. Several lines of evidence now suggest that the isoforms may have unique roles, which may be mediated in part by the highly divergent, variably lipidated hypervariable domains. For example, only the K-Ras4B isoform has been shown to be essential for mouse embryogenesis (Umanoff *et al.*, 1995; Johnson *et al.*, 1997), and H-Ras is the only isoform activated by Ras-GRF (Ras guanine nucleotide release factor) *in-vivo* (Jones and Jackson, 1998). In addition, the isoforms vary in their ability to activate Raf-1 and phosphatidylinositol 3-kinase effectors (Yan *et al.*, 1998), and appear to regulate MAP kinase (mitogen-activated protein kinase) activity by distinct mechanisms *in-vivo* (Hamilton and Wolfman, 1998). In at least one example, the unique modifications within the K-

Ras4B hypervariable domain are believed to be responsible for its association with SmgGDS, a guanine nucleotide exchange factor (Kawamura *et al.*, 1993; Mizuno *et al.*, 1991). SmgGDS translocates small G proteins, including K-Ras4B, from the membrane to the cytoplasm, which may allow interaction of this isoform with disinct effectors not shared by the other isoforms.

1.7 Ras Lipidation

Early studies of Ras initially localized it to the plasma membrane (Willingham *et al.*, 1980) and this association is essential for Ras function (Willumsen *et al.*, 1984a; Willumsen *et al.*, 1984b). However, recent studies of Ras trafficking have revealed that a significant proportion of Ras is also associated with distinct intracellular membranes (Choy *et al.*, 1999; Apolloni *et al.*, 2000). Ras mRNA is initially translated on free ribosomes, producing the soluble 21.5 kDa Ras precursor designated cytoplasmic p21 (c-p21) (Shih *et al.*, 1982; Gutierrez *et al.*, 1989). Prenylation, proteolysis and carboxymethylation produces the 21 kDa intermediate form of c-p21. This c-p21 intermediate becomes palmitoylated (on H-Ras, N-Ras and K-Ras4A) to become the mature, membrane-associated membrane-p21 (m-p21) (Grand *et al.*, 1987; Gutierrez *et al.*, 1989). It is the fully lipid-modified, processed form of m-p21 which stably associates with the plasma membrane to mediate cellular signalling.

The post-translational incorporation of tritiated palmitate onto H-Ras by an alkali sensitive linkage was the first indication that p21 Ras was covalently lipidated (Sefton *et al.*, 1982; Buss and Sefton, 1986). Because earlier studies had determined that H-Ras Cys186 was essential for fatty acylation, membrane association and transformation (Willumsen *et al.*, 1984a; Willumsen *et al.*,

1984b), it was generally accepted that Ras was palmitoylated at Cys186. However, a number of other studies soon provided contrary evidence proving that Ras was in fact prenylated on Cys186, and processed in a fashion similar to the *Tremella* fungal mating peptides and the *S.cerevisiae* a-factor (see Section 1.3). The first of these studies independently established that the mammalian N-Ras –aaX tripeptide was proteolysed, and that H-Ras was carboxymethylated at the C-terminus (Gutierrez *et al.*, 1989; Clarke *et al.*, 1988). This provided the first suggestion that the common CaaX motifs found in mammalian H-Ras, a-factor and the known prenylated *Tremella* peptides may similarly direct prenylation in addition to the observed proteolysis and methylation of these proteins.

Within a year of these studies, several reports emerged which clearly demonstrated that the major Ras isoforms were farnesylated on Cys186, and that palmitoylation occurred on upstream cysteines in the H-Ras, N-Ras and K-Ras4A isoforms, but not in the K-Ras4B isoform (Hancock *et al.*, 1989; Casey *et al.*, 1989). This was supported by kinetic studies of *S.cerevisiae* RAS palmitoylation, which suggested that this modification was a late event in RAS processing (Tamanoi *et al.*, 1988). Ultimately, it was established that all Ras isoforms were farnesylated on Cys186 and required a second membrane binding signal to anchor them in the plasma membrane (Hancock *et al.*, 1989; Hancock *et al.*, 1990).

N-Ras, K-Ras4A and H-Ras incorporate a palmitate second signal upstream of the prenylcysteine only after the prerequisite farnesylation. N-Ras is palmitoylated on Cys181, K-Ras4A on Cys180 and H-Ras on Cys181 and 184.

K-Ras4B contains no cysteines upstream of the prenylcysteine and is not palmitoylated; instead, a polybasic region functionally substitutes for palmitate as the second signal (Hancock *et al.*, 1989; Casey *et al.*, 1989). The palmitate moieties on N-Ras and H-Ras turn over with a half-life respectively estimated at 20 and 90 minutes, which may enable both dynamic membrane association and regulation of function (Magee *et al.*, 1987; Lu and Hofmann, 1995). Establishment of Ras lipid modifications enabled more detailed examinations of the role of variable Ras lipidation on membrane association, function and very recently, trafficking of the Ras isoforms to the plasma membrane.

1.8 The Role of Prenylation and the Second Signal in Ras Membrane Association, Function and Trafficking

Although farnesylation and subsequent proteolysis and methylation of the Ras isoforms greatly increases their overall hydrophobicity, these modifications are not sufficient to enable sustained membrane association (Gutierrez *et al.*, 1989; Silvius and L'Heureux, 1994). Consequently, the concept of a polybasic or palmitate second signal directing Ras plasma membrane association was established (Hancock *et al.*, 1990; Hancock *et al.*, 1991a). Section 1.7 and Table 2.1 describe the specific second signals found within the major Ras isoforms.

1.8.1 Membrane Association

The presence of both the C-terminal CaaX motif and a palmitate or polybasic second signal are absolutely required for efficient Ras membrane association, specifically plasma membrane association. Farnesylation and the second signal synergize to localize approximately 90% of each isoform to cellular membranes. When appended to heterologous reporter proteins, the Ras hypervariable domains

localize these otherwise soluble proteins to the plasma membrane (Hancock *et al.*, 1991a; Choy *et al.*, 1999; Stokoe *et al.*, 1994).

Mutation of the Ras CaaX cysteine to serine inhibits prenylation, processing and consequently, palmitoylation of N-Ras and H-Ras, resulting in soluble, non-transforming Ras proteins (Hancock *et al.*, 1990; Hancock *et al.*, 1991a). The second signal is specifically required for sustained plasma membrane association of Ras, as determined by immunofluorescence and confocal microscopy (Hancock *et al.*, 1990; Hancock *et al.*, 1991a). Abrogation of the second signal does not affect farnesylation and processing but results in soluble Ras proteins which, surprisingly, are capable of inducing diminished but significant levels of transformation in NIH 3T3 cells despite a lack of plasma membrane association (Hancock *et al.*, 1989; Hancock *et al.*, 1990; Hancock *et al.*, 1991a). Recent studies indicate that prenylation alone suspends mutant Ras on endoplasmic reticulum and Golgi membranes, and suggests that the second signal allows the exit of Ras from the endomembrane system and delivery to the plasma membrane (Choy *et al.*, 1999; Apolloni *et al.*, 2000). Progressive mutation of each of the six contiguous lysine residues comprising the K-Ras4B polybasic second signal to neutral glutamine residues results in a parallel increase in the solubility of each mutant protein. A minimum of three lysine residues must remain to maintain any plasma membrane association; mutation of five or six of the lysine residues produces mutants which are almost entirely soluble (Hancock *et al.*, 1990). Replacement of the polybasic domain lysine residues with arginine does not affect plasma membrane association or transforming ability (Hancock *et al.*, 1991a). Similarly, conservative mutation of the palmitoylated cysteine residues to serine within the N-Ras and H-Ras second signals produces

farnesylated, soluble mutant proteins. Mutation of either of the two palmitoylated cysteines in the H-Ras second signal (Cys181 and Cys184) results in variable membrane association of these mutants (Hancock *et al.*, 1989). Upon cell fractionation, the H-RasC181S mutant demonstrates a greater association with the P100 fraction as compared with the H-RasC184S mutant. Metabolic labelling of these mutants with radiolabelled palmitate shows that the C181S mutant labels to 70% of wild-type H-Ras levels while the C184S mutant labels to 40% of wild-type levels (Hancock *et al.*, 1989). These studies demonstrate that while specific plasma membrane association of Ras requires both farnesylation and a second signal, cellular transformation only requires farnesylation.

In addition to farnesyl and palmitate, the amino acid residues surrounding the lipidated cysteine residues within the Ras hypervariable domains have been suggested to contribute targeting information (Willumsen *et al.*, 1996). Willumsen and coworkers concluded that mutation of residues adjacent to palmitoylated cysteines within H-Ras redirected the mutant proteins from the plasma membrane to internal membranes and abolished transformation. However, the mutations were engineered into a H-RasC181S mutant which lacks one of the two palmitates comprising the second signal and is compromised for efficient plasma membrane association. Furthermore, the mutations introduced negatively charged amino acids into the hypervariable domain, close to the prenylcysteine and palmitoylated cysteine residues, which may act to repel the mutant protein from the plasma membrane. Therefore, a series of more conservative mutations within the wild-type hypervariable domain may address the role of intervening residues more clearly.

1.8.2 Function

Ras plasma membrane association does not strictly require the native combination of farnesyl and a palmitate/polybasic second signal *per se*. Ras transforming ability and plasma membrane association may be promoted by either farnesylation or geranylgeranylation in combination with the second signals. Geranylgeranylation of K-Ras4B and H-Ras has been demonstrated by substituting a CaaL motif for the native CaaX motifs (Hancock *et al.*, 1991a; Cox *et al.*, 1992). In addition, K-Ras4B has been shown to be alternately geranylgeranylated in cells treated with farnesyltransferase inhibitor (Rowell *et al.*, 1997), and K-Ras4A, K-Ras4B and N-Ras are all substrates for geranylgeranyltransferase *in-vitro* (Zhang *et al.*, 1997). While geranylageranylation of Ras is less efficient than farnesylation, geranylgeranylated p21Ras bind to membranes with greater avidity and induce comparable levels of tranformation (Hancock *et al.*, 1991a; Cox *et al.*, 1992). Subcellular fractionation of differentially prenylated Ras localized the geranylgeranylated form primarily to the P100 fraction, and the farnesylated form to the S100 fraction (Hancock *et al.*, 1991a). Specific plasma membrane localization of K-Ras4B is maintained by geranylgeranylation when combined with a second signal (Hancock *et al.*, 1991a). However, while geranylgeranylation retains the transforming ability of oncogenic H-Ras, it appears to inhibit the growth-promoting function of normal H-Ras (Cox *et al.*, 1992).

Alternative membrane binding signals have been utilized to elucidate the precise roles of farnesylation and the second signal in Ras plasma membrane association and function. These alternative signals include N-terminal myristoylation (Buss

et al., 1988; Lacal *et al.*, 1988; Buss *et al.*, 1989; Cadwallader *et al.*, 1994), N-terminal palmitoylation (Coats *et al.*, 1999), incorporation of an N-terminal transmembrane domain (Hart and Donoghue, 1997) and a polybasic C-terminal extension on H-Ras which abolishes prenylation but retains the upstream palmitoylation sites (Booden *et al.*, 1999). These studies established that all four alternative signals restored palmitoylation of the normally unmodified H-RasC186S mutant, which requires farnesylation of Cys186 before upstream palmitoylation can occur. They also established that, when combined with an authentic Ras palmitate or polybasic second signal in the absence of farnesylation, all alternative signals could facilitate plasma membrane association, and all but N-terminal palmitoylation induced potent transformation of transfected cells. However, the combination of N-terminal myristoylation or transmembrane signals with C-terminal farnesylation, in the absence of a second signal, inhibited transformation and mislocalized the myristoylated/farnesylated chimeras to intracellular membranes. Similarly, combined N- and C-terminal palmitoylation inhibits transformation. The combination of N-terminal palmitoylation and farnesylation was not studied (Coats *et al.*, 1999). Therefore, either C-terminal farnesylation/ polybasic extension, or N-terminal myristoylation/transmembrane sequences may effectively combine with a palmitate or polybasic second signal to facilitate cellular transformation as well as plasma membrane localization.

In the absence of native farnesylation and palmitoylation, Ras membrane association and transforming ability were restored by a transmembrane sequence but not by myristoylation alone. Prenylated Ras isoforms which lack the second signal retain a modest transforming activity; however, the additional presence of

any N-terminal modification (except palmitoylation) on these prenylated forms inhibits that activity. Combinations of specific N- and C-terminal modifications may create steric or conformational restrictions within the chimeric proteins which inhibits normal function. Alternatively, it has been suggested that the presence of a second signal is essential for p21Ras exit from the endomembrane system and delivery to the plasma membrane (Apolloni *et al.*, 2000). The main conclusions which may be drawn from the current collective data are that (1) the specific lipid modifications of p21Ras serve as general plasma membrane association signals which may be replaced by functionally equivalent signals, (2) that a specific combination of these signals is required for plasma membrane localization, and (3) the presence of a palmitate or polybasic signal is an essential determinant of plasma membrane localization. This implies that farnesyl and/or palmitate may not be specifically required for the interaction of p21Ras with activator or effector proteins, or with a recently reported putative H-Ras plasma membrane docking protein (Siddiqui *et al.*, 1998).

1.8.3 Trafficking

The variable lipidation of the major Ras isoforms has very recently been demonstrated to direct the differential trafficking of these isoforms to the plasma membrane (Choy *et al.*, 1999; Apolloni *et al.*, 2000). Utilizing full-length and truncated GFP-Ras chimeras, Choy and coworkers and Apolloni and coworkers demonstrated distinct differences in the subcellular distribution and delivery of the Ras isoforms based on the second signal that was present. When palmitate was the second signal, as in the N-Ras and H-Ras isoforms, the proteins were associated with the plasma membrane, Golgi apparatus and vesicular structures. Palmitoylated chimeras trafficked from the endoplasmic reticulum (ER) to the

Golgi, where they associated with motile, coalescing vesicles which delivered them to the plasma membrane. Brefeldin A (BFA) treatment or 15° temperature block inhibited delivery of newly synthesized chimeras to the plasma membrane and instead suspended them on ER and ER-Golgi intermediate compartment membranes. The motile vesicles were not recycling endosomes, but were determined to transport anterograde to the secretory pathway along linear tracks.

Conversely, when a polybasic domain was the second signal, as in the K-Ras4B isoform, the protein associated almost exclusively with the plasma membrane and showed limited, diffuse perinuclear localization. The polybasic domain appeared to divert protein delivery from the classical secretory pathway, as BFA treatment had no effect on GFP-K-Ras4B delivery to the plasma membrane. K-Ras4B had been previously shown to associate with microtubules in a prenylation-dependent fashion *in-vitro*, and taxol treatment of intact cells induced mislocalization of K-Ras4B, but not H-Ras (Thissen *et al.*, 1997). Apolloni and coworkers (2000) confirmed that taxol treatment of transfected cells greatly diminished the plasma membrane association of truncated GFP-K-Ras4B, but not truncated GFP-H-Ras. Despite this, they also determined that the GFP-K-Ras4B chimera remained exclusively associated with the P100 membrane fraction. Confocal analysis of these cells revealed that most of the GFP-K-Ras4B chimera was found in irregular structures unlike ER or Golgi in appearance. Colocalization studies indicated that these structures were not tubulin or microtubule bundles but did not identify them. Additional data indicating that the cytoplasmic half life of K-Ras4B was one-third that of N-Ras (Choy *et al.*, 1999) and that farnesyltransferase has a 50-fold higher affinity for

K-Ras4B than H-Ras *in-vitro* (James *et al.*, 1995) support the concept of differential trafficking of the Ras isoforms.

1.9 Thesis Objective

The objective of the current was to investigate the role of the variable lipid modifications within the major cellular Ras isoforms, N-Ras, H-Ras and K-Ras4B. Specifically, we sought to determine whether variable Ras lipidation could direct the differential endomembrane association of each isoform. The role of variable Ras isoform lipidation in membrane association has previously been extensively studied. Surprisingly, however, trafficking of the Ras isoforms to the plasma membrane was not. In addition to plasma membrane association, the differential, isoform-specific endomembrane associations which were observed in these same studies were not pursued. As a result, their relevance to Ras trafficking remained obscure, and until the recent work of Choy and coworkers (1999) and Apolloni and coworkers (2000), the trafficking of Ras isoforms to the plasma membrane remained uncharacterized. Therefore, the initial goal of this project was to determine whether variable lipid modification within the N-Ras, H-Ras and K-Ras4B hypervariable domains directed their differential membrane association.

To facilitate this, chimeric proteins were created which incorporated the last 14 amino acids from the hypervariable domains of these three isoforms onto the C-terminus of red-shifted green fluorescent protein (GFP). These 14 amino acids were sufficient to direct appropriate Ras-like lipid modification and subcellular localization of the chimeric proteins. Only hypervariable domain sequences were included to avoid potential contributions of other binding domains found in

p21Ras including guanine nucleotide binding domains, effector binding domains, and putative caveolin binding domains (Barbacid, 1987; Song *et al.*, 1996; Couet *et al.*, 1997).

Table 1.1 Summary of Covalent Lipid Modifications of Proteins.

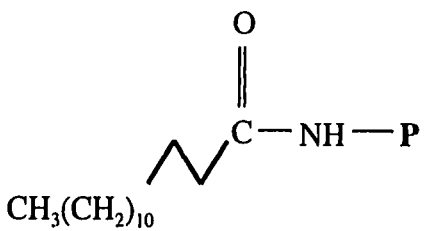
Modification	Localization	Consensus	Enzyme	Linkage	Timing
Acylation					
•Myristoylation	N-terminal glycine	MGXXXS/T	NMT	amide (irreversible)	cotranslational
•Palmitoylation	divergent cysteines	none	PAT	thioester (reversible)	posttranslational
Prenylation					
•Farnesylation	C-terminal cysteine	**C**AAX motif	FTase	thioether (irreversible)	posttranslational
•Geranylgeranylation	C-terminal cysteine	**C**AAL/F motif	GGTase I	thioether (irreversible)	posttranslational
	C-terminal cysteines	**CC** or **CxC**	GGTase II	thioether (irreversible)	posttranslational
Glypiation					
•Glycosyl phosphatidyl inositol	C-terminus	none	several	amide (irreversible)	cotranslational

Table 1.2 Examples of Isoprenylated Proteins and Their Functions.

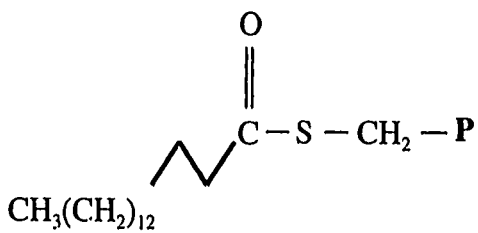
Farnesylated Proteins	**Function**
H-Ras N-Ras K-Ras4A K-Ras4B	Cellular growth and proliferation
Prelamin A Lamin B	Nuclear envelope structure
Rhodopsin kinase Transducin cGMP phosphodiesterase α	Retinal signalling
Paralemmin	Cellular morphology
Rhodotorucine A a-factor 1 mating pheromone	Fungal mating *S.cerevisiae* mating
Geranylgeranylated Proteins	**Function**
Rap1A Rap1B	Negative growth control
Rac1 Rac2	Membrane ruffling
RhoA RhoC	Stress fiber formation
Rab1A Rab1B Rab2	Vesicular transport
cGMP phosphodiesterase β	Retinal signalling
Heterotrimeric G-protein γ subunits	Signal transduction

Acylation

Myristoylation (amide bond)

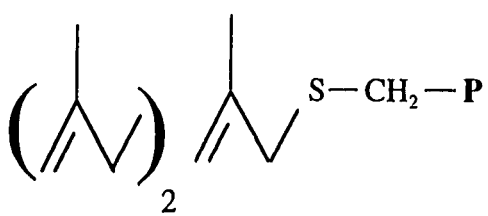


Palmitoylation (thioester bond)



Prenylation

Farnesylation (thioether bond)



Geranylgeranylation (thioether bond)

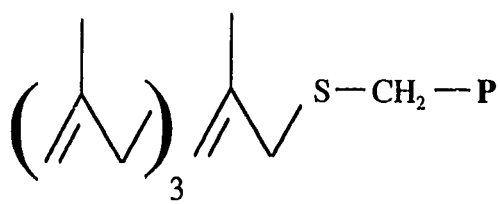


Figure 1.1 Covalent Linkages of Acyl Groups and Isoprenoids to the Protein Backbone.
The **P** in each individual figure represents the modified amino acid residue within the context of the protein backbone.

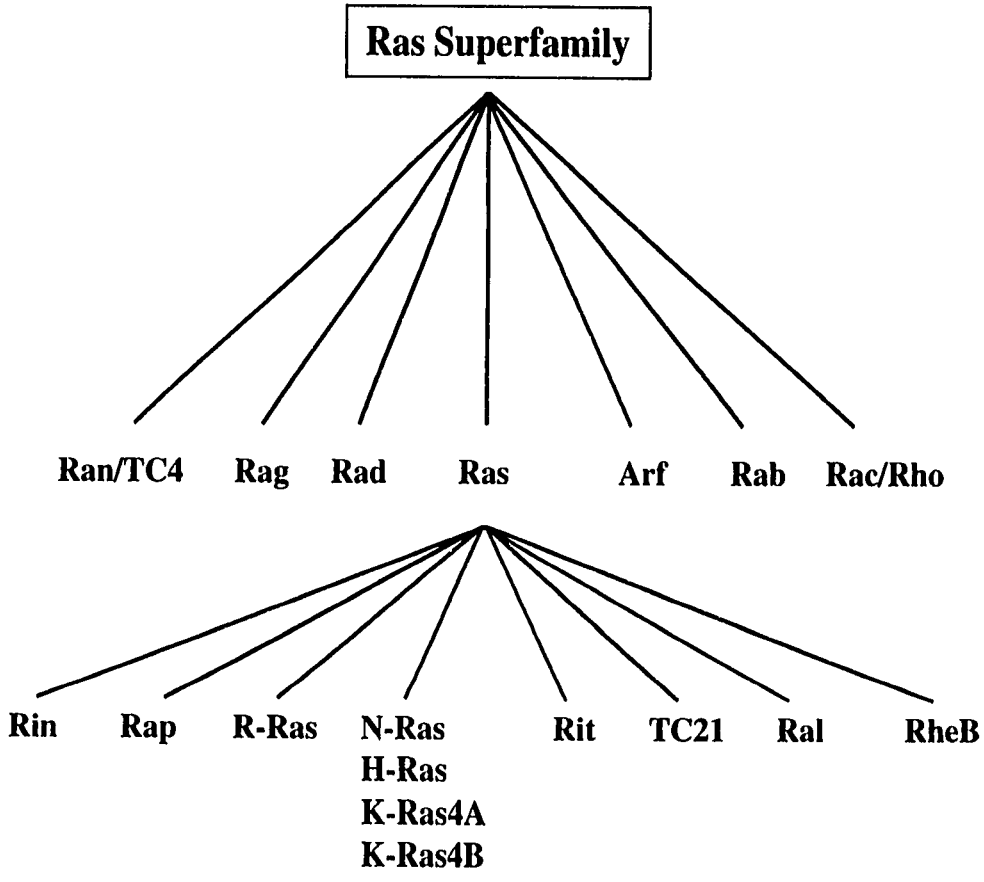


Figure 1.2 The Ras Superfamily of Small GTP-binding Proteins. Adapted from Herrmann and Nassar (1996) and Bos (1997).

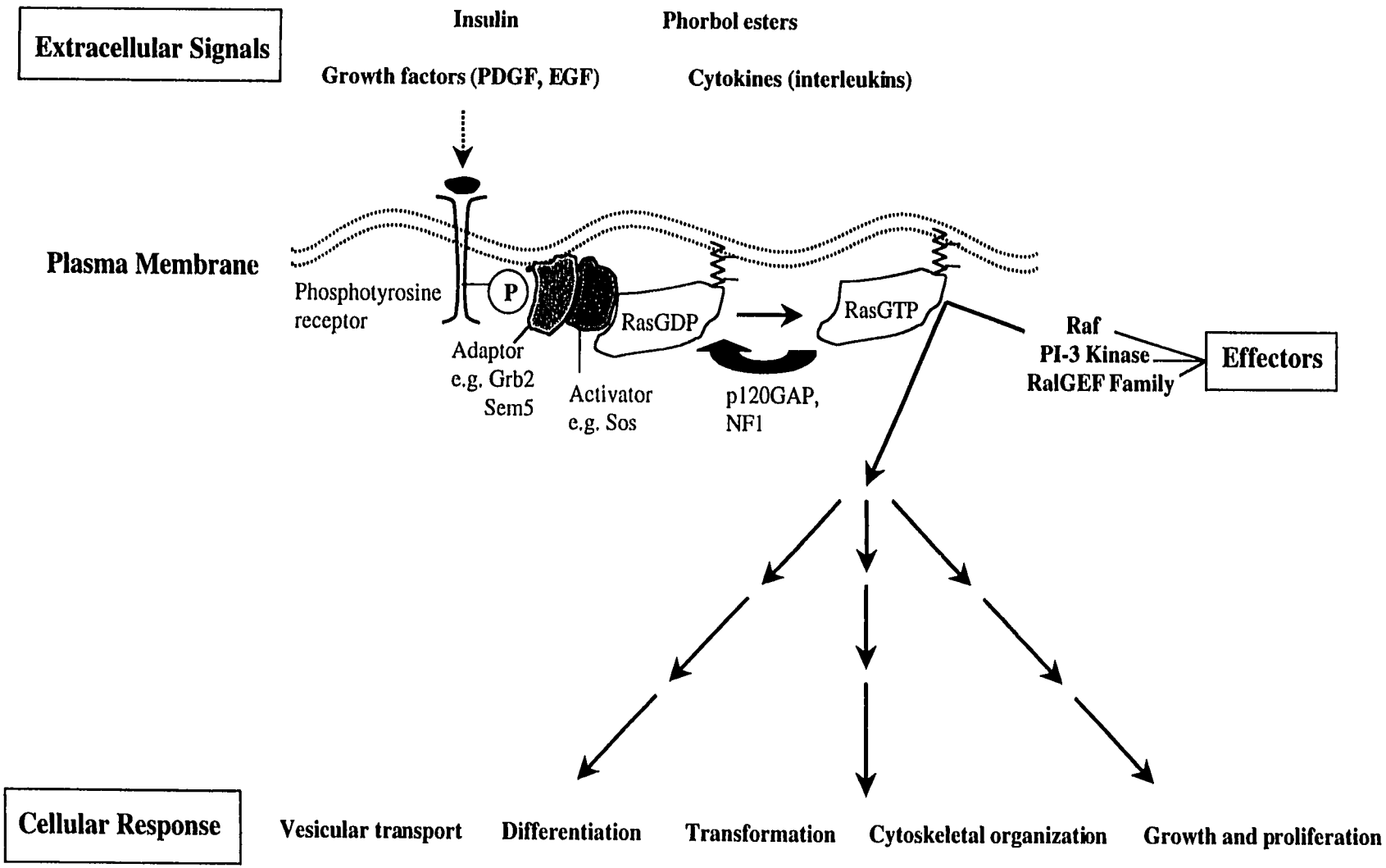


Figure 1.3 Multiple Ras-Mediated Pathways in Response to Various Extracellular Signals.
Adapted from Denhardt (1996) and Katz and McCormick (1997).

2.0 MATERIALS AND METHODS

2.1 Cell Lines, Media and Culture Conditions

COS-7 cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in maintenance medium (Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS; v/v), 100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, all purchased from Life Technologies). Cells were passed using trypsin-EDTA (0.25% trypsin, 1 mM ethylenediaminetetraacetate (EDTA); Life Technologies). COS-7 cells were maintained at 37°C, 5 % CO_2 humidified atmosphere and were passed every three to four days until passage 16, at which time they were discarded. To prepare frozen stocks, sufficient trypsin-EDTA was added to each confluent 100 mm dish to cover the monolayer, then aspirated. Dishes were incubated at 37°C until cell sloughing began; 1 ml freezing media (70 % DMEM, 20 % FBS, 10 % dimethylsulfoxide) was added to resuspend the cells and the entire volume was aliquoted into a cryovial (Wheaton) and immediately frozen at -80°C. To thaw cells, vials were rapidly thawed in a 37°C waterbath, and contents were transferred to a 100 mm dish containing maintenance medium. Two passages were allowed before cells were utilized in experiments.

2.1.1 Bacterial Strains and Media

Escherichia coli DH5α (*supE*44Δ*lac*U169(θ80*lacZ*ΔM15)*hsdR*17*rec*A1*end*A1*gyr* A96*thi*-1*rel*A1; Hanahan, 1983) was obtained from laboratory stocks and grown in Luria-Bertani (LB) medium at 37°C (Sambrook *et al.*, 1989). Transformed *E. coli* DH5α was grown in LB medium supplemented with either 50 µg/ml kanamycin or 100 µg/ml ampicillin (Sigma). Solid media was prepared by the

addition of 1.5 % bacto-agar (Difco). Frozen stocks of *E. coli* were prepared by adding sterile glycerol to the cell suspension to a final concentration of 15 %. Preparation of competent cells was as described by Hanahan (1983) with modifications. A 5 ml overnight starter culture of *E. coli* in SOB (Super Optimal Broth) medium was used to seed 500 ml of SOB and grown to OD_{600} 0.4-0.5. The culture was divided into two aliquots, centrifuged at 2600xg and the pellets each resuspended in 25 ml of cold 0.1 M $CaCl_2$. The cell suspensions were chilled on ice for 30 minutes, centrifuged at 2600xg for five minutes, and cell pellets were each resuspended in 5 ml 0.1 M $CaCl_2$/10 % glycerol. Competent cells were aliquoted and frozen at -80°C.

2.2 Molecular Biology

Unless otherwise stated, methods used were essentially as described in Sambrook *et al.* (1989).

2.2.1 Plasmids, DNA Isolation and Purification

The pRSGFP-C1 vector (Figure 2.1; Clontech, Palo Alto, CA) encodes a red-shifted variant of wild-type GFP (Prasher *et al.*, 1992; Inouye and Tsuji, 1994). RSGFP contains three amino acid substitutions; wild-type Phe-64 to Met, wild-type Ser-65 to Gly and wild–type Gln-69 to Leu (Delagrave *et al.*, 1995). The RSGFP variant produces a fluorescence which is four to six times more intense than that of wild-type GFP (GFP Application Notes, Clontech). An extensive multiple cloning site (MCS) has been engineered at the C-terminus of the RSGFP gene, allowing C-terminal fusions. This vector incorporates a SV40 origin of replication for mammalian cell propagation and expression, as well as f1 and pUC origins of replication for filamentous phage and bacterial

propagation, respectively. In addition, the cytomegalovirus (CMV) promoter, SV40 poly-adenylation signal and kanamycin/neomycin resistance gene are also incorporated (Figure 2.1).

The various engineered GFP-Ras fusion sequences were subcloned into the pCMV5 mammalian expression vector (Figure 2.1; Andersson *et al.*, 1989). This vector incorporates a SV40 origin of replication enabling propagation in SV40- and SV40 large T-transformed cell lines, and is especially efficient in COS cells. It also possesses a f1 origin of replication, the CMV promoter, the human growth hormone termination and poly-adenylation signals and an ampicillin resistance gene.

The pEGFP-N-Ras, pEGFP-H-Ras and pEGFP-K-Ras4B vectors were gifts of Dr. P. Casey (Duke University Medical Center, Durham, NC). These constructs encode fusion proteins in which the enhanced GFP (EGFP; Cormack *et al.*, 1996) is fused to the N-terminus of full-lenth N-Ras, H-Ras or K-Ras4B. The fusion genes were subcloned into the pEGFP-C1 vector (Clontech). EGFP is a red-shifted variant of wild-type GFP which has been optimized for brighter fluorescence in mammalian cells by incorporating the double mutations Phe-64 to Leu and Ser-65 to Thr (Cormack *et al.*, 1996). It has also been optimized for human codon-usage to increase translation efficiency in eukaryotic cells (Haas *et al.*, 1996).

Plasmid DNA for PCR and sequencing was isolated from *E. coli* DH5α by modified alkaline lysis (Birnboim and Doly, 1979; Sambrook *et al.*, 1989), followed by purification on a glassmilk matrix (Vogelstein and Gillespie, 1979). Small-scale preparations (5-10 ml) were performed using the RPM Miniprep kit

(Bio101), while large-scale preparations (≥ 1L) were performed using the RPM 4G Mini Monster Kit (Bio101). All DNA solutions were prepared in tris-EDTA (TE) buffer (10 mM tris pH 8.0, 1 mM EDTA) and stored at -20°C.

2.2.2 Oligonucleotide Design and Preparation

The method of splicing by overlap extension (SOE; Horton *et al.*, 1989) was utilized to create fusion genes in which either wild-type or mutant hypervariable domain sequences of N-Ras, H-Ras or K-Ras4B isoforms were appended to the C-terminus of RSGFP (Table 2.1). The same sense (5′) primer was used for all PCR reactions (Table 2.2); this 33 base primer incorporated the first seven amino acid codons of RSGFP as well as a 5′ *Mlu* I restriction site to allow directional cloning into pCMV5. Fourteen different antisense (3′) primers were designed to append either wild-type or various mutant forms of the last 14 amino acids of each Ras isoform to the C-terminus of RSGFP (Table 2.2). These C-terminal 14 amino acids represent the divergent hypervariable domain sequences which are unique to each isoform (Lowy and Willumsen, 1993). An additional antisense primer was designed to amplify unmodified RSGFP for ligation into pCMV5 as a control. To enable SOE, all C-terminal primers incorporated the last seven amino acids of RSGFP immediately upstream of the Ras hypervariable sequences, and a 3′ *Bam*H I restriction site was included to allow directional cloning into pCMV5. All primers were synthesized at the DNA Core Facility (Department of Biochemistry, University of Alberta) using the phosphoramidite method (Beaucage and Caruthers, 1981) at the 40 nmole scale. Oligonucleotides were prepared by resuspending the purified oligonucleotide in 200 µl TE, heating to 65°C for ten minutes, then cooling on ice. The OD_{260} of an initial 1/100 dilution of oligonucleotide stock was determined using a quartz cuvette. The

OD_{260} was multiplied by the dilution factor and the conversion factor 30 OD_{260}=1 mg/ml single-stranded DNA (Sambrook *et al.*, 1989) to determine the oligonucleotide concentration. The molecular weight of the oligonucleotide was then used to establish the molarity of the solution. Working solutions of 10 pmol/µl were prepared in TE, and all oligonucleotide solutions were stored at -20°C. All primers were designed and prepared by L. Berthiaume, M. Nishiwaki and I. Balan prior to C. Mattar's involvement in the project.

2.2.3 Polymerase Chain Reaction

Polymerase chain reactions (PCR) were conducted in 50 µl volumes and included 100 ng of pRSGFP-C1 template DNA, 20-40 pmol of each sense and antisense oligonucleotide primer costituting the appropriate primer pair (Table 2.3), 5 µl of 10X ThermoPol reaction buffer (New England Biolabs; NEB), 1 U Vent DNA polymerase (NEB) and 2.5 mM of each dNTP (dATP, dTTP, dCTP, dGTP; Promega). Amplification was allowed to proceed for thirty-five cycles, with each cycle incorporating successive steps at 94°C for 30 seconds, 45-55°C for 30 seconds to one minute, and 72°C for 90-120 seconds. The first cycle was preceeded by an initial denaturing step of 94°C for five minutes, and the last cycle was followed by a final elongation step of 72°C for ten minutes.

2.2.4 General Cloning Methods

Restriction digests of pCMV5 vector and GFP-Ras amplification fragments were carried out at the appropriate temperature(s) for 1-2 hours in a final volume of 50 µl using restriction enzymes and 10X buffers obtained from NEB. Bovine serum albumin (BSA) was added to a final concentration of 400 µg/ml.

Digested pCMV5 vector was dephosphorylated using calf intestinal alkaline phosphatase (NEB) in two stages; 2.5U of enzyme was added to the digestion mixture and incubated at 37°C for 30 minutes, then an additional 2.5U of enzyme was added and the mixture was incubated for a further 30 minutes. Reactions were terminated by heating the reaction mixture at 75°C. The dephosphorylated vector was then gel purified.

Agarose and acrylamide gel purification of amplified PCR products, digested inserts and pCMV5 vector used in cloning was done using a modified "crush and soak" method (Sambrook *et al*, 1989) adapted from Maxam and Gilbert (1977). After separation of the DNA on 6 % polyacrylamide (digested vector) or 0.8 % agarose (PCR products or digested inserts) and staining with ethidium bromide, the appropriate bands were excised and crushed in an Eppendorf tube using a Kontes pellet pestle and 25 µl TE. The mixture was incubated at 37°C for one hour, then centrifuged through a Spin-X tube (Costar) at 13 200xg to separate the gel matrix from the DNA filtrate.

Directional ligation of the digested GFP-Ras inserts into digested pCMV5 was carried out in a 50 µl mixture containing an approximate 10:1 insert to vector molar ratio, 5 µl of 10X ligase buffer (Gibco) and 1 U of T4 DNA ligase (Gibco). Ligation was carried out overnight at 16°C. Competent *E. coli* DH5α were transformed with ligation mixture volumes corresponding to 1 ng and 5 ng of vector DNA.

2.2.5 Generation of Chimeric GFP-Ras Constructs

To create the GFP-Ras fusion sequences, all PCR reactions utilized pRSGFP-C1 as the template and the same 5′ (sense) primer, RSGFP33S (Table 2.3). The 3′

(antisense) primers determined which wild-type or mutant Ras hypervariable domain sequence would be appended to the C-terminus of RSGFP. Table 2.3 summarizes the primer pairs used to generate the individual wild-type and mutant constructs, and conditions of the PCR reactions were as previously described in this section. The PCR amplification fragments of approximately 0.7-0.8 Kbp were isolated from 6 % acrylamide gel, digested with *Mlu* I and *Bam*H I to generate cohesive ends, then repurified from acrylamide, as previously described in this section. The receiving pCMV5 vector was also digested with *Mlu* I and *Bam*H I to generate cohesive ends and purified from 0.8 % agarose. Approximated quantities of the individual inserts and vector, corresponding to a 10:1 insert to vector ratio, were ligated as described in Section 2.2.4 and the mixture transformed into *E. coli* DH5α. After confirmatory digestion with *Mlu* I and *Bam*H I, a single clone for each construct was chosen for dideoxy chain termination based-sequencing (Sanger *et al.*, 1977) at the DNA Core Facility (Department of Biochemistry, University of Alberta). The final construct (pRSGFP-Ras) appears in Figure 2.1. All constructs were generated by L. Berthiaume, M. Nishiwaki and I. Balan prior to C. Mattar's involvement in the project.

2.3 Cell Transfection

10 mg/ml (25X) working solutions of DEAE (diethylaminoethyl)-dextran (chloride form, 500 000 MW; Sigma) were prepared by slowly dissolving the dextran in sterile PBS (phosphate buffered saline pH 7.4; 2.68 mM potassium chloride, 1.47 mM potassium dihydrogen phosphate, 137 mM sodium chloride, 4.27 mM sodium phosphate) heated to 80°C. Once dissolved, the solution was heated to boiling using a microwave, then stored at 4°C. Chloroquine

(diphosphate salt; Sigma) was prepared as a 100 mM stock in sterile PBS and stored under tinfoil at -20°C. 1 ug/ml working solutions of each RSGFP-Ras construct were prepared from maxi-prep DNA in sterile TE and sterile filtered through a Spin-X tube (Costar). DMSO (dimethylsulfoxide; Caledon) was prepared as a 10 % solution in warm sterile PBS just before use.

2.3.1 Modified DEAE-dextran/DMSO Method

COS-7 cells below passage 16 were transfected using a modified DEAE-dextran/DMSO method (Hancock *et al.*, 1988). This method differs from the traditional dextran/DMSO method in that the cells to be transfected are seeded to culture dishes two to three hours before transfection instead of one to several days. Confluent 100 mm dishes of COS-7 cells, no more than three days old, were trypsinized and the resulting 10 ml cell suspension from each dish was passed at 1:10 to each 100 mm dish to be transfected. Newly seeded dishes were incubated at 37°C, 5 % CO_2 for two to three hours to allow cell adhesion, washed twice in PBS and overlayed with 4 ml of transfection mix per dish. The transfection mix consists of 3.9 ml DMEM supplemented with 10 % NuSerum (Becton-Dickinson), 160 µl of 25X (10 mg/ml) DEAE-dextran (400 µg/ml C_f), 4 µl of 100 mM chloroquine (0.1 mM C_f) and 5 µg of construct DNA. The transfection mix was left on the cells for 2.5 hours at 37°C, 5 % CO_2, then aspirated before 4 ml of 10 % DMSO was added to each dish for two minutes at room temperature. Cells were washed twice in PBS and 10 ml maintenance medium was added. Cells were allowed to express chimeric GFP-Ras proteins for 41-44 hours post-transfection before use in experiments in order to correlate observable cellular fluorescence with protein expression.

2.4 Protein Immunoprecipitation

Chimeric GFP-Ras proteins were immunoprecipitated from either whole cell lysates (after detergent lysis) or from soluble and particulate subcellular fractions (after Dounce homogenization and subcellular fractionation). Table 2.4 describes the components, preparation and storage of all solutions used in immunoprecipitation (IP) of GFP-Ras chimeras from whole cell lysates and subcellular fractions.

2.4.1 Immunoprecipitation of GFP-Ras Chimeras

Dishes to be immunoprecipitated were washed twice in ice-cold STE (salt-tris-EDTA) buffer (Table 2.4). Cells were lysed by the addition of 2.5 ml of ice-cold 1X cold lysis buffer (CLB) plus phenylmethylsulfonylfluoride (PMSF) and aprotinin/leupeptin (Table 2.4) to each dish for 10 minutes (on ice) with frequent rocking. Lysates were harvested by scraping each plate with a Costar cell lifter, then aliquoting the lysates into 1.5 ml screw-cap Eppendorf tubes. An additional one hour lysis was carried out at 4°C with rocking. Tubes were then centrifuged for ten minutes at 13 200xg (4°C) to pellet cell debris. Supernatants were transferred to clean tubes and 2 µl (10 µg) of rabbit polyclonal anti-GFP H184 antibody (raised in our laboratory against purified recombinant GFP expressed in *E. coli*) was added to each tube. For preimmune controls, 2 µl of preimmune serum was added, and for protein A sepharose (PAS) controls, only PAS was added. Tubes were returned to 4°C for 1-2 hours with rocking, then 28 µl of a 50% slurry of protein A sepharose CL-4B (PAS; Table 2.4) was added to each tube. Immune complex formation and precipitation was allowed to occur overnight (16 hours) at 4°C with rocking. After precipitation, PAS-antibody-protein complexes were washed three times in 0.5 ml of 1X CLB (minus

protease inhibitors) by pelleting the complexes at 600xg for two minutes between each wash. After the final wash, 28 µl of 1X SDS sample buffer (with 20 mMdithiothreitol (DTT); Table 2.4) was added to each tube and heated at 96°C for two minutes. Samples were either frozen immediately at -80°C, or supernatants were loaded onto SDS-PAGE after cooling on ice and pelleting PAS beads at 600xg for two minutes.

2.4.2 Subcellular Fractionation

For each fractionation experiment, four 100 mm dishes of transfected COS-7 cells were fractionated and immunoprecipitated for every GFP-Ras construct; two dishes were fractionated for the GFP control. Subcellular fractionation of COS-7 cells was carried out essentially as described in Alland *et al.* (1994) with the exception that sodium vanadate was replaced with PMSF (Table 2.4). Two other modifications to the protocol were made. A total (T) fraction, representing half of the homogenate volume (approximately 1.2 ml), was aliquoted from the homogenate before centrifugation. Centrifugation of the remainder (approximately 1.2 ml) was carried out for one hour and 4°C at 100 000xg in a Beckman TL100 Ultracentrifuge, using polycarbonate thick wall tubes (3.2 ml capacity) and the TLA 100.4 rotor. The supernatant (S100) represented the soluble fraction and the pellet (P100) represented the particulate fraction. The pellets were resuspended in the S100 volume of hypotonic lysis buffer (HLB, plus protease inhibitors and sucrose/EDTA; Table 2.4) and homogenized with 5-10 strokes using a Dounce homogenizer with tight-fitting pestle. All tubes were then adjusted to 1 ml with 5X CLB (Table 2.4) and further solubilized for one hour at 4°C with rocking. Immunoprecipitation of the chimeric GFP-Ras proteins was carried out as described in Section 2.4.1.

2.5 Protein (Western) Blot Analysis

All methods utilized were modifications of those presented in Sambrook *et al.* (1989). Proteins were separated on 12 % SDS-PAGE (1.5 mm thickness) in tris-glycine buffer (25 mM tris, 225 mM glycine, 0.1 % SDS). Western blot analysis (Towbin *et al.*, 1979; Burnette, 1981) was performed by transferring separated proteins onto polyvinylidene difluoride (PVDF) membrane (Millipore) for five hours at 100 volts in tris-glycine-methanol buffer (25 mM tris-Cl, 192 mM glycine, 20 % methanol; pH 8) cooled to -17°C. The transfer sandwich consisted of sponges, Whatman thick chromatography paper, polyacrylamide gel and PVDF membrane arranged within a BioRad transfer cassette as shown in Figure 2.2. Before incorporation into the sandwich, the sponges and chromatography paper were thoroughly soaked in transfer buffer, and the PVDF membrane was wetted in methanol just before overlay onto the gel.

After transfer, membranes were stained in ponceau S (3% ponceau S (w/v), 30% trichloracetic acid (w/v), 30% sulfosalicylic acid (w/v)) to visualize transferred proteins. Membranes were rinsed in distilled water and blocked for one hour at room temperature, with agitation, in blotto/5% milk blocking solution (14 mM NaCl, 2 mM tris, 0.15% HCl (v/v), pH 7.6, with 5% powdered skim milk (w/v) added). Blocking solution was replaced with 50 ml of primary antibody solution (1:2500 dilution of rabbit polyclonal anti-GFP H184 antibody in blocking solution) and incubated for two hours at room temperature with agitation. Membranes were washed three times with PBS, ten minutes per wash, and overlayed with 50 ml secondary antibody solution (1:5000 dilution of donkey anti-rabbit-horseradish peroxidase conjugate (Amersham) in blocking solution) for one hour at room temperature with agitation. Membranes were washed with

PBS as described and chemiluminescence analysis was performed using ECL-Plus (Enhanced Chemiluminescence; Amersham) and Kodak imaging film.

2.6 Confocal Microscopy

Live cell fluorescence studies were performed using a Leitz Aristoplan fluorescence scope with argon/krypton laser at 488 nm emission, and either a x63 (1.4 numerical aperature (NA)) or x100 (1.32 NA) oil immersion objective (Department of Cell Biology, University of Alberta). Fixed cell immunofluorescence (immunocytochemistry) studies were carried out using a Zeiss LSM510 laser scanning confocal microscope mounted on a Zeiss Axiovert M100 inverted scope equipped with a x63 (1.4 NA) oil immersion lens (Cross Cancer Institute, Edmonton, Alberta).

FITC (fluorescein isothiocyanate) or TR (Texas Red) filters were used to collect the data and minimize bleed-through. Scans were optimized for chromophore detection. Manipulation of the final images was conducted using Adobe Photoshop 5.0.

2.6.1 Immunocytochemistry Reagents

The methods described below are modifications of those found in Harlow and Lane (1988). For both live and fixed cell microscopy studies, flame-sterilized 22x22 mm glass coverslips (No.1 thickness; Fisher) were coated with 20 μg/ml poly-L-lysine solution (Sigma). Poly-L-lysine was diluted in water and added to coverslips for five minutes at room temperature with agitation. Coverslips were then rinsed in distilled water and allowed to dry for at least two hours before use. For cell fixation, 4 % paraformaldehyde pH 7.4 (Sigma) was prepared by adding paraformaldehyde to distilled water while stirring, then heating to 60°C. 1 N

NaOH was added dropwise while swirling until the white precipitate dissolved. The solution was cooled to room temperature and 10X PBS was added to a final concentration of 1X. The solution was prepared just before use. For cell permeabilization, 0.1 % triton X-100 (ICN) was prepared in PBS just before use. The solution could be stored for several weeks at room temperature providing it was protected from light. Blocking solution was a 4 % normal donkey serum (Jackson Immunoresearch) in PBS; this solution was also used as antibody diluent. For immunofluorescence studies, mouse monoclonal anti-GFP MAB2510 (Chemicon) was used at 1:200, goat polyclonal anti-calreticulin (gift of Dr. M. Michalak (University of Alberta)) was used at 1:150, and rabbit polyclonal anti-giantin (gift of Dr. E.K. Chan (Scripps Institute, LaJolla, CA)) was used at 1:2000. All secondary antibodies (donkey anti-mouse IgG-FITC conjugate, donkey anti-goat IgG-TR conjugate and donkey anti-rabbit IgG-TR conjugate) were purchased from Jackson Immunoresearch and used at 1:100. DiI-LDL (Molecular Probes) was diluted to a working solution of 10 μg/ml in serum-free DMEM. For Golgi and endosome colocalization studies, 4 mM stocks of nocodazole in DMSO (stored at -20°C) were diluted to a working solution of 20 μM in serum-free DMEM. Coverslips were mounted onto glass slides (Corning) using Prolong Antifade reagent (Molecular Probes) and stored in the dark at room temperature until viewed.

2.6.2 Cell Preparation: Live Cell Fluorescence and Immunocytochemistry

Poly-L-lysine coated coverslips were placed into six-well culture dishes (Costar) and each coverslip was seeded with 130, 000-200, 000 cells, which were transfected as described in Section 2.3.1, except that 1 ml transfection mix was prepared for each coverslip using 1 μg of construct DNA and 40 μl of dextran,

and chloroquine was omitted. Coverslips were prepared for live cell fluorescence or immunocytochemistry at 41-44 hours post-transfection. For live cell fluorescence, the cells were rinsed in warm PBS and mounted onto glass slides in PBS using nail polish as a sealant. For immunocytochemistry, coverslips were washed in PBS, fixed for ten minutes at room temperature in 4% paraformaldehye, washed twice in PBS, then permeabilized in 0.1% triton X-100 for two minutes at room temperature. For endosome colocalization studies, coverslips were initially incubated with DMEM/diI-LDL for one hour at 37°C, 5% CO_2; duplicate coverslips were then treated with nocodazole by the addition of 3 µl of nocodazole stock to each well for one hour prior to fixation. For Golgi colocalization studies, duplicate coverslips were incubated in DMEM/20 µM nocodazole at 37°C, 5 % CO_2 for one hour prior to fixation. After permeabilization, coverslips were washed twice in PBS and 80 µl of blocking solution was dripped onto each coverslip and incubated for one hour at 37°C, 5% CO_2. All subsequent incubations were carried out at 37°C, 5% CO_2. Blocking solution was replaced with 80 µl of primary antibody solution and incubated for one hour; coverslips were washed four times over five minutes with PBS and 80 µl of secondary antibody was applied for one hour. Coverslips were mounted after washing four times over five minutes with PBS.

2.7 Metabolic Labelling

Transiently transfected COS-7 cells were metabolically labelled 24 hours post-transfection with either the isoprenoid precursor RS-[2-^{14}C] mevalonic acid lactone (MVA) or [^{125}I]iododpalmitate. RS-[2-^{14}C] mevalonic acid lactone in toluene (51 mCi/mmol SA) and [^{125}I]NaI (2-3 Ci/mmol SA) were purchased from Amersham Pharmacia. Iodopalmitic acid (gift of Dr. M. Resh, Sloan-Kettering

Cancer Center, New York, NY) was dissolved in acetone to produce a 10 mM stock and stored at -20°C.

2.7.1 Radioiodination of Palmitate

Preparation of the iodopalmitate analogue (Figure 2.3) was carried out as described by Berthiaume *et al.* (1995) without the HPLC purification step. In a glass reaction vessel, 200 µl (2 µmol) of iodopalmitate stock was evaporated and treated with 3 µl glacial acetic acid. The [^{125}I]NaI vial was rinsed twice with 150 µl of acetone, and each rinse transferred to the reaction vessel. The vessel was capped with a rubber septum, and a charcoal trap inserted. The radioiodination reaction proceeded at 55°C for 16 hours. Distilled water (0.5 ml) was added to the reaction tube and the aqueous layer extracted twice with 1.2 ml of chloroform, which was transferred to a clean vial and evaporated under nitrogen. The residue was resuspended in 1070 µl of 95% ethanol and ten 100 µl aliquots were dispensed into glass vials, dried under nitrogen and stored at -20°C. The remainder of the original suspension was diluted and counted in a scintillation counter, with typical specific activity of the final [^{125}I]iodopalmitate preparation at 2-3 Ci/mmol. Each vial of [^{125}I]iodopalmitate was resuspended in 15-20 µl of 95% ethanol before use. Iodopalmitate for experimentation was prepared by Zhao Yang, technologist in the Berthiaume Lab.

2.7.2 [^{125}I]Iodopalmitate Labelling of COS-7 Cells

One 100 mm dish of transfected COS-7 cells was metabolically labelled with [^{125}I]iodopalmitate for each GFP-Ras construct according to Alland *et al.* (1994) and McCabe and Berthiaume (1999). Cells were washed in PBS and starved of fatty acids for one hour in 3 ml depletion medium (DMEM, 10% fatty-acid free

BSA (w/v)) per dish. Depletion medium was replaced with 3 ml labelling medium (DMEM/10%BSA with approximately 80 μCi of [^{125}I]iodopalmitate added per dish). Cells were metabolically labelled for 16 hours at 37°C, 5% CO_2 before radiolabelled GFP-Ras proteins were recovered by immunoprecipitation as described in Sections 2.4 and 2.4.1.

2.7.3 [^{14}C]Mevalonic Acid Lactone Labelling of COS-7 Cells

One 100 mm dish of transfected COS-7 cells was metabolically labelled with [^{14}C]mevalonic acid lactone (MVA) for each GFP-Ras construct according to an adaptation of the method of Jones and Speigel (1990) and Rowell *et al.* (1997). Cells were washed in PBS and starved of mevalonate for six hours in 3 ml depletion medium (DMEM, 10% FBS and 30 μM mevastatin (dissolved in acetone; Sigma) per dish. Depletion medium was replaced with 3 ml labelling medium (depletion medium with 50 μCi [^{14}C]MVA added per dish). Labelling medium was prepared by evaporating RS-[2-^{14}C]MVA in toluene to dryness under dry nitrogen at 56°C and resuspending the residue in fresh depetion medium. Cells were metabolically labelled for 16 hours at at 37°C, 5% CO_2 before radiolabelled GFP-Ras proteins were recovered by immunoprecipitation as described in Section 2.4 and 2.4.1.

2.7.4 Autoradiography of Radiolabelled Chimeras

Western blot analysis of radiolabelled GFP-Ras chimeras recovered by immunoprecipitation was carried out as described in Section 2.5. After chemiluminescence analysis, the PVDF membranes were allowed to air dry for 24 hours before autoradiography was initiated as residual chemiluminescence may remain for up to 24 hours. For [^{14}C]MVA labelling studies, membranes

were initially exposed to a phosphorimager screen (Molecular Dynamics) for 5-7 days, and autoradiography was then carried out for two weeks at -80°C using a Kodak Biomax low energy (LE) transcreen and Kodak Biomax maximum sensitivity (MS) film. For [^{125}I]iodopalmitate labelling studies, autoradiography of membranes was carried out for 27 days at -80°C using a Kodak Biomax high energy (HE) transcreen and Kodak MS film.

Table 2.1 Ras Isoform Hypervariable Domain Sequences* Appended to the C-terminus of Red-Shifted GFP

Construct	Hypervariable Sequence	Prenyl	Second Signal
	181 186		
GFP-N-RasWT	DGTQG**C**MGLP**C**VVM	farnesyl	palmitate@C181
GFP-N-RasC181S	DGTQG**S**MGLP**C**VVM	farnesyl	none
GFP-N-RasC186S	DGTQG**C**MGLP**S**VVM	none	none
GFP-N-RasC181,6S	DGTQG**S**MGLP**S**VVM	none	none
	+ + + + + + + + 185		
GFP-K-RasWT	**KKKKKKS**K**T**K**C**VLS	farnesyl	polybasic region
GFP-K-RasKQ	**QQQQQQS**Q**T**Q**C**VLS	farnesyl	none
GFP-K-RasC185S	**KKKKKKS**K**T**K**S**VLS	none	polybasic region
GFP-K-RasKQ,C185S	**QQQQQQS**Q**T**Q**S**VLS	none	none
	181 184 186		
GFP-H-RasWT	ESGPG**C**MS**C**K**C**VIM	farnesyl	palmitate@C181,184
GFP-H-RasC181S	ESGPG**S**MS**C**K**C**VIM	farnesyl	palmitate@C184
GFP-H-RasC184S	ESGPG**C**MS**S**K**C**VIM	farnesyl	palmitate@C181
GFP-H-RasC186S	ESGPG**C**MS**C**K**S**VIM	none	none
GFP-H-RasC181,4S	ESGPG**S**MS**S**K**C**VIM	farnesyl	none
GFP-H-RasC181,4,6S	ESGPG**S**MS**S**K**S**VIM	none	none

* The N-Ras DNA sequence was obtained from GenBank, Accession No.X02571; the K-Ras4B and H-Ras DNA sequences were derived from amino acid sequences published in Glomset and Farnsworth (1994)

Table 2.2 DNA Sequences of Oligonucleotide Primers used to create the GFP-Ras Fusion Sequences

Primer	Sequence (5′→3′)
Sense (5′) Primer	
•RSGFP-33S	CCC CAC GCG TCC ATG GGT AAA GGA GAA GAA CTT
Antisense (3′) Primers	
•N-RasWT-AS	CGC GGA TCC TTA CAT CAC CAC **ACA** TGG CAA TCC CAT **ACA** ACC CTG AGT CCC ATC TTT GTA TAG TTC ATC CAT GCC
N-RasC181S-AS	CGC GGA TCC TTA CAT CAC CAC ACA TGG CAA TCC CAT **AGA** ACC CTG AGT CCC ATC TTT GTA TAG TTC ATC CAT GCC
N-RasC186S-AS	CGC GGA TCC TTA CAT CAC CAC **AGA** TGG CAA TCC CAT ACA ACC CTG AGT CCC ATC TTT GTA TAG TTC ATC CAT GCC
N-RasC181,6S-AS	CGC GGA TCC TTA CAT CAC CAC **AGA** TGG CAA TCC CAT **AGA** ACC CTG AGT CCC ATC TTT GTA TAG TTC ATC CAT GCC
•K-Ras(4B)WT-BamHI-75-AS	CGC GGA TCC TTA AGA CAG AAC **GCA TTT** GTT **TTT** AGA **TTT TTT TTT TTT TTT TTT** TTT GTA TAG TTC ATC CAT GCC
K-RasKQ-AS	CGC GGA TCC TTA AGA CAG AAC GCA **CTG** GTT **CTG** AGA **CTG CTG CTG CTG CTG CTG** TTT GTA TAG TTC ATC CAT GCC
K-RasC185S-AS	CGC GGA TCC TTA AGA CAG AAC **AGA** TTT GTT TTT AGA TTT TTT TTT TTT TTT TTT TTT GTA TAG TTC ATC CAT GCC
K-RasKQ,C185S-AS	CGC GGA TCC TTA AGA CAG AAC **AGA CTG** GTT **CTG** AGA **CTG CTG CTG CTG CTG CTG** TTT GTA TAG TTC ATC CAT GCC
•H-RasWT-BamHI-75-AS	CGC GGA TCC TTA CAT GAT AAC **GCA** TTT **GCA** AGA CAT **GCA** ACC CGG ACC AGA TTC TTT GTA TAG TTC ATC CAT GCC
H-RasC181S-AS	CGC GGA TCC TTA CAT GAT AAC GCA TTT GCA AGA CAT **AGA** ACC CGG ACC AGA TTC TTT GTA TAG TTC ATC CAT GCC
H-RasC184S-AS	CGC GGA TCC TTA CAT GAT AAC GCA TTT **AGA** AGA CAT GCA ACC CGG ACC AGA TTC TTT GTA TAG TTC ATC CAT GCC
H-RasC186S-AS	CGC GGA TCC TTA CAT GAT AAC **AGA** TTT GCA AGA CAT GCA ACC CGG ACC AGA TTC TTT GTA TAG TTC ATC CAT GCC
H-RasC181,4S-AS	CGC GGA TCC TTA CAT GAT AAC GCA TTT **AGA** AGA CAT **AGA** ACC CGG ACC AGA TTC TTT GTA TAG TTC ATC CAT GCC
H-RasC181,4,6S-AS	GGC GGA TCC TTA CAT GAT AAC **AGA** TTT **AGA** AGA CAT **AGA** ACC CGG ACC AGA TTC TTT GTA TAG TTC ATC CAT GCC
•GFP-BamHI-30-AS	CGC GGA TCC TTA TTT GTA TAG TTC ATC CAT

Underlined sequences represent restriction sites; MluI in the RSGFP-33S primer and BamHI in all of the antisense primers. The CGC codon immediately 5′ to the restriction sites is a stuffer sequence to facilitate optimum enzyme activity at the DNA ends.
The N-Ras cDNA sequence was obtained from GenBank, Accession No. X02571; the K-Ras4B and H-Ras DNA sequences were derived from amino acid sequences published in Glomset and Farnsworth (1994).

Table 2.3 Primer Pairs Used in PCR to create RSGFP-Ras Fusion Sequences

RSGFP-Ras Construct (in pCMV5)	Primer Pair
GFP-N-RasWT	RSGFP-33S/N-RasWT-AS
GFP-N-RasC181S	RSGFP-33S/N-RasC181S-AS
GFP-N-RasC181,6S	RSGFP-33S/N-RasC181,6S-AS
GFP-N-RasC186S	RSGFP-33S/N-RasC186S-AS
GFP-K-RasWT	RSGFP-33S/K-Ras(4B)WT-BamHI-75AS
GFP-K-RasC185S	RSGFP-33S/K-RasC185S-AS
GFP-K-RasKQ	RSGFP-33S/K-RasKQ-AS
GFP-K-RasKQ,C185S	RSGFP-33S/K-RasKQ,C185S-AS
GFP-H-RasWT	RSGFP-33S/H-RasWT-BamHI-75AS
GFP-H-RasC181S	RSGFP-33S/H-RasC181S-AS
GFP-H-RasC184S	RSGFP-33S/H-RasC184S-AS
GFP-H-RasC186S	RSGFP-33S/H-RasC186S-AS
GFP-H-RasC181,4S (C2/S2)	RSGFP-33S/H-RasC181,4S-AS
GFP-H-RasC181,4,6S (C3/S3)	RSGFP-33S/H-RasC181,4,6S-AS
RSGFP control	RSGFP-33S/GFP-BamHI-30AS

Table 2.4 Solutions Used in Immunoprecipitation of GFP-Ras from Whole Cell Lysates and Subcellular Fractions

Solution	Component	Source	Instructions
STE (salt-tris-EDTA)	100 mM NaCl 10 mM Tris pH 7.4 1 mM EDTA	BDH Sigma Sigma	Store at 4 degrees
1X CLB (cold lysis buffer)	50 mM Tris-Cl pH 8.0 150 mM NaCl 2 mM MgCl 2 mM EDTA 1% NP-40 0.5%sodium deoxycholate 1 mM PMSF (in isopro-panol) 10 µg/ml aprotinin and leupeptin (in water)	Sigma BDH BDH BDH BDH Sigma Sigma Boehringer Manheim	Add PMSF and aprotinin/ leupeptin to aliquot just before use (these are stored at –20°C). Leupeptin, not aprotinin, may be refrozen. Incomplete buffer stored at 4°C; protect from light
5X CLB	components identical to 1X CLB except that five times the concentration of each is incorporated into the same final volume		As for 1X CLB
HLB (hypotonic lysis buffer)	10 mM Tris-Cl pH 7.4 0.2 mM MgCl 1 mM PMSF 10 µg/ml aprotinin and leupeptin	Sigma BDH Sigma Boehringer Manheim	Add PMSF and aprotinin/ leupeptin to aliquot just before use.Incomplete buffer stored at 4°C
PAS (protein A sepharose)	50% slurry in 1X CLB (without PMSF and aprotinin/leupeptin)	Amersham Pharmacia	50 mg PAS swelled in water for one hour on ice, then washed twice in 1X CLB minus inhibitors. Beads resuspended in equal volume 1X CLB (by estimation). Store at 4°C
1X SDS loading buffer	50 mM Tris-Cl pH 6.8 100 mM dithiothreitol (DTT) 2% SDS 0.1% bromphenol blue 10% glycerol	Sigma Sigma Caledon Sigma BDH	Store incomplete buffer at RT add DTT just before use. Complete buffer may be frozen at -20°C

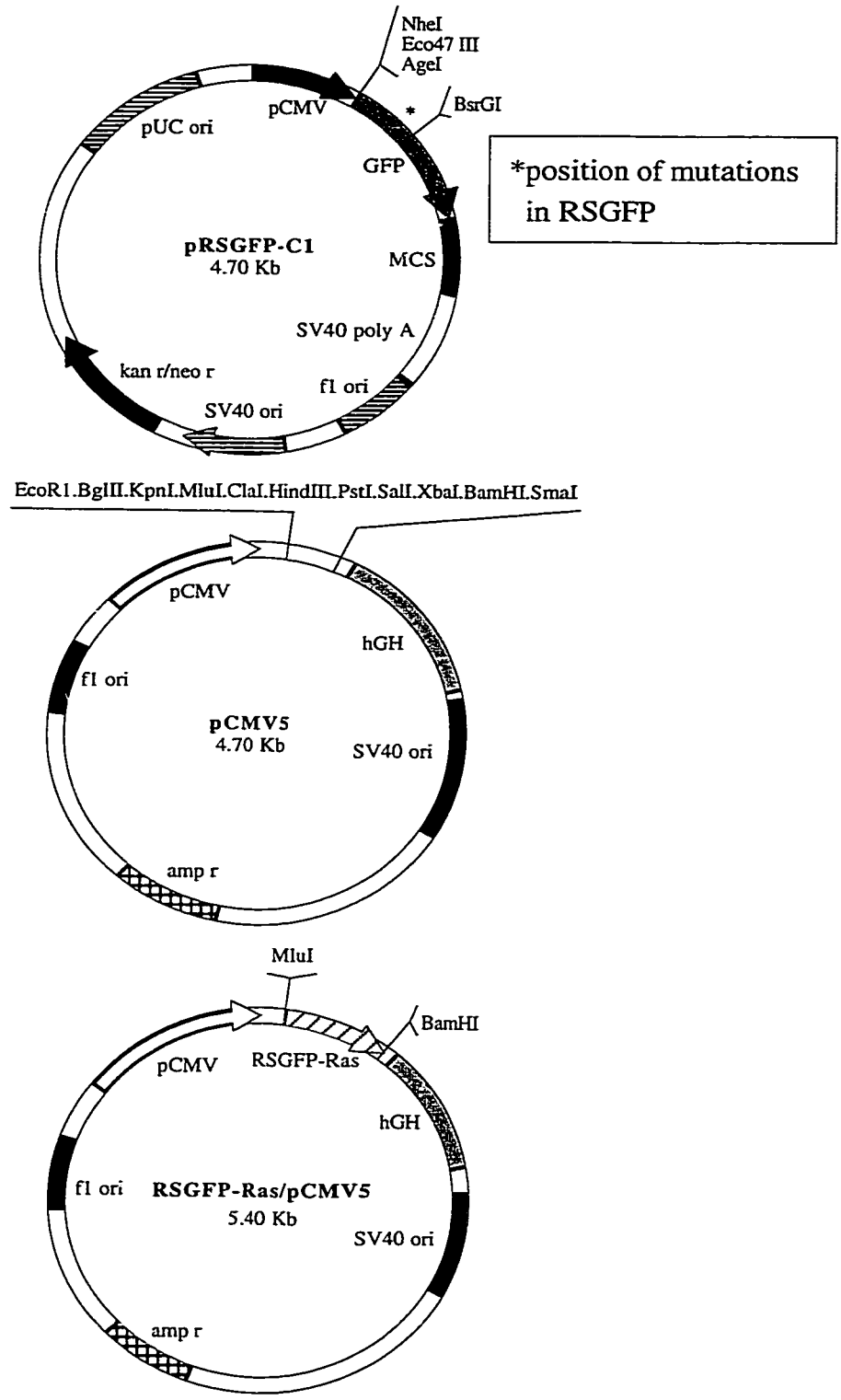


Figure 2.1 Plasmids Used in this Study

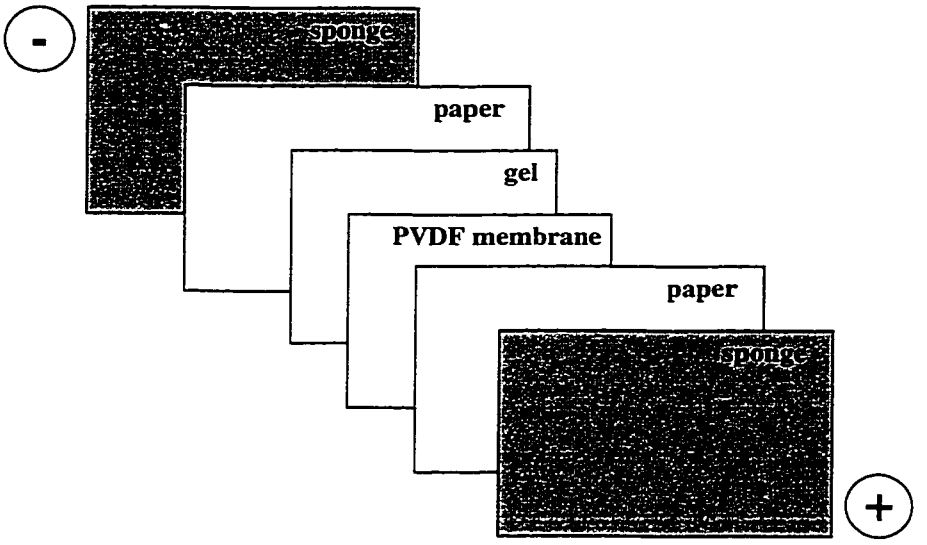
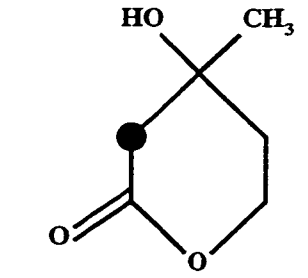


Figure 2.2 Orientation of Transfer Cassette Components for Western Blot Analysis

The (-) and (+) symbols represent the cathode and anode, respectively, within the transfer unit.



[^{14}C]Mevalonic Acid Lactone

[^{125}I]Iodopalmitate

Figure 2.3 Structures of [^{14}C]Mevalonic Acid Lactone and [^{125}I]Iodopalmitate used in Metabolic Labelling Studies

The [^{14}C] in the mevalonic acid lactone structure is represented by the solid circle.

3.0 RESULTS

3.1 Rationale for GFP-Ras Chimera Design and Characterization

We sought to establish whether the variably lipidated C-terminal hypervariable domains of the major Ras isoforms (N-Ras, K-Ras4B and H-Ras) may direct their differential subcellular localization. To investigate this, a series of GFP-Ras hypervariable domain fusion genes were created using the technique of splicing by overlap extension (SOE; Horton *et al.*, 1989). This PCR-mediated mutagenesis technique was used to append wild-type or lipidation-mutant forms of the last 14 amino acids from each major isoform to the C-terminus of RSGFP (Table 2.1). This approach enabled assessment of the contribution of each lipid modification within the hypervariable domains to the subcellular localization of each chimera. Only the Ras hypervariable domain sequences were appended to RSGFP to avoid potential contributions to membrane association by other functional domains or protein-protein interaction modules present in full-length Ras.

To design the appropriate oligonucleotides (Table 2.2), the N-Ras cDNA sequence was obtained from GenBank (Accession No. X02571), and the DNA sequences for the K-Ras4B and H-Ras hypervariable domains were derived from amino acid sequences published in Glomset and Farnsworth (1994). It was later discovered that the farnesylation consensus sequences (CaaX motifs) of these isoforms were erroneously transposed in this review. As a result, the GFP-K-Ras chimera possesses the H-Ras CaaX motif (CVLS), while the GFP-H-Ras chimera possesses the K-Ras4B CaaX motif (CVIM), but the remainder of the hypervariable domain sequences present in these constructs are authentic for the respective isoforms (Table 2.1).

Processing of the CaaX motif is a prerequisite for palmitoylation of upstream cysteines within the Ras hypervariable domains, and inhibition of this processing also inhibits palmitoylation, resulting in unmodified and cytosolic proteins (Hancock *et al.*, 1989; Willumsen *et al.*, 1984a). Therefore, substitution of the prenylated cysteine residue with a serine residue within the CaaX motif abolishes all lipid modifications of p21 Ras. To assess the role of the individual lipid modifications, various GFP-N-Ras, GFP-K-Ras and GFP-H-Ras mutants were created in which lipidated cysteines within the hypervariable domains were mutated to serines, either singly or in combination (Table 2.1). To analyze the contribution of the K-Ras4B polybasic region, lysine residues were substituted with glutamine residues. The resulting fusion proteins were predicted to lack the second signal (N-RasC181S, K-RasKQ and H-RasC181,4S), be completely unmodified (N-RasC186S, N-RasC181,6S, K-RasC185S, K-RasKQ,C185S, H-RasC186S and H-RasC181,4,6S) or to be singly palmitoylated at either of the modified cysteines comprising the second signal (H-RasC181S and H-RasC184S). The GFP-K-RasC185S chimera is unique in that it retains the intrinsic polybasic second signal in the absence of farnesylation.

3.2 Metabolic Labelling of GFP-Ras Chimeras

In order to confirm the correct prenylation and palmitoylation states of each GFP-Ras fusion protein, COS-7 cells were transiently transfected with the various chimeric constructs and metabolically labelled with either the isoprenoid precursor [^{14}C]mevalonic acid lactone (MVA) or [^{125}I]iodopalmitate, respectively (Figure 2.4).

Mevalonic acid is an intermediate in the cholesterol biosynthetic pathway which

is formed by the reduction of hydroxymethylglutaryl-coenzyme A (HMG-CoA) by HMG-CoA reductase (Figure 3.1). Intermediate products derived from mevalonic acid by this pathway include farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate, which are used in part for protein isoprenylation (Figure 3.1). Metabolic labelling of prenylated proteins is accomplished by the addition of exogenous MVA to cultured cells. MVA is a stable, cyclic form of mevalonic acid which may be more readily taken up by cultured cells than the charged acid form; exogenous radiolabelled MVA is taken up by cells and converted to mevalonic acid (Faust and Krieger, 1987), from which all products of the cholesterol pathway are derived. The HMG-CoA reductase inhibitor mevastatin (Figure 3.1) (Endo, 1992) is included in the labelling medium to suppress the cholesterol biosynthesis pathway by inhibiting endogenous mevalonic acid production. Addition of exogenous radiolabelled MVA enables the pathway to proceed, and the resulting radiolabelled farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate are utilized for protein prenylation.

GFP-Ras fusion proteins immunoprecipitated from metabolically labelled COS-7 cells were subjected to both Western blot and autoradiographic analysis in order to assess label incorporation (Figure 3.2). Prenylation was evident in all chimeras possessing an intact CaaX motif, as demonstrated by the appearance of the autoradiographic signal overlapping that of the corresponding Western blot analysis (Figure 3.2, Panels A and B, GFP-N-RasWT, GFP-N-RasC181S, GFP-K-RasWT, GFP-H-RasWT, GFP-H-RasC181S, GFP-H-RasC184S and GFP-H-RasC181,4S). Prenylation was abolished when the prenylated cysteine was mutated to serine (Figure 3.2, Panel B; GFP-N-RasC186S, GFP-N-RasC181,6S, GFP-K-RasC185S, GFP-K-RasKQ,C185S, GFP-H-RasC186S and

GFP-H-RasC181,4,6S). Consequently, palmitoylation of the N-Ras and H-Ras chimeras, as monitored by [^{125}I]iodopalmitate labelling, was also abolished (Figure 3.2, Panel D). Unlike the N-Ras and H-Ras isoforms, K-Ras4B is prenylated but not palmitoylated, possessing a polybasic region as the second signal, and therefore is not labelled by [^{125}I]iodopalmitate (Figure 3.4, Panel D; Hancock *et al.*, 1989, Hancock *et al.*, 1991, Casey *et al.*, 1989).

The GFP-H-RasC181S and GFP-H-RasC184S single cysteine mutants demonstrated differences in iodopalmitate incorporation as judged from the apparent ratios of the Western blot and [^{125}I] autoradiographic signals (Figure 3.2, Panels C and D). The GFP-H-RasC181S mutant was more extensively labelled than the GFP-H-RasC184S mutant. This is in agreement with previously published data which determined that the full-length forms of these mutants were palmitoylated to 70% and 40% of wild-type levels, respectively (Hancock *et al.*, 1989). Interestingly, the GFP-H-RasC184S and GFP-N-RasWT chimeras, which are prenylated and palmitoylated in the same positions within their hypervariable domains, showed a similar level of [^{125}I]iodopalmitate incorporation. Mutant chimeras in which all palmitoylated cysteine residues were mutated to serine did not incorporate [^{125}I]iodopalmitate (Figure 3.2, Panel D; GFP-N-RasC181S, GFP-H-RasC181,4S). The GFP control did not incorporate [^{14}C] or [^{125}I] label (Figure 3.2, Panels B and D).

Western blot analysis (Figure 3.2, Panels A and C) indicated that expression levels of the various chimeras did not correlate with the presence or intensity of [^{14}C] or [^{125}I] label incorporation, which may suggest differences in processing efficiency or stability of the attached palmitate. The prenylated chimeras (N-

RasWT, N-RasC181S, H-RasWT, H-RasC181S, H-RasC184S and H-RasC181,4S GFP chimeras) produced a slightly faster mobility on SDS-PAGE than non-prenylated chimeras (N-RasC186S, N-RasC181,6S, H-RasC186S and H-RasC181,4,6S chimeras) as a result of the –aaX proteolysis which occurs during processing of prenylated proteins (Gutierrez *et al.*, 1989). The GFP-K-RasWT and GFP-K-RasKQ chimeras are both prenylated and processed, yet the K-RasKQ chimera demonstrated faster mobility in SDS-PAGE due to neutralization of the polybasic region. This enabled increased association of SDS with the GFP-K-RasKQ chimera, resulting in increased mobility toward the anode.

The metabolic labelling results indicate that the C-terminal 14 amino acids derived from the Ras hypervariable domain are sufficient to direct appropriate "Ras-like" lipidation of a heterologous GFP reporter protein. The CaaX motif cysteine was required to enable both prenylation and subsequent palmitoylation of chimeras incorporating these lipids. Prenylation of a chimera was correlated with an increased mobility in SDS-PAGE, indicating that the appropriate post-translational processing had taken place. In addition, the transposition of the K-Ras4B and H-Ras CaaX motifs had no apparent effect on the lipid modification of these chimeras, as they were determined to be appropriately lipidated.

3.3 Subcellular Fractionation of Transfected COS-7 Cells

Subcellular fractionation of COS-7 cells expressing the various GFP-Ras chimeras was performed in order to examine the distribution of the variably lipidated wild-type and mutant chimeras between cytosolic and membrane fractions (Figure 3.3). Cells were fractionated into post-nuclear total (T), soluble (S100) and particulate or membrane-associated (P100) fractions and the

GFP-Ras chimeras immunoprecipitated from each fraction as described in the Methods.

Of the wild-type chimeras, GFP-K-RasWT and GFP-H-RasWT were membrane-associated and localized almost exclusively to the P100 fraction, but GFP-N-RasWT consistently demonstrated approximately equal partitioning between the S100 and P100 fractions (Figure 3.3). Pulse-chase analysis of full-length GFP-Ras chimeras by Choy and coworkers (1999) also identified a sustained, soluble pool of a full-length GFP-N-Ras chimera which they determined to be prenylated. Unlike the P100-associated form, the GFP-N-RasWT species found in the S100 fraction was not palmitoylated (Figure 3.4).

All chimeric proteins that were farnesylated but lacked the second signal were soluble and were found in the S100 fraction (Figure 3.3, GFP-N-RasC181S, GFP-K-RasKQ and GFP-H-RasC181,4S). The singly palmitoylated H-Ras chimeras (GFP-H-RasC181S, GFP-H-RasC184S) were primarily membrane-associated but demonstrated an increased solubility by their presence in the S100 fraction as compared with the dually palmitoylated GFP-H-RasWT chimera. These mutants are palmitoylated at different positions upstream of the prenylcysteine and demonstrated differential efficiency of membrane association, with GFP-H-RasC181S more prevalent in the P100 fraction than GFP-H-RasC184S. This coincides with the more extensive [^{125}I]iodopalmitate incorporation into the H-RasC181S chimera indicated by the metabolic labelling data (Figure 3.2).

Mutation of the prenylated cysteine to serine resulted in unmodified, soluble fusion proteins that, like the GFP control, were present exclusively in the S100

fraction (Figure 3.3, GFP-N-RasC181,6S, GFP-N-RasC186S, GFP-H-RasC181,4,6S and GFP-H-RasC186S). The exception was GFP-K-RasC185S, which consistently fractionated nearly equally into both the S100 and P100 fractions. Fractionation studies performed by Hancock and coworkers (1991a) localized a similar chimeric protein primarily to the S100 fraction.

These fractionation data confirm the requirement for farnesylation in combination with a palmitate or polybasic second signal for efficient membrane association of the Ras isoforms. The results also suggest that the position of the palmitate second signal in relation to the prenylcysteine may affect the affinity of membrane association, as demonstrated by the distributions of the singly palmitoylated GFP-H-Ras chimeras.

3.4 Subcellular Localization of GFP-Ras Chimeras in Live COS-7 Cells

The intracellular localization of the GFP-Ras chimeras was examined in order to determine whether variable Ras lipidation might direct differential membrane association of the N-Ras, K-Ras4B and H-Ras isoforms. The subcellular localization was initially determined in live COS-7 cells to avoid potential artifacts produced by fixation and permeabilization (Brock *et al.*, 1999) (Figure 3.5). Transiently transfected, live COS-7 cells were observed by confocal microscopy at 41-44 hours post-transfection, as preliminary experiments determined this to be optimal for detecting GFP fluorescence.

Distinct differences were observed in the localization patterns produced by N-RasWT, K-RasWT and H-RasWT GFP chimeras, which are both farnesylated and contain a second signal. GFP-N-RasWT, GFP-K-RasWT and GFP-H-RasWT all localized to the plasma membrane, although the N-RasWT chimera

consistently demonstrated a weaker association than the H-RasWT and K-RasWT chimeras in the majority of cells observed (Figure 3.5). The GFP-H-RasWT and GFP-K-RasWT chimeras also appeared in distinctive filopodial membrane structures which were typically absent in cells transfected with GFP-N-RasWT. GFP-N-RasWT and GFP-H-RasWT were also found in dense, polarized perinuclear structures but GFP-K-RasWT inconsistently produced a very weak, diffuse perinuclear signal. This difference in perinuclear localization was also demonstrated for a full-length GFP-K-Ras4B chimera (Thissen *et al.*, 1997) and for a truncated GFP-K-Ras4B hypervariable domain chimera (Choy *et al.*, 1999). In addition, GFP-N-RasWT, and to a lesser extent GFP-H-RasWT, produced a distinctive, irregular punctate pattern in the cytoplasm of most cells observed.

Loss of either the palmitate or polybasic second signal prevented plasma membrane association and resulted in intracellular accumulation, often concentrated in a dense perinuclear region (Figure 3.5, GFP-N-RasC181S, GFP-K-RasKQ and GFP-H-RasC181,4S). These farnesylated chimeras were excluded from the nucleus in live cells, a phenomenon previously observed by Hancock and coworkers (1991), who observed this using a truncated protein A-K-Ras4B chimera. In some cells these farnesylated chimeras also produced a reticular pattern suggestive of the endoplasmic reticulum (ER). The singly palmitoylated GFP-H-RasC181S and GFP-H-RasC184S chimeras demonstrated perinuclear and variable plasma membrane localization as well as a diffuse cytoplasmic staining (Figure 3.5). It was noted that GFP-H-RasC184S, which is farnesylated and palmitoylated at the same positions within the hypervariable domain as GFP-N-RasWT, demonstrated greater plasma membrane association

than GFP-H-RasC181S. However, unlike GFP-N-RasWT, GFP-H-RasC184S did not display a punctate phenotype, which may suggest that the unique amino acid sequences within these hypervariable domains are involved in determining this differential membrane association.

Unmodified chimeras in which both the farnesylation and second signal were abolished were uniformly distributed throughout the cell, as was the GFP control (Figure 3.5, GFP-N-RasC186S, GFP-H-RasC186S, GFP; not shown: GFP-N-RasC181,6S, GFP-K-RasKQ,C185S, GFP-H-RasC181,4,6S). The GFP-K-RasC185S chimera is unusual in that it retains its polybasic second signal despite the loss of farnesylation. This chimera appeared in the cytoplasm but also produced an intense concentration in discrete areas within the nucleus suggestive of nucleoli, and in some cells also appeared at the plasma membrane and in reticulated endomembranes (Figure 3.5).

In conclusion, the combination of farnesylation and palmitoylation localized the N-Ras and H-Ras chimeras to the plasma membrane, perinuclear region and punctate structures. In contrast, the combination of farnesylation and a polybasic region localized the K-Ras chimera primarily to the plasma membrane. Farnesylation alone appeared to trap the mutant chimeras in a dense perinuclear region, and also served to exclude them from the nucleus. These results also indicate that the transposition of the K-Ras4B and H-Ras CaaX motifs did not affect their ultimate subcellular localizations, as studies conducted in a variety of cell types demonstrated similar differential localizations among the isoforms (Thissen *et al.*, 1997, Choy *et al.*, 1999; Apolloni *et al.*, 2000).

3.5 Colocalization of GFP-Ras Chimeras with Intracellular Membranes

Initial studies of GFP-Ras chimera localization in live COS-7 cells demonstrated that variably lipidated chimeras differentially associated with distinct intracellular membrane structures (Section 3.4). In particular, the wild-type chimeras (GFP-N-RasWT, GFP-K-RasWT and GFP-H-RasWT) and those which were prenylated but lacked a second signal (GFP-N-RasC181S, GFP-K-RasKQ and GFP-H-RasC181,4S) produced variable patterns of localization suggestive of association with the endoplasmic reticulum (ER), Golgi and potentially endocytic and/or exocytic vesicles. To establish the identity of these intracellular structures, colocalization studies were conducted in COS-7 cells using markers specific for the ER, Golgi and endosomes. The GFP-H-RasC186S chimera was chosen to represent all non-lipidated, unmodified chimeras (GFP-N-RasC181,6S, GFP-N-RasC186S, GFP-K-RasKQ,C185S, GFP-H-RasC181,4,6S). GFP-K-RasC185S was unique in that it was also non-lipidated but retained the polybasic second signal. It was included in colocalization studies as a result of its apparent membrane association in live COS-7 cells and presence in the P100 fraction in subcellular fractionation studies.

3.5.1 Colocalization of GFP-Ras Chimeras with the Endoplasmic Reticulum Marker Calreticulin

To determine association with the ER, GFP-Ras chimeras were colocalized with the lumenal ER protein calreticulin (Michalak *et al.*, 1992, Sonnichsen *et al.*, 1994) in COS-7 cells (Figures 3.6a and 3.6b). Calreticulin is found in both perinuclear and reticulated peripheral structures in COS-7 cells as demonstrated in Figures 3.6a and 3.6b.

In fixed cells, the wild-type GFP-Ras chimeras demonstrated differential localizations similar to those found in live cells, as assessed by confocal microscopy. GFP-N-RasWT and GFP-H-RasWT associated with the plasma membrane, perinuclear and irregular punctate structures in the cytoplasm, while GFP-K-RasWT was primarily associated with the plasma membrane (Figures 3.6a and 3.6b). As in live COS-7 cells, GFP-N-RasWT produced a weaker plasma membrane association than the H-RasWT and K-RasWT chimeras in fixed COS-7 cells. However, the perinuclear structure produced by GFP-N-RasWT and GFP-H-RasWT often appeared more diffuse in fixed cells than in live cells. Though all wild-type chimeras did show a diffuse cytoplasmic staining, none appeared to colocalize with either the peripheral or perinuclear calreticulin signals. The GFP (green) and calreticulin (red) signals appeared discrete, and the merged images produced an orange as opposed to yellow color, suggesting the signals were juxtaposed but not truly colocalized (Figures 3.6a and 3.6b). It was noted that the calreticulin signal was weaker in transfected cells than in untransfected cells, a phenomena noted in a similar colocalization study (Roy *et al.*, 1999), but differences in red and green signal intensities did not appear to account for the resulting orange color in the merged image.

Abolition of the second signal prevented plasma membrane association but had variable effects on the intracellular localization of the chimeras. Abolishing palmitoylation of the N-Ras and H-Ras fusion proteins resulted in prenylated chimeras which were localized to a perinuclear structure and demonstrated a grainy or reticulated cytoplasmic pattern (Figures 3.6a and 3.6b, GFP-N-RasC181S and GFP-H-RasC181,4S). These chimeras were partially colocalized with the peripheral calreticulin signal, showing a limited overlap of the green

and red signals. No colocalization with the perinuclear calreticulin signal was observed. Abolishing the K-Ras4B polybasic second signal produced the neutral prenylated GFP-K-RasKQ chimera which was not perinuclear but demonstrated a weaker colocalization with peripheral calreticulin than the prenylated N-RasC181S and H-RasC181,4S chimeras (Figure 3.6a). In contrast to their variable plasma membrane associations in live COS-7 cells, in fixed COS-7 cells the singly palmitoylated GFP-H-RasC181S and GFP-H-RasC184S chimeras both showed the same extent of weak plasma membrane association, as well as perinuclear and diffuse cytoplasmic localizations (Figure 3.6b). Neither of these chimeras appeared to colocalize with peripheral or perinuclear calreticulin.

The non-lipidated chimeras and the GFP control produced a diffuse cytoplasmic signal which did not colocalize with calreticulin (Figures 3.6a and 3.6b, GFP-K-RasC185S and GFP; not shown: GFP-H-RasC186S).

The study of GFP-Ras chimera colocalization with the ER marker calreticulin demonstrated that the combination of farnesylation and either a palmitate or polybasic second signal enabled plasma membrane localization of the wild-type chimeras but did not result in detectable association of these chimeras with peripheral or perinuclear calereticulin. Prenylation alone appeared to mediate a partial colocalization with peripheral calreticulin, and loss of a palmitoylation second signal appeared to promote only a slightly greater colocalization with calreticulin than the loss of a polybasic second signal. Overall, only prenylated chimeras which lacked their respective second signals colocalized with the ER to any significant extent, as determined by colocalization with the ER marker calreticulin.

3.5.2 Colocalization of GFP-Ras Chimeras with the Golgi Marker Giantin

The GFP fluorescence observed as a dense, polarized perinuclear structure in live COS-7 cells expressing certain GFP-Ras chimeras was suggestive of the Golgi apparatus (Figure 3.5). To confirm the identity of this perinuclear structure, colocalization experiments between the GFP-Ras chimeras and the resident medial Golgi protein giantin (Linstedt and Hauri, 1993) were carried out in the absence and presence of nocodazole, a microtubule disrupting agent (De Brabander *et al.*, 1976). The structural integrity of the Golgi is dependent on an intact microtubule network (Thyberg and Moskalewski, 1985); nocodazole disrupts this network by causing depolymerization of microtubules, resulting in Golgi fragmentation and dispersion of the fragments throughout the cytoplasm (Wilson and Jordan, 1994; Thyberg and Moskalewski, 1999). Comparing the distributions of the Golgi fragments with those of the unidentified signal in the presence of nocodazole is useful in confirming the absence or presence of colocalization with a given marker.

Colocalization of the wild-type chimeras with giantin revealed a lipidation-dependent association with this Golgi marker (Figure 3.7a and 3.7b). The prenylated and palmitoylated GFP-N-RasWT and GFP-H-RasWT chimeras presented a striking colocalization with the giantin signal, both in the presence and absence of nocodazole (Figures 3.7a, 3.7b and 3.7c). Additional peripheral and perinuclear structures which were distinct from the giantin signals were also observed. Unlike the wild-type N-Ras and H-Ras chimeras, the prenylated and polybasic GFP-K-RasWT showed no significant perinuclear localization, and did not demonstrate colocalization with giantin in the absence (Figure 3.7a) or presence (not shown) of nocodazole.

Loss of palmitoylation resulted in prenylated chimeras (GFP-N-RasC181S and GFP-H-RasC181,4S) which produced a more diffuse perinuclear signal than observed in live cells (Figure 3.5); these chimeras did not colocalize with giantin either in the absence (Figures 3.7a and 3.7b) or presence (Figure 3.7c, GFP-H-RasC181,4S; not shown: GFP-N-RasC181S) of nocodazole. Neutralization of the K-Ras4B polybasic second signal produced the prenylated GFP-K-RasKQ chimera which was uniformly distributed throughout the cytoplasm and was not colocalized with giantin (Figure 3.7a); nocodazole treatment produced discrete GFP-K-RasKQ and giantin signals (not shown). Therefore farnesylation alone does not appear to mediate Golgi localization, as determined by colocalization with giantin, but must be combined specifically with palmitoylation to confer this association. This is supported by colocalization data from the singly palmitoylated GFP-H-RasC181S and GFP-H-RasC184S chimeras which both show extensive colocalization with giantin in the absence and presence of nocodazole (Figures 3.7b and 3.7c). The intensity of the cytoplasmic and weak plasma membrane signals were reduced to show the fine perinuclear detail and degree of colocalization with giantin. However, like the wild-type chimeras, these mutants presented distinct signals which did not colocalize with giantin.

Non-lipidated chimeras were evenly distributed throughout the cell and did not colocalize with giantin, as was the case for the GFP control (Figures 3.7a and 3.7b, GFP-H-RasC186S (not shown), GFP-K-RasC185S, and GFP; nocodazole images not shown).

This colocalization data proposes that Golgi association of the chimeric GFP-Ras proteins, as assessed by colocalization with giantin, depends upon both the

presence and nature of the second signal. In the absence of a second signal, farnesylation alone did not confer colocalization with giantin. Chimeras which were farnesylated and possessed a palmitate second signal colocalized with giantin (GFP-N-RasWT, GFP-H-RasWT), while the chimera bearing farnesylation and a polybasic second signal did not (GFP-K-RasWT). Notably, the mutant H-Ras chimeras which possessed only one of the two palmitates comprising the complete second signal colocalized with giantin (GFP-H-RasC181S and GFP-H-RasC184S). Of those chimeras demonstrating giantin colocalization, the unidentified peripheral and perinuclear GFP-Ras signals which remained distinct from the giantin signal may represent the asssociation of these chimeras with *cis*- or *trans*-Golgi subcompartments, or with endocytic or exocytic vesicles.

3.5.3 Colocalization of GFP-Ras Chimeras with the Endosome Marker diI-LDL

Giantin colocalization studies demonstrated an extensive but imperfect colocalization between this Golgi marker and the GFP-N-RasWT, GFP-H-RasWT, GFP-H-RasC181S and GFP-H-RasC184S chimeras (Section 3.5.2). In addition, in both live and fixed COS-7 cells, the GFP-N-RasWT and GFP-H-RasWT chimeras produced punctate structures throughout the cytoplasm which did not colocalize with giantin (Sections 3.3 and 3.5.2). To identify these other perinuclear and peripheral structures, colocalization between various GFP-Ras chimeras and the endosome marker diI-LDL was performed in COS-7 cells, both in the absence and presence of nocodazole (Figures 3.8a, 3.8b and 3.8c).

DiI-LDL is prepared by the incorporation of intrinsically-fluorescent diI molecules into the core of low density lipoprotein (LDL) particles (Pitas *et al.*, 1981). Internalization of diI-LDL occurs by receptor-mediated endocytosis

within clathrin coated pits, generating endosomes which ultimately fuse with lysosomes, releasing the core cholesterol (Brown and Goldstein, 1974; Goldstein and Brown, 1974; Anderson *et al.*, 1977). The diI-LDL-containing endosomes present both peripheral and perinuclear signals in COS-7 cells (Figures 3.8a and3.8b). An intact microtubule network is required for the intracellular trafficking of endocytic and exocytic vesicles (Cole and Lippincott-Schwartz, 1995; Lane and Allan, 1998), and nocodazole may be used to redistribute these structures by disrupting the microtubule network. Comparison of the redistributed diI-LDL-endosome signals with the peripheral and perinuclear GFP-Ras signals facilitates the characterization of these unidentified structures.

The perinuclear and punctate structures illuminated by GFP-N-RasWT and GFP-H-RasWT did not colocalize with diI-LDL in the absence of nocodazole (Figures 3.8a, 3.8b). Although the perinuclear GFP-N-RasWT and GFP-H-RasWT signals were in close proximity to the diI-LDL signals, and showed an inconsistent and limited juxtaposition with perinuclear diI-LDL, the signals appeared exclusive and were not colocalized. Nocodazole treatment resulted in dissimilar redistributions of the diI-LDL, GFP-N-RasWT and GFP-H-RasWT signals, and confirmed the absence of colocalization (Figure 3.8c). Similarly, the GFP-K-RasWT chimera did not colocalize with diI-LDL (Figure 3.8a; nocodazole image not shown).

Mutant chimeras which were farnesylated but lacked either the palmitate or polybasic second signal did not colocalize with the peripheral or perinuclear diI-LDL signal in the absence (Figures 3.8a and 3.8b, GFP-N-RasC181S, GFP-K-RasKQ, GFP-H-RasC181,4S) or presence (Figure 3.8c, GFP-H-RasC181,4S;

not shown: GFP-N-RasC181S and GFP-K-RasKQ) of nocodazole. In the absence of nocodazole the singly palmitoylated GFP-H-RasC181S and GFP-H-RasC184S chimeras produced a very limited signal overlap with peripheral structures containing diI-LDL (Figure 3.8b). However, nocodazole treatment produced dispersed GFP-Ras and diI-LDL signals which were clearly distinct (Figure 3.8c). Therefore, the incomplete H-Ras second signal did not appear to promote colocalization with the peripheral or perinculear diI-LDL signals.

Like the GFP control, the non-lipidated chimeras were evenly distributed throughout the cytoplasm and did not colocalize with peripheral or perinuclear diI-LDL (Figures 3.8a and 3.8b, GFP-H-RasC186S (not shown), GFP-K-RasC185S and GFP; nocodazole images not shown).

Table 3.1 summarizes the endomembrane association(s) of each GFP-Ras chimera studied, based on the preceeding colocalization results.

3.6 Time Course Studies of Full-length EGFP-Ras and Truncated RSGFP-Ras Chimeras

The truncated GFP-Ras chimeras were designed to determine the contribution of the unique Ras isoform lipid modifications to their respective endomembrane associations, in the absence of amino acid sequences outside of the hypervariable domain. However, the same amino acid sequences not represented in these chimeric proteins may variably affect the observed diffferential endomembrane association. It was therefore necessary to determine whether the differential subcellular localizations observed among the wild-type GFP-Ras chimeras was representative of the behaviour of the full-length Ras isoforms.

To this end, EGFP-Ras chimeras were obtained in which the full-length, wild-type form of N-Ras, K-Ras4B and H-Ras were appended to the C-terminus of EGFP and subcloned into pEGFP-C1 (Clontech). The subcellular distributions of the EGFP-Ras chimeras in fixed COS-7 cells was determined using confocal microscopy at various time points after transfection. Parallel subcellular distribution studies of the truncated wild-type GFP-Ras hypervariable domain chimeras used throughout this study were also carried out at the same time points. These time-course studies enabled comparison of the final subcellular localizations of the truncated and full-length chimeras observed at 42 hours post-transfection, when all experiments were conducted. For clarity, the truncated GFP-Ras hypervariable domain chimeras will be referred to as "RSGFP-Ras", while the full-length EGFP-Ras chimeras will be referred to as "EGFP-Ras".

3.6.1 Subcellular Distribution of EGFP-Ras Chimeras at Various Time Points

Figure 3.9 demonstrates the progression of the full-length EGFP-Ras chimeras from an initial perinuclear region to the plasma membrane. At eight hours post-transfection, EGFP-N-Ras and EGFP-H-Ras produced a weak but defined perinuclear signal as compared with EGFP-K-Ras4B, which produced a weak but diffuse perinuclear signal. By 12 hours post-transfection, all three chimeras demonstrated a similar, prominent plasma membrane association but only the N-Ras and H-Ras EGFP chimeras produced a dense, brilliant perinuclear signal (Figure 3.9). The perinuclear signal observed with EGFP-K-Ras4B remained weak and diffuse at 12 hours. 24 hours after transfection, EGFP-N-Ras and EGFP-H-Ras developed punctate structures in addition to perinuclear and plasma membrane association. At 24 hours post-transfection, EGFP-K-Ras4B

developed a distinct perinuclear signal in the majority of the cells observed but did not illuminate punctate structures. The extent of perinuclear, plasma membrane and punctate localizations observed for EGFP-N-Ras and EGFP-H-Ras remained relatively constant over the 30, 36 and 42 hour post-transfection time points (36 hours not shown); only an increase in the intracellular and nuclear signals was noted with time. The plasma membrane and perinuclear localization of EGFP-K-Ras4B also remained constant over the 30, 36 and 42 hours post-transfection time points (36 hours not shown), and an increase in the diffuse intracellular and nuclear signals was also observed. By 42 hours post-transfection, all three chimeras demonstrated similar plasma membrane and perinuclear localization.

3.6.2 Subcellular Distribution of RSGFP-Ras Chimeras at Various Time Points

The time course study of the truncated RSGFP-Ras chimeras began at 24 hours post-transfection to parallel the first appearance of RSGFP fluorescence in live COS-7 cells. At 24 hours post-transfection, RSGFP-N-RasWT and RSGFP-H-RasWT appeared in a bright, compact perinuclear region and in scant punctate structures, but no plasma membrane association was observed (Figure 3.10). In contrast, no signal could be detected in cells expressing RSGFP-K-RasWT. The bright perinuclear localization of the N-RasWT and H-RasWT chimeras remained constant over 30, 36 and 42 hours post-transfection, but only a weak perinuclear signal was inconsistently detected for the K-RasWT chimera at these time points. Plasma membrane localization of RSGFP-H-RasWT and RSGFP-K-RasWT, but not RSGFP-N-RasWT, became evident at 30 hours post-transfection. In addition, the N-RasWT and H-RasWT chimeras produced more numerous punctate structures at 30 hours as compared with 24 hours, and at 30

hours all three wild-type chimeras also showed diffuse intracellular and nuclear localizations. By 36 hours post-transfection, RSGFP-N-RasWT had localized to the plasma membrane, but this association was relatively weak compared with the extent of RSGFP-H-RasWT and RSGFP-K-RasWT plasma membrane association at this time point. Both the diffuse intracellular and nuclear signals observed for all three chimeras continued to increase over 30, 36 and 42 hours post-transfection, and by 42 hours the cytoplasmic signal diminished the plasma membrane signal in some cells.

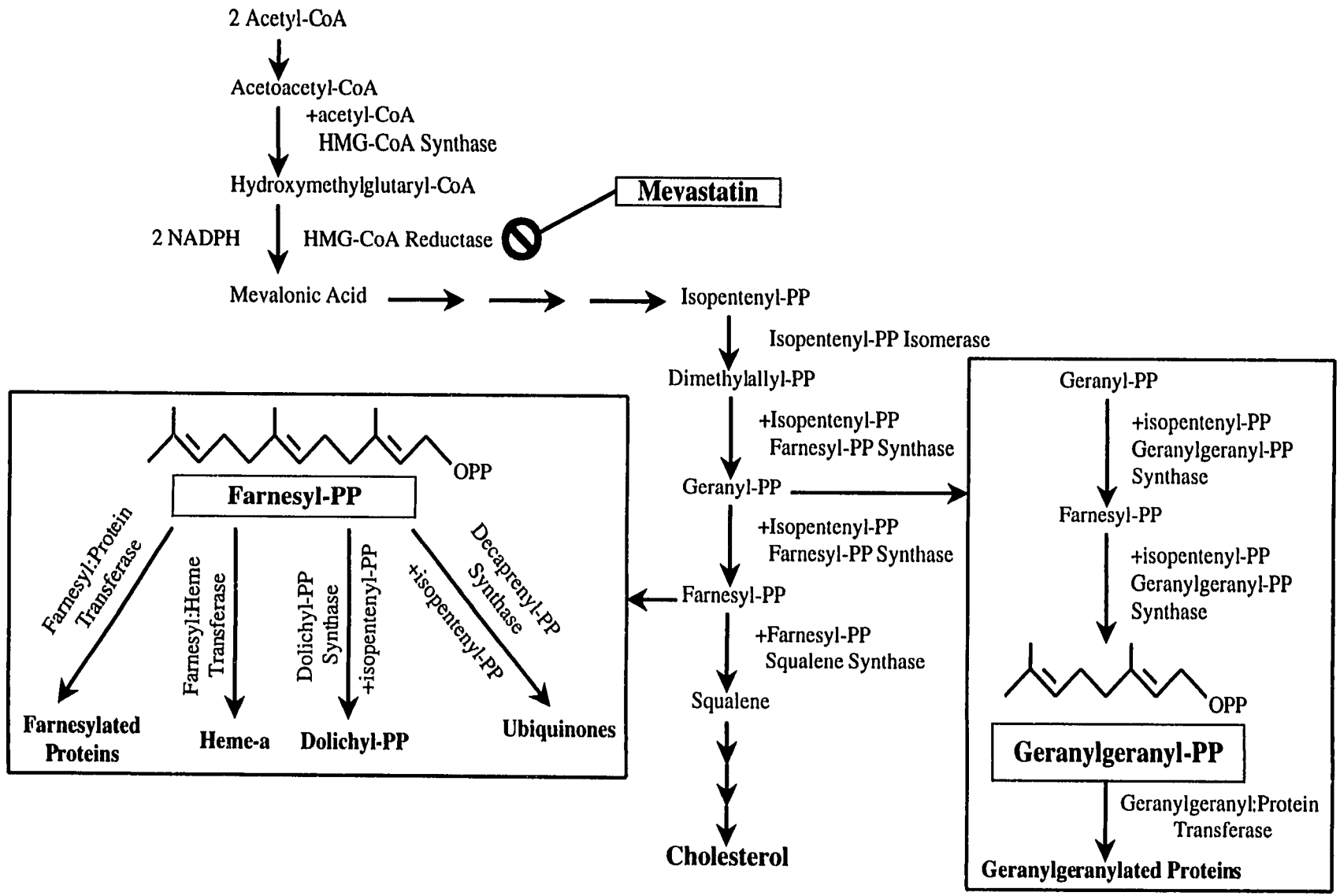
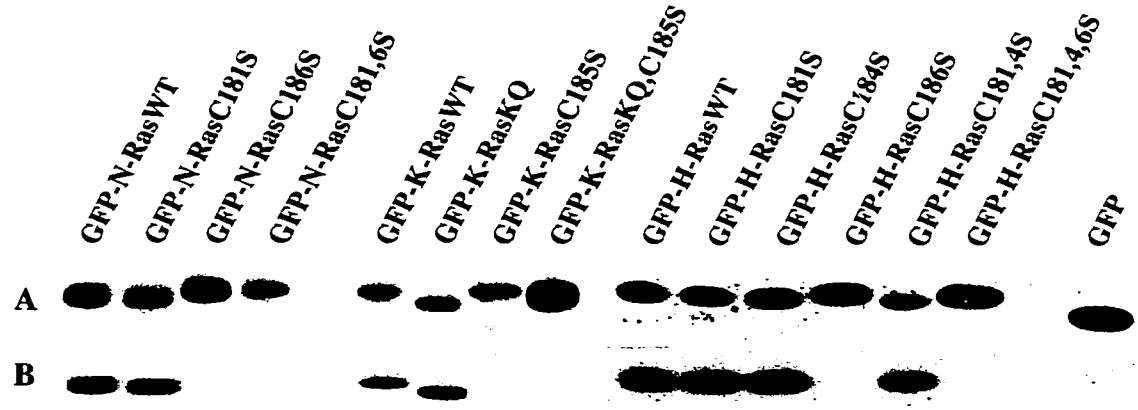


Figure 3.1 Cholesterol Biosynthetic Pathway in Mammalian Cells
Major products are in bold. Adapted from Vance and Vance (1991).

[^{14}C]Mevalonic Acid Lactone Labelling



[^{125}I]Iodopalmitate Labelling

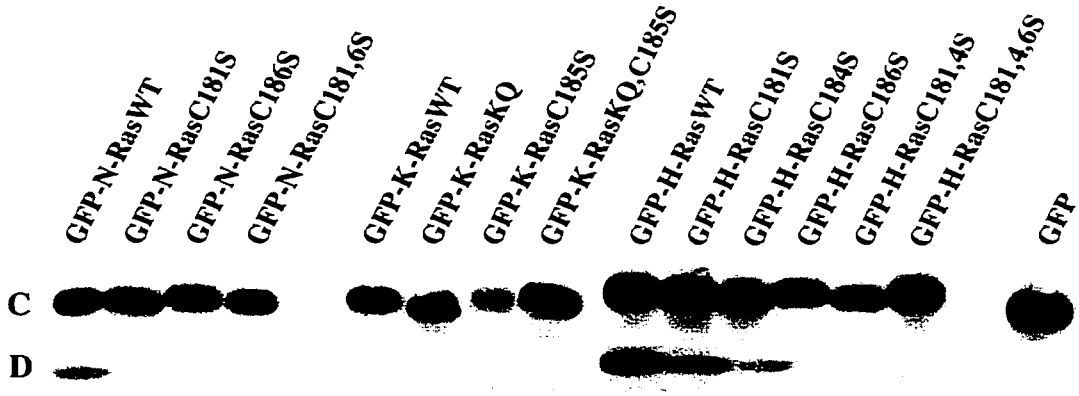


Figure 3.2 Metabolic Labelling of GFP-Ras Chimeras with [^{14}C]Mevalonic Acid Lactone and [^{125}I]Iodopalmitate

Each band represents the respective GFP-Ras chimeras immunoprecipitated from transfected, metabolically labelled COS-7 cells. Panels A and C: Western blot; Panels B and D: autoradiograph.

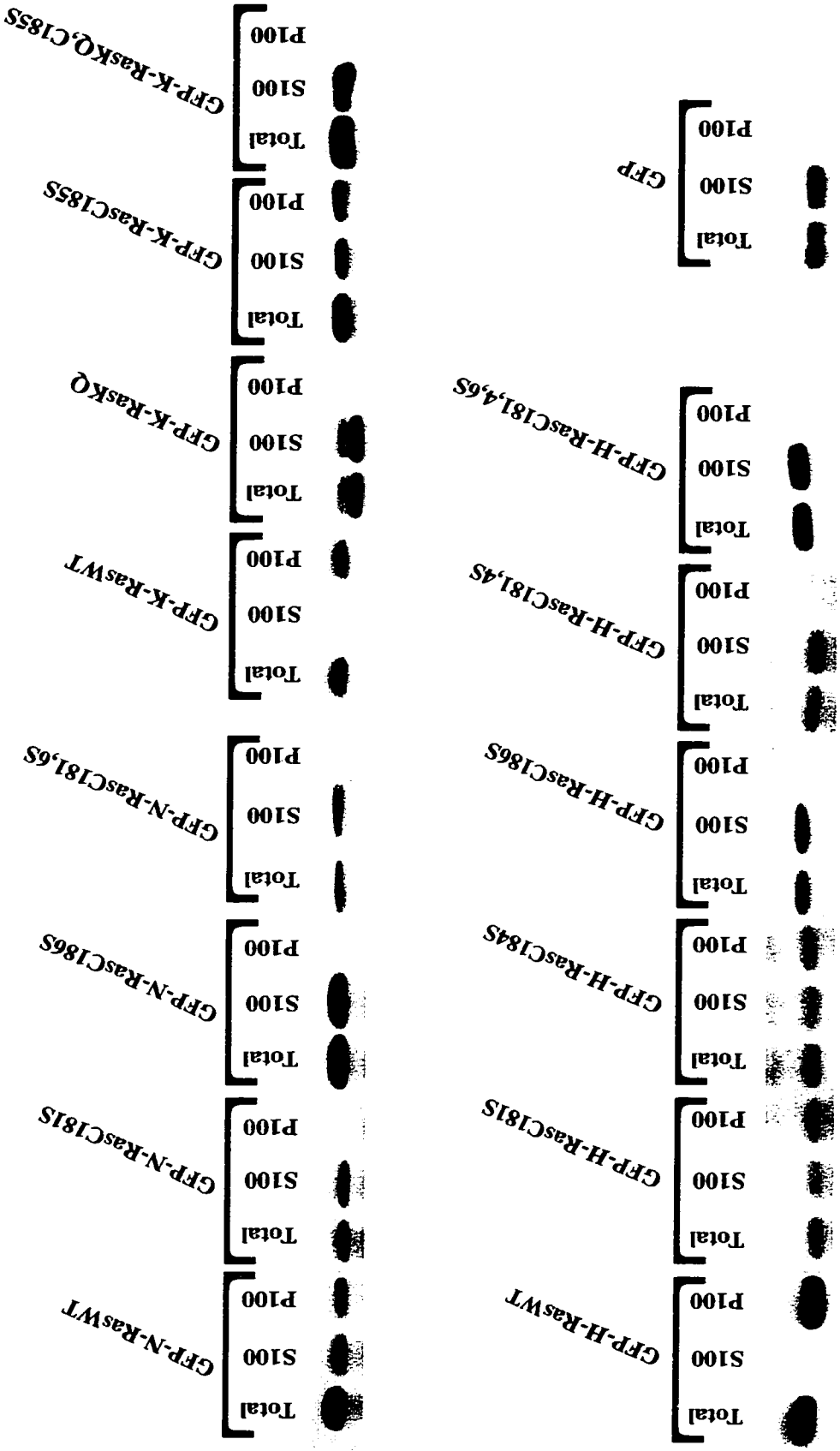


Figure 3.3 Western blot Analysis of GFP-Ras Chimeras Immunoprecipitated from Total, S100 and P100 Fractions Prepared from Transfected COS-7 Cells

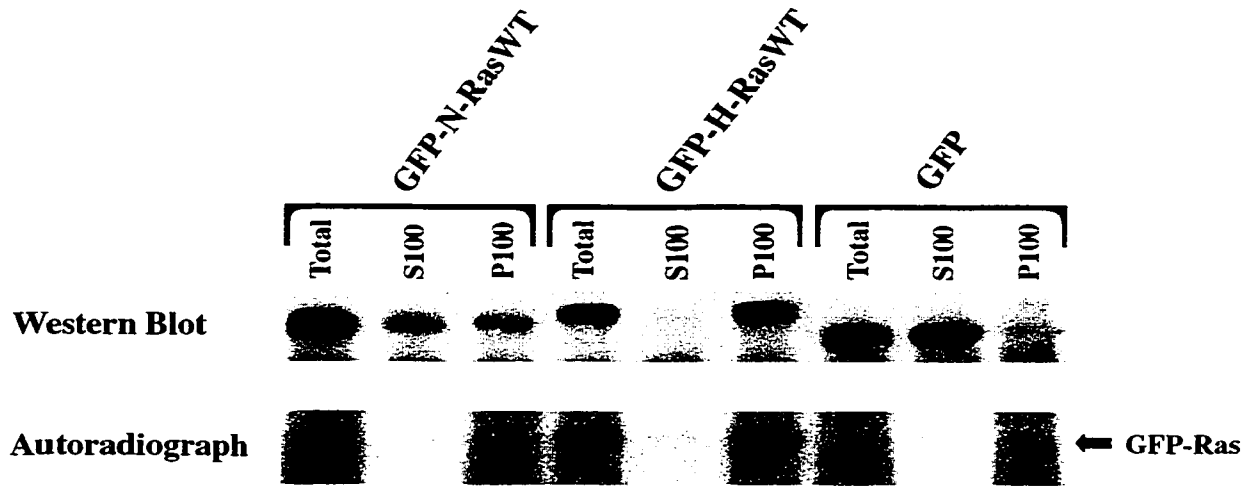


Figure 3.4 Western Blot and Autoradiographic Analysis of GFP-Ras Chimeras and GFP Immunoprecipitated from Total, S100 and P100 Fractions Prepared from Transfected, [^{125}I]Iodopalmitate-Labelled COS-7 Cells

The prenylated and dually palmitoylated GFP-H-RasWT chimera was included as a positive iodopalmitate labelling control. This control also established that no soluble cellular proteins were labelled with iodopalmitate. The GFP protein was included as a negative iodopalmitate labelling control to demonstrate that soluble GFP protein was not labelled with iodopalmitate.

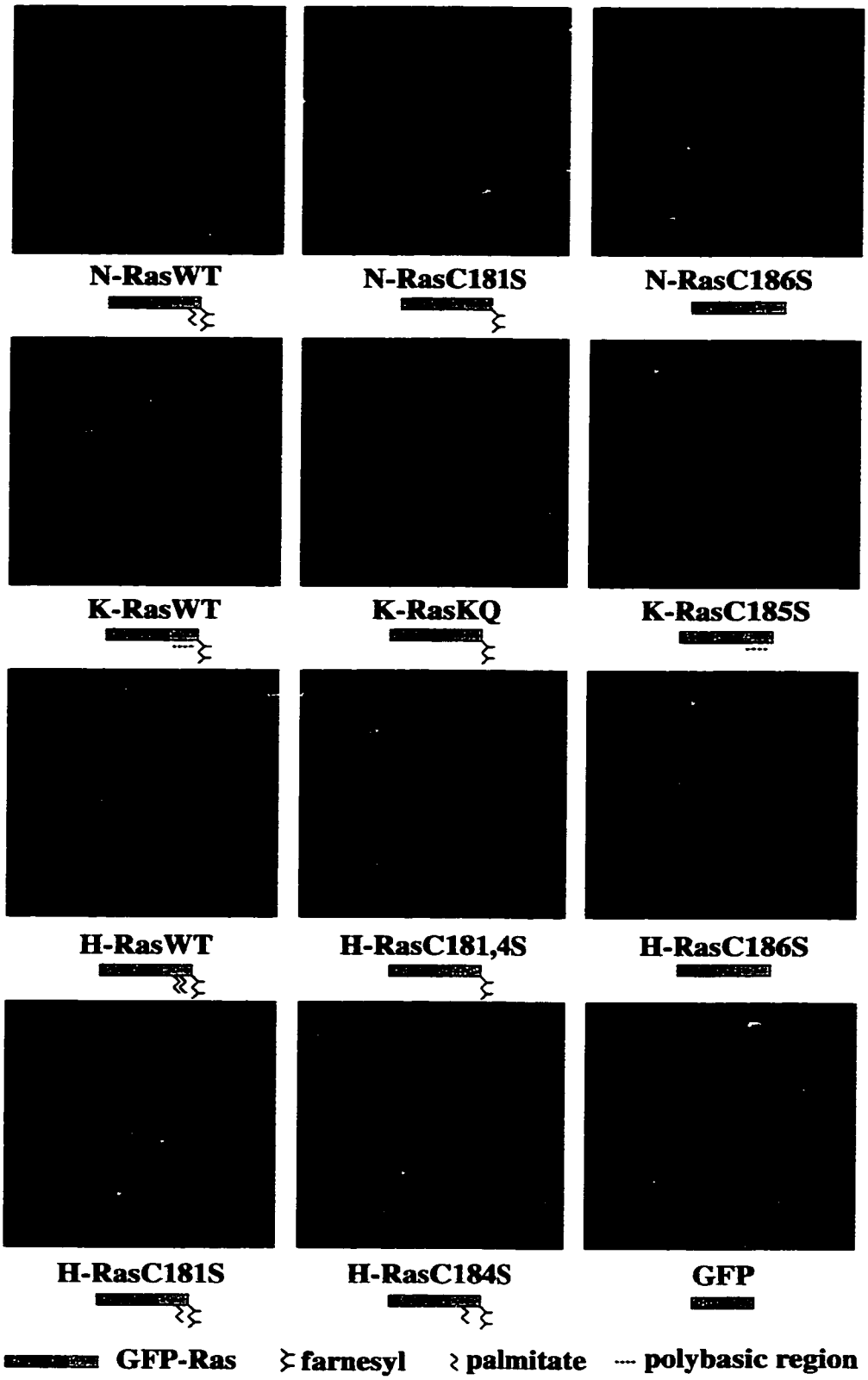


Figure 3.5 Subcellular Localization of GFP-Ras Chimeras in Transiently Transfected Live COS-7 Cells

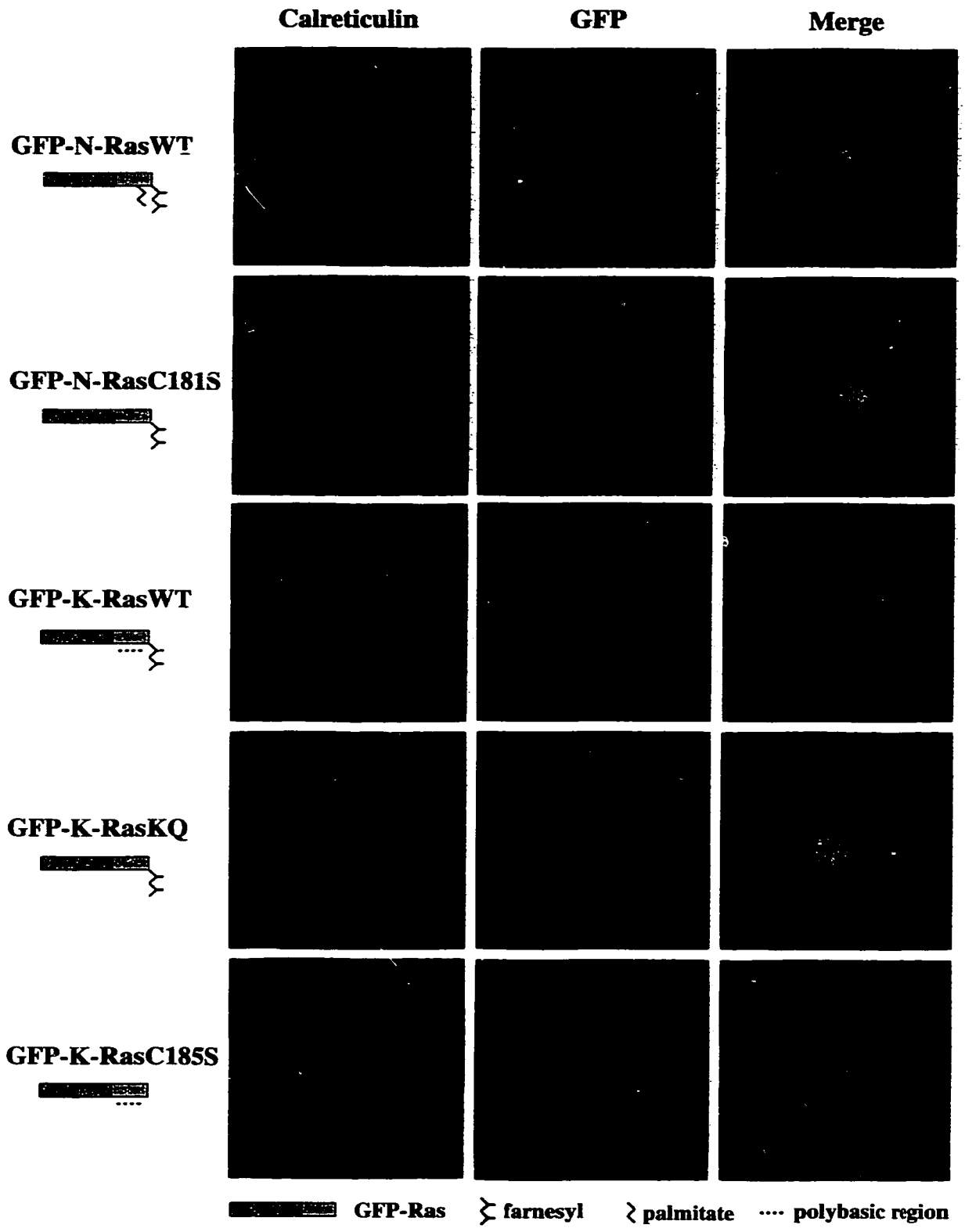


Figure 3.6a Colocalization of GFP-Ras Chimeras with the Endoplasmic Reticulum Marker Calreticulin in COS-7 Cells

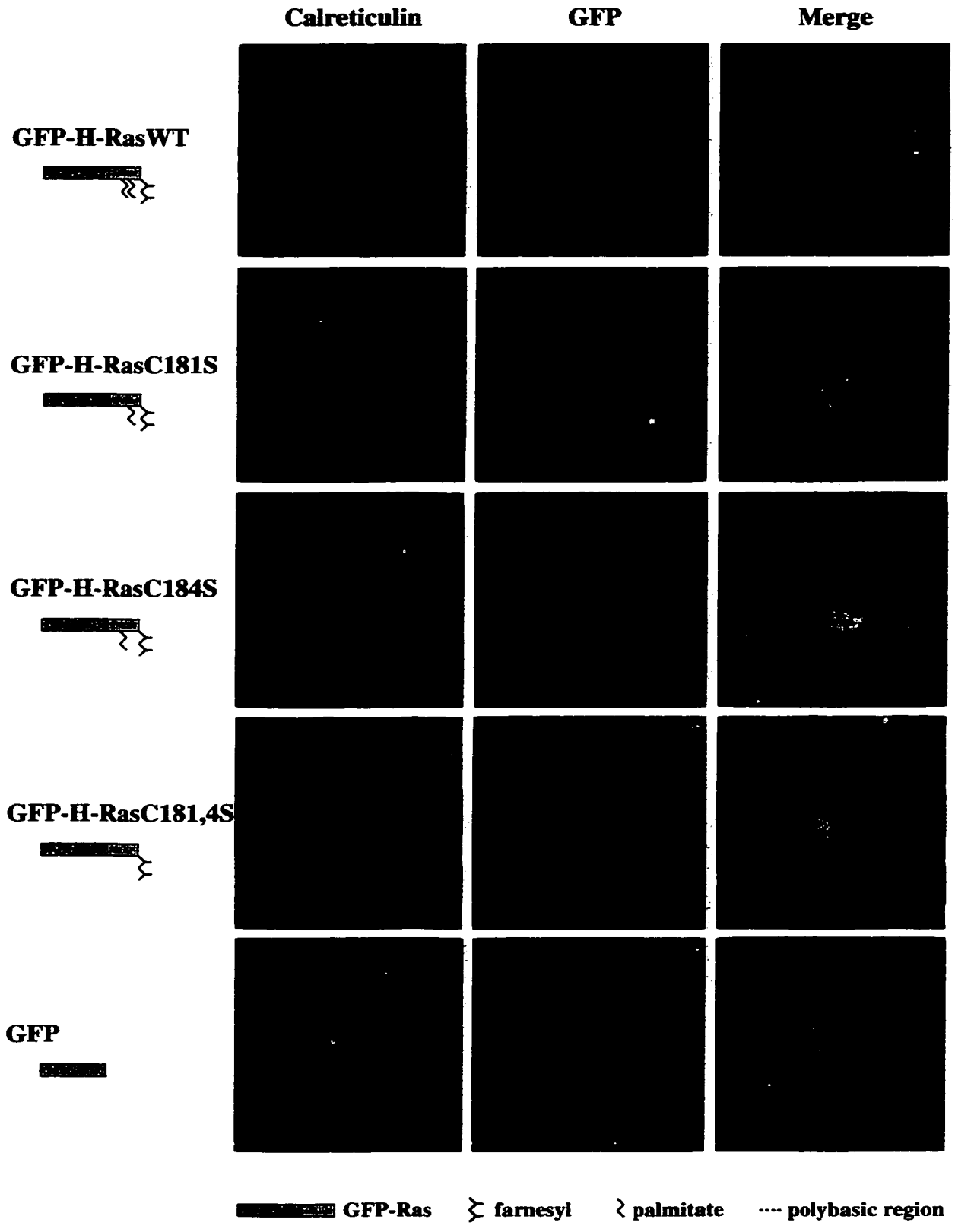


Figure 3.6b Colocalization of GFP-Ras Chimeras with the Endoplasmic Reticulum Marker Calreticulin in COS-7 Cells

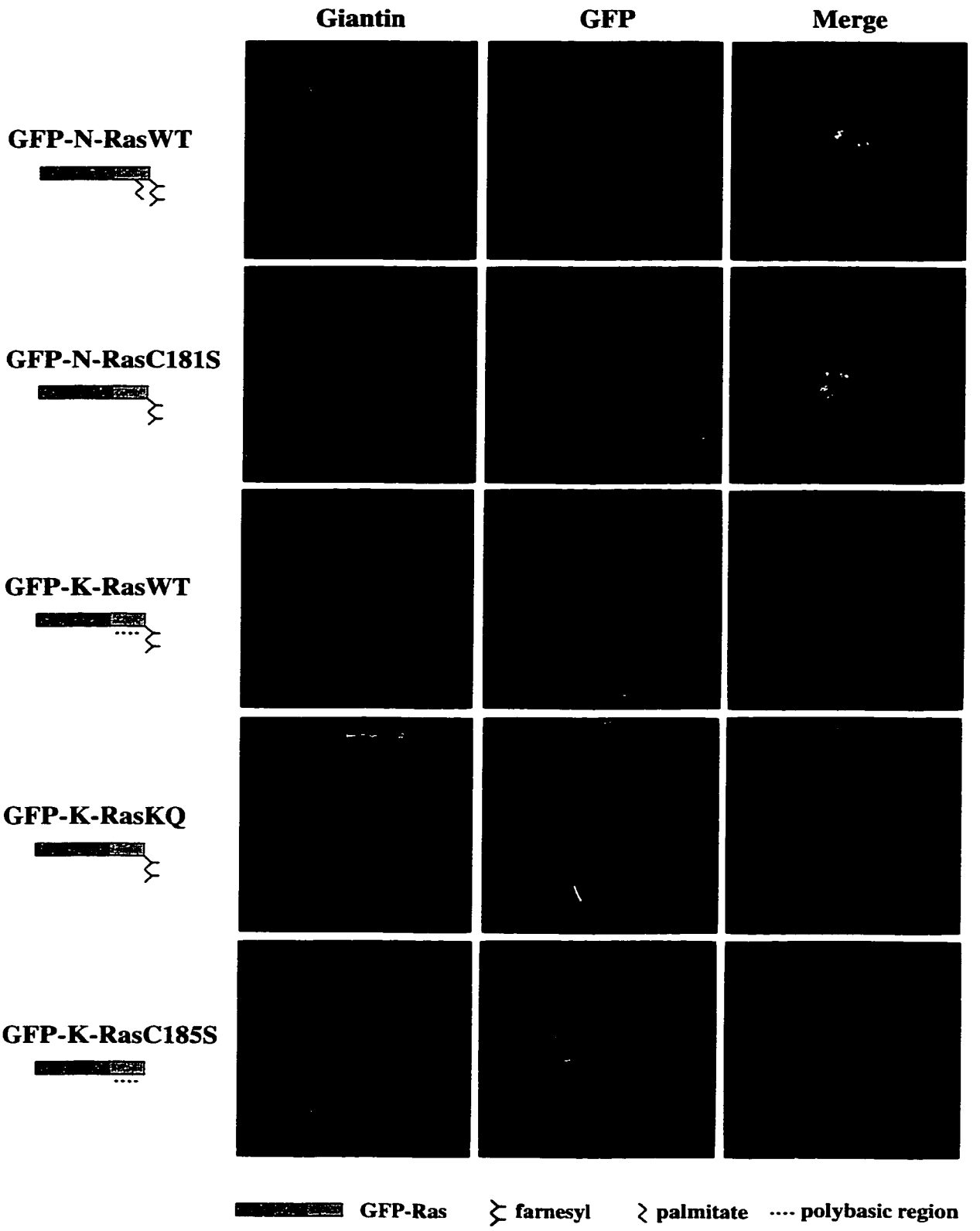


Figure 3.7a Colocalization of GFP-Ras Chimeras with the Golgi Marker Giantin in COS-7 Cells

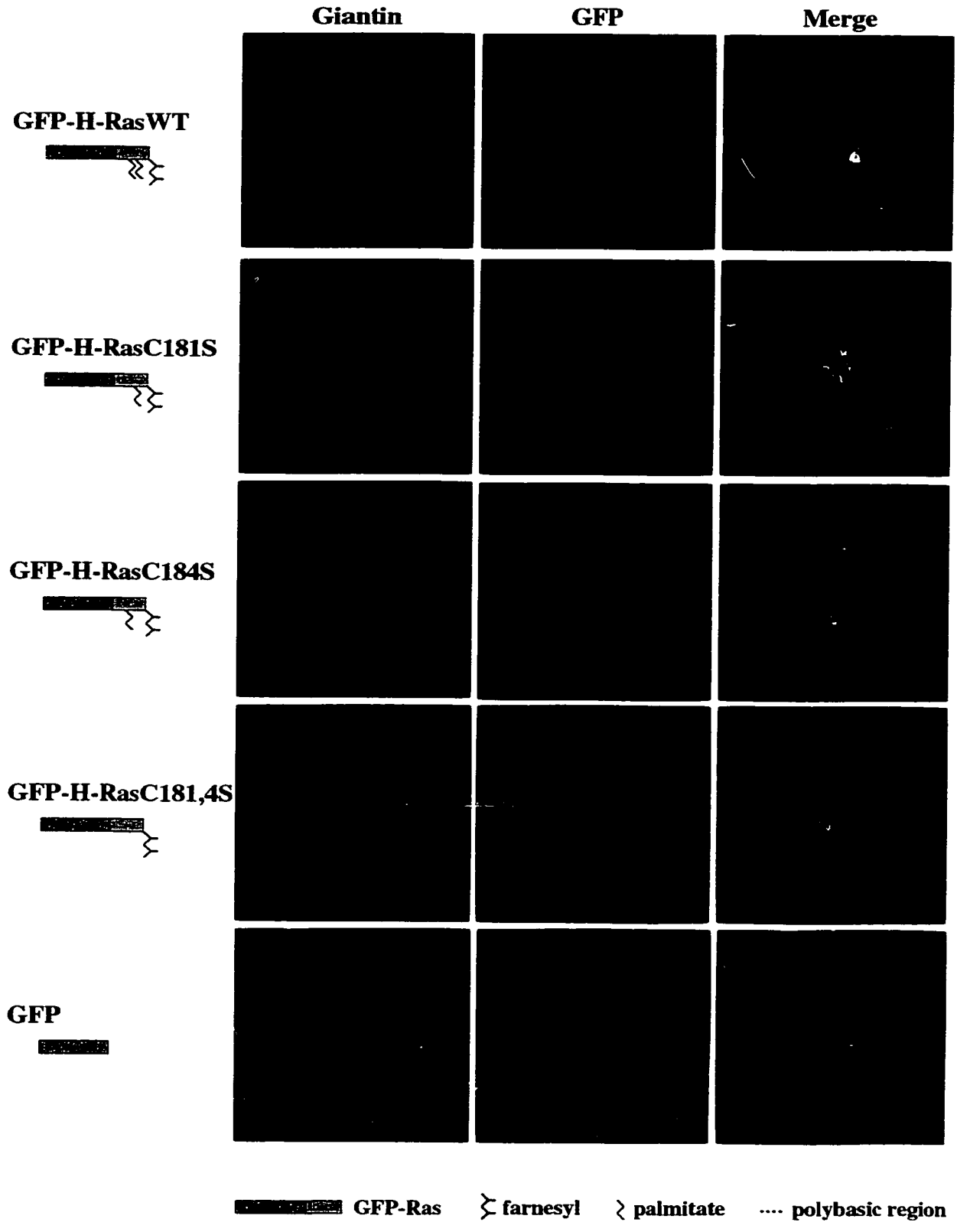


Figure 3.7b Colocalization of GFP-Ras Chimeras with the Golgi Marker Giantin in COS-7 Cells

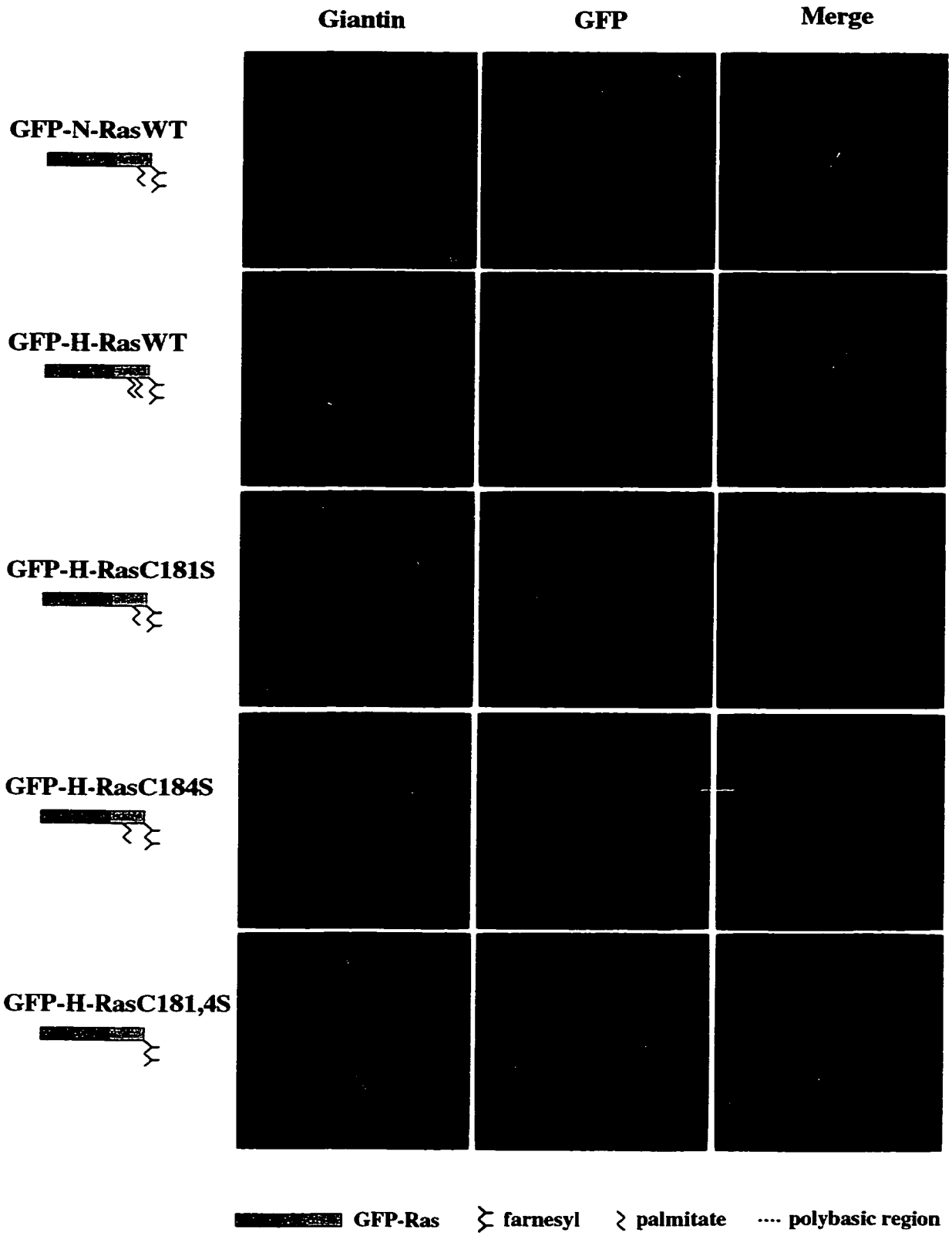


Figure 3.7c Colocalization of GFP-Ras Chimeras with the Golgi Marker Giantin in COS-7 Cells in the Presence of Nocodazole

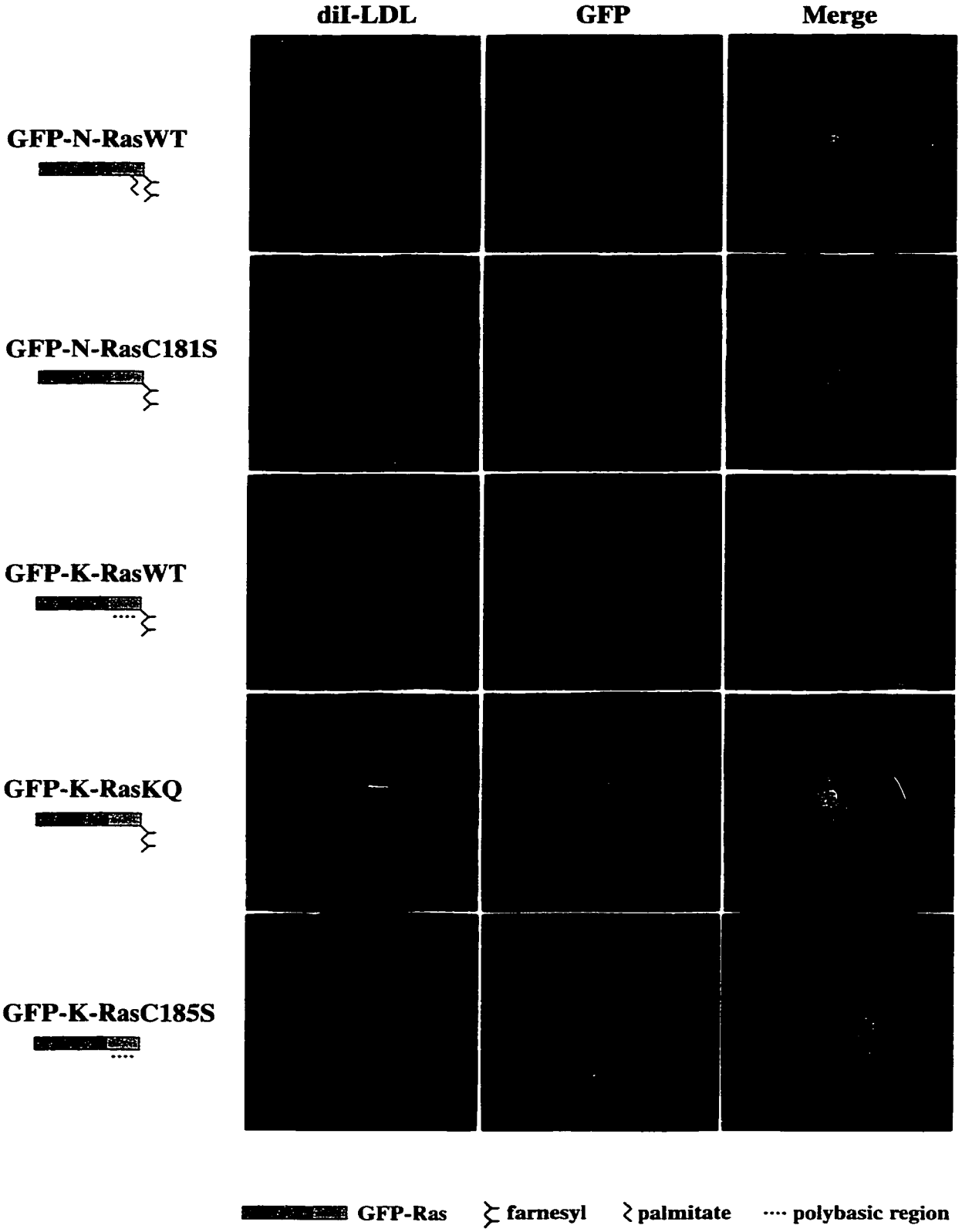


Figure 3.8a Colocalization of GFP-Ras Chimeras with the Endosome Marker diI-LDL in COS-7 Cells

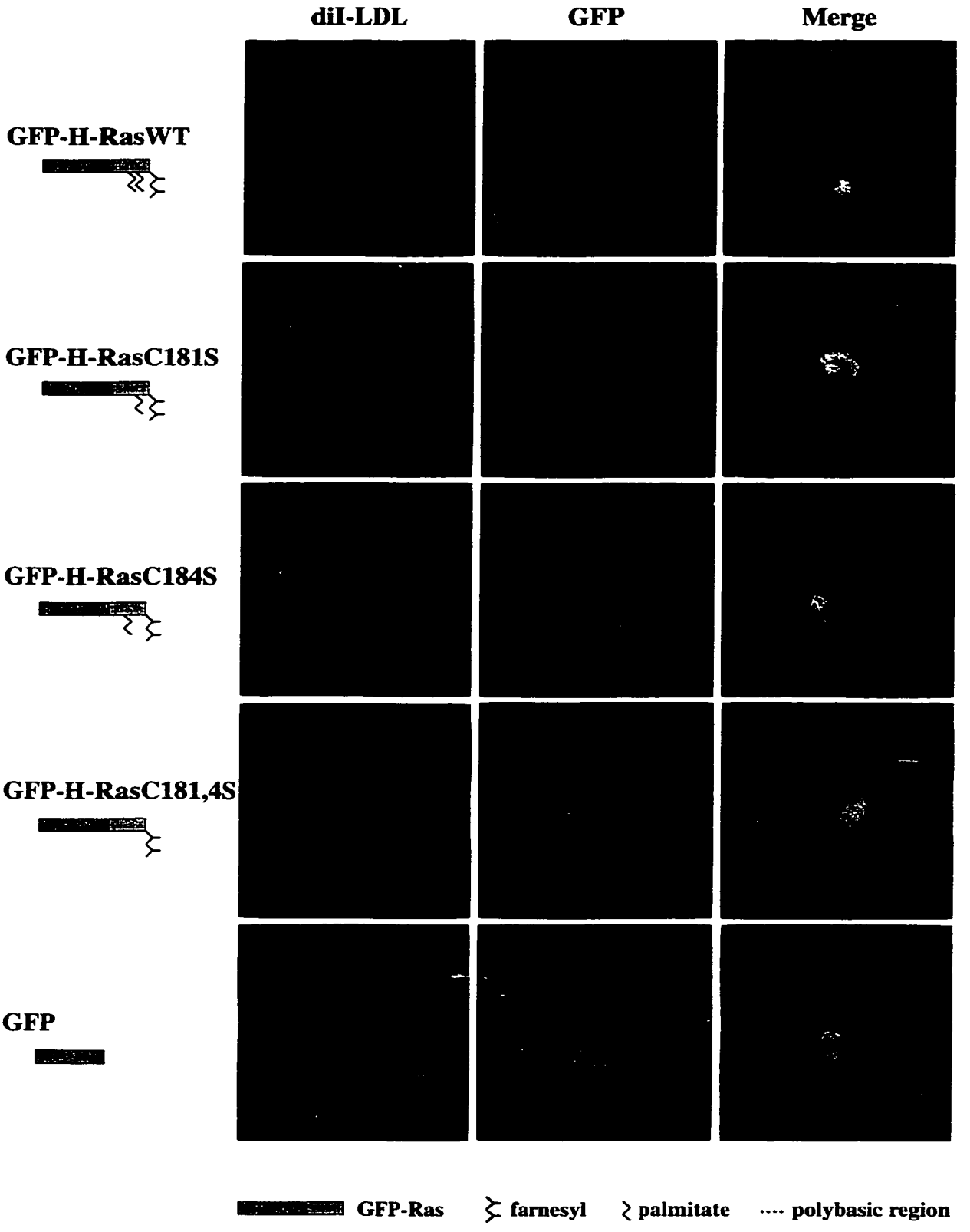


Figure 3.8b Colocalization of GFP-Ras Chimeras with the Endosome Marker diI-LDL in COS-7 Cells

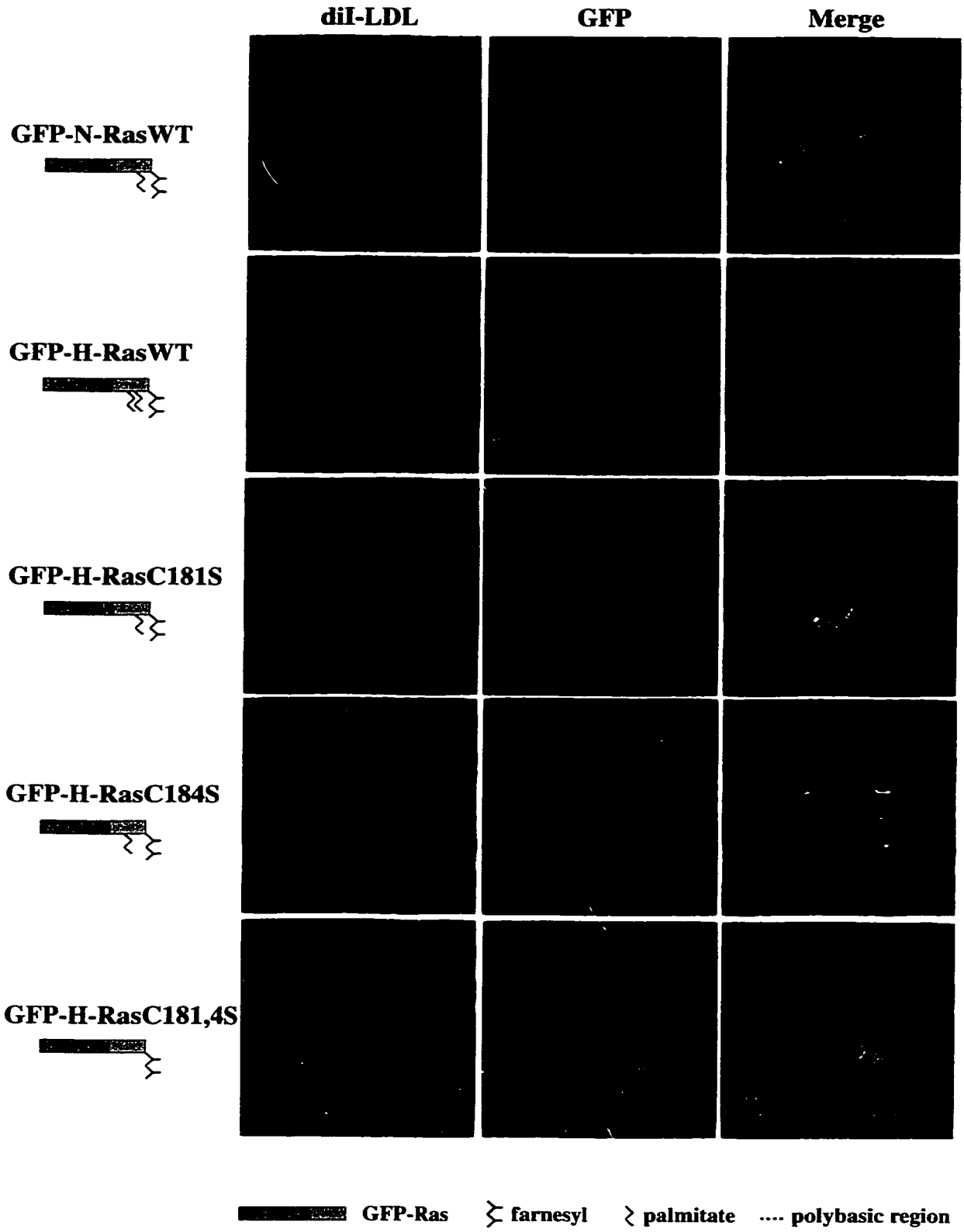


Figure 3.8c Colocalization of GFP-Ras Chimeras with the Endosome Marker di I-LDL in COS-7 Cells in the Presence of Nocodazole

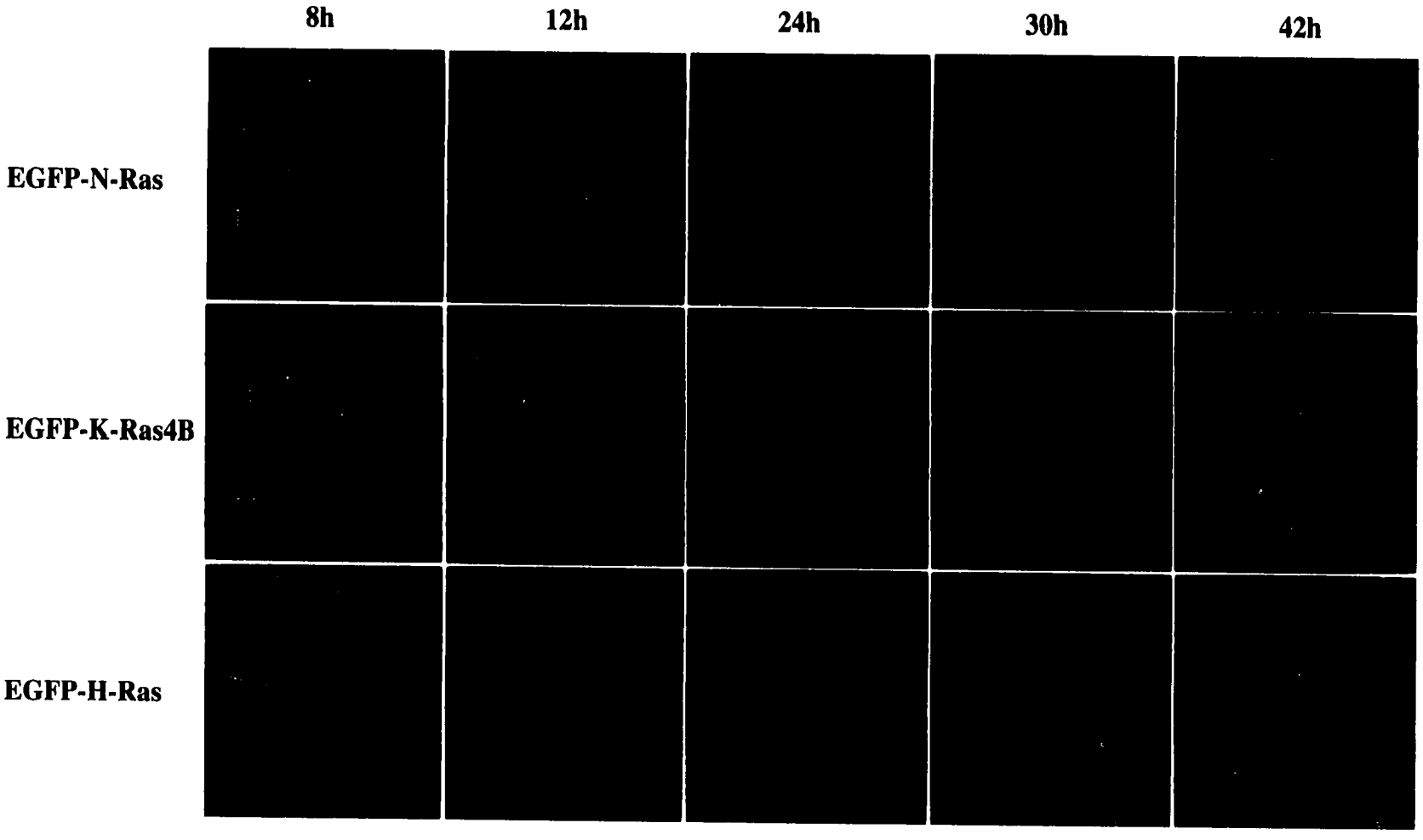


Figure 3.9 Subcellular Distribution of Full-length EGFP-Ras Chimeras in Fixed COS-7 Cells at Various Time Points Post-transfection

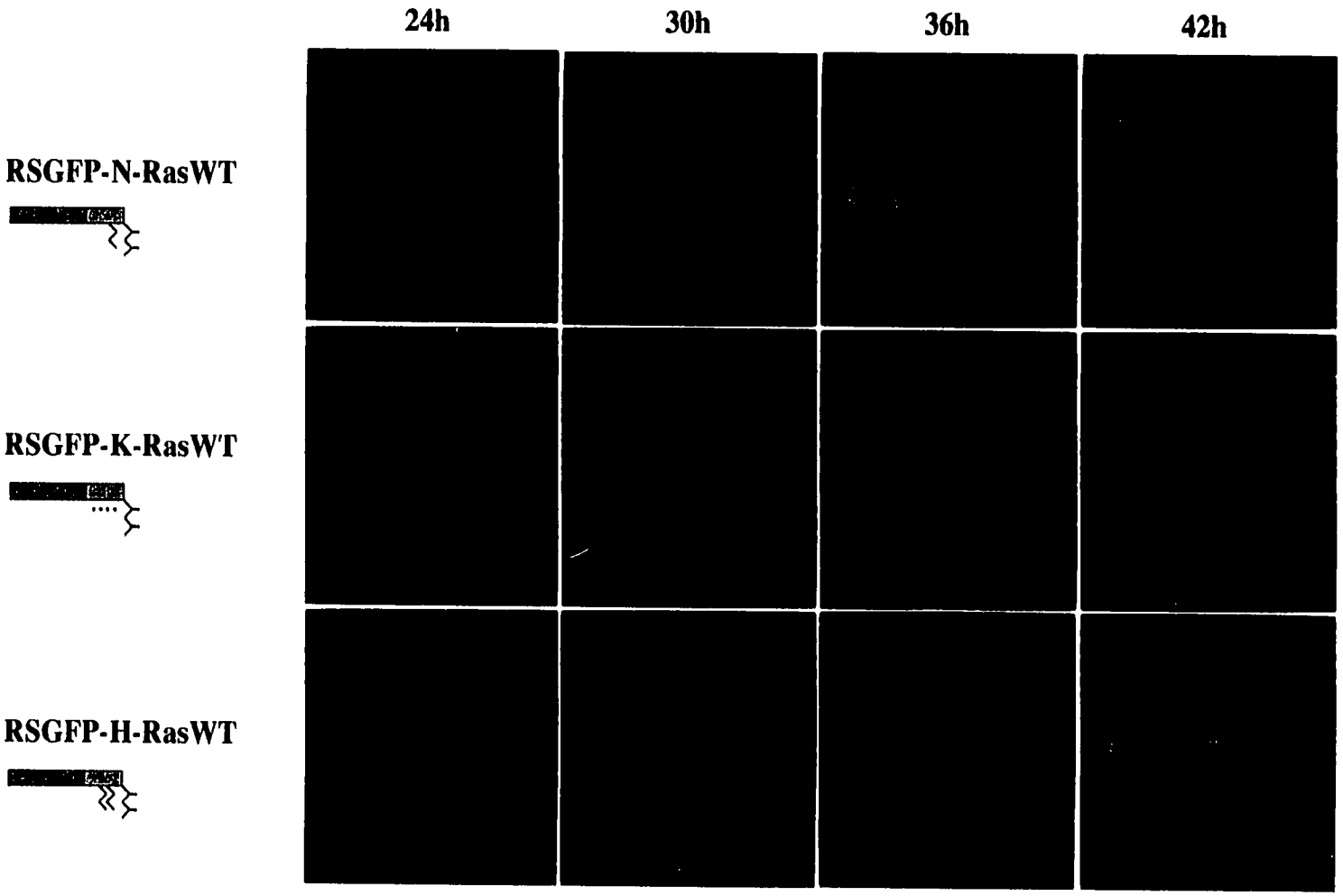


Figure 3.10 Subcellular Distribution of Truncated RSGFP-Ras Chimeras in Fixed COS-7 Cells at Various Time Points Post-transfection

Table 3.1 Summary of GFP-Ras Chimera Endomembrane Association Determined by Colocalization Studies.

Chimeric Protein	Membrane Binding Signal	Endomembrane Association
GFP-N-RasWT	Prenyl@Cys186, Palmitate@Cys181	medial Golgi, PM, punctate structures
GFP-N-RasC181S	Prenyl@Cys186	ER, PN
GFP-K-RasWT	Prenyl@Cys185, Polybasic region	PM
GFP-K-RasKQ	Prenyl@Cys185	ER, PN (most distinct in live cells)
GFP-K-RasC185S	Polybasic region	none
GFP-H-RasWT	Prenyl@Cys186, Palmitate@Cys181 & 184	medial Golgi, PM, punctate structures
GFP-H-RasC181S	Prenyl@Cys186, Palmitate@Cys184	medial Golgi, PM
GFP-H-RasC184S	Prenyl@Cys186, Palmitate@Cys181	medial Golgi, PM
GFP-H-RasC181,4S	Prenyl@Cys186	ER, PN
GFP-H-RasC186S	none	none
RSGFP	none	none

Endomembrane association was determined in transiently transfected, fixed COS-7 cells using confocal microscopy to assess the colocalization of each chimeric GFP-Ras protein with organelle markers described in the text. PM; plasma membrane, ER; endoplasmic reticulum as represented by calreticulin colocalization, PN; unidentified perinuclear structure(s) not colocalized with medial Golgi protein giantin or endosome marker diI-LDL, punctate structures; unidentified cytoplasmic punctate structures which did not colocalize with medial Golgi protein giantin or endosome marker diI-LDL.

4.0 DISCUSSION

4.1 Summary

The data presented in this thesis describe the differential behaviour of variably lipidated GFP-Ras hypervariable domain chimeras, as assessed using radiolabelling, subcellular fractionation and confocal microscopy techniques. The scope of this study is unique in that it collectively compares all major Ras isoform lipidation mutants. The chimeras demonstrated lipidation-dependent differences in the extent of radiolabel incorporation (representing covalent lipid modification), in subcellular fractionation behaviour, and in endomembrane association as determined by colocalization studies. The data are supported by very recent work which correlates differential plasma membrane trafficking of the major Ras isoforms with the unique lipid combinations within their respective hypervariable domains. The results presented here confirm and augment the emerging model of differential Ras trafficking. Our results provide among the first evidence of lipidation-dependent protein trafficking, and suggest that other lipidated proteins may utilize unique trafficking routes to reach their ultimate membrane destinations.

4.2 Radiolabelling Data Suggest Position of Cysteine Preceding the Prenylcysteine Affects Palmitoylation Efficiency

The radiolabelling data confirmed that the Ras hypervariable domain sequences are sufficient within themselves to direct both appropriate "Ras-like" lipidation and plasma membrane association of a heterologous reporter protein, namely GFP. It also confirms that prenylation of the CaaX cysteine in the N-Ras and H-Ras isoforms is essential for subsequent palmitoylation (Hancock *et al.*, 1989).

It is noteworthy that the GFP-N-RasWT chimera and the GFP-H-RasC184S chimera, which are both palmitoylated at cysteine 181 within their hypervariable domains, incorporate iodopalmitate to a similar limited extent. In contrast, the GFP-H-RasC181S chimera, which is palmitoylated at cysteine 184, incorporates iodopalmitate to a much greater extent than either of these chimeras. A similar difference in iodopalmitate incorporation between the H-RasC181S and H-RasC184S single cysteine mutants was previously observed (Hancock *et al.*, 1989). Hancock and coworkers determined that palmitate incorporation onto these H-Ras mutants relative to wild-type H-Ras was 70% for the C181S mutant, and 40% for the C184S mutant. These observations suggest that the cysteine in position 184 is more efficiently palmitoylated than the cysteine in position 181. This may be due to greater accessibility of cysteine 184 to the membrane-associated PAT enzyme. It may also indicate that the palmitate on cysteine 181 has a shorter half-life than the palmitate on cysteine 184. Ultimately, these results suggest that the position of the palmitate second signal in relation to the prenylcysteine may affect the efficiency of palmitoylation or depalmitoylation. In addition, the results demonstrate that the transposition of the K-Ras4B and H-Ras CaaX motifs does not alter the processing of these GFP chimeras, consistent with previous demonstrations that either CaaX motif is sufficient to direct protein prenylation (Casey *et al.*, 1989; Hancock *et al.*, 1989; Kato *et al.*, 1992).

4.3 Subcellular Fractionation Reveals Unusual Distributions

In S100/P100 fractionation experiments, most of the GFP-Ras chimeras produced distributions consistent with published data, with the exception of the GFP-N-RasWT and GFP-K-RasC185S chimeras. Previous pulse-

chase/subcellular fractionation experiments involving expression of full-length N-Ras, H-Ras and K-Ras4B isoforms in various cell types had established that (1) wild-type isoforms associated almost exclusively with the P100 fraction, and (2) mutation of the CaaX cysteine or abolition of the second signal rendered the mutant proteins soluble (Hancock *et al.*, 1989; Hancock *et al.*, 1990; Gutierrez *et al.*, 1989). The data presented here are largely in agreement with the conclusions drawn from the literature. Mutation of the CaaX cysteine to serine or loss of the second signal produced soluble, S100-associated GFP-Ras proteins. Sequential mutation of the cysteine residues comprising the H-Ras second signal produced chimeras which demonstrated increased solubility, but which were differentially associated with the P100 fraction. The H-RasC181S mutant reproducibly exhibited greater association with the P100 fraction than the C184S mutant, in agreement with published data derived using full-length H-Ras mutants (Hancock *et al.*, 1989).

A similar conclusion may also be derived from the labelling and fractionation results: the more proximate the palmitate to the prenylcysteine, the greater the membrane association, which in turn may promote more efficient palmitate incorporation by the putative, membrane-associated PAT enzyme. Conversely, it may result in a less efficient deacylation by the putative, cytosolic acyl-protein thioesterase enzyme (APT1; Duncan and Gilman, 1998). Mutational studies of the myristoylated, dually palmitoylated Fyn tyrosine kinase analogously reveal that palmitoylation of the cysteine nearest the myristate facilitates more efficient association with cellular membranes (Alland *et al.*, 1994). Despite greater iodopalmitate incorporation and membrane association of GFP-H-RasC181S, Willumsen and coworkers estimated the transformation efficiency of full-length

H-RasC181S to be lower than that of H-RasC184S (Willumsen *et al.*, 1996). This may result from a lower apparent plasma membrane association of the H-RasC181S chimera as compared with the H-RasC184S chimera. This is supported by our live confocal data which suggests that the H-RasC184S chimera demonstrates greater plasma membrane association than the H-RasC181S chimera.

Two striking differences in subcellular distribution, as compared with the literature, were evident in the fractionation behaviour of the GFP-N-RasWT and GFP-K-RasC185S chimeras. The N-RasWT chimera was the only wild-type chimera present in both the soluble and particulate fractions, and was equally distributed in both. The soluble GFP-N-RasWT species appeared to be fully processed, as it demonstrated the same electrophoretic mobility as the species found in the P100 fraction, which is known to be prenylated and palmitoylated (Magee *et al.*, 1987; Gutierrez *et al.*, 1989). Choy and coworkers have identified a similar soluble pool of full-length GFP-N-Ras protein which had a half-life of two hours (Choy *et al.*, 1999). While this soluble GFP-N-RasWT species was shown to be prenylated (Choy *et al.*, 1999), we demonstrated that our soluble GFP-N-RasWT chimera was not palmitoylated. That such a large soluble species of incompletely modified N-RasWT chimera exists suggests that palmitoylation and subsequent plasma membrane delivery of N-Ras may be less efficient than that of H-Ras. It could also imply that palmitate turnover is comparatively higher on N-Ras. The half-life of palmitate on N-Ras has been estimated at 20 minutes, as compared with 90 minutes for H-Ras (Magee *et al.*, 1987; Liu and Hoffman, 1995). In addition, the study by Choy and coworkers determined that only 10% of endogenous Ras associates with cellular

membranes, with the remaining cytosolic pool being degraded before membrane association. Within this context, our results also suggest that membrane association of N-Ras is inefficient as compared to K-Ras4B and H-Ras.

The unexpected association of the non-prenylated GFP-K-RasC185S chimera with the P100 fraction may represent an electrostatic interaction which was not disrupted by the magnesium concentration present in the fractionation buffers. S100/P100 fractionation of a full-length K-RasC185S protein by Hancock and coworkers localized the protein exclusively to the S100 fraction (Hancock *et al.*, 1990). The hypotonic lysis buffer utilized in that study contained 5mM magnesium chloride, which was 25-fold higher than the 0.2mM magnesium chloride concentration used in our study. The low magnesium chloride concentration in our lysis buffer may not have been sufficient to disrupt electrostatic interactions with cellular membranes mediated by the intact polybasic domain present in this chimera. However, biophysical studies of K-Ras4B hypervariable domain peptides have suggested that the interaction of this isoform with cellular membranes is largely electrostatic as opposed to hydrophobic, and that it may discriminate between membranes of differing anionic lipid composition, preferentially interacting with those rich in anionic phospholipids (Leventis and Silvius, 1998). In an analagous study, a non-palmitoylated $G_{12\alpha}$ mutant was still found to be associated with the particulate fraction. The polybasic nature of the sequences surrounding the palmitoylation site were suggested to contribute electrostatic binding interactions (Jones and Gutkind, 1998). Fractionation of the GFP-K-RasC185S chimera using lysis buffers of varying magnesium chloride concentrations may establish the extent of electrostatic interaction.

4.4 Live Confocal Microscopy and Colocalization Studies Indicate Differential, Lipidation-dependent Membrane Association

The subcellular localization of the various wild-type and mutant GFP-Ras chimeras was initially established in live COS-7 cells to avoid potential artifacts induced by fixation and permeabilization, such as protein redistribution (Brock *et al.*, 1999) or nuclear concentration (Hancock *et al.*, 1990). Distinct, lipidation-dependent differences in localization were observed for the wild-type and second signal-deficient GFP-Ras chimeras observed in live and fixed COS-7 cells.

4.4.1 Distinct, Lipidation-Dependent Membrane Associations are Apparent in Live COS-7 Cells

The wild-type GFP-Ras chimeras demonstrated distinct, reproducible differences in subcellular distribution in live COS-7 cells. In addition to plasma membrane localization, only the N-RasWT and H-RasWT chimeras demonstrated punctate cytoplasmic and compact perinuclear signals. In contrast, the K-RasWT chimera was present primarily at the plasma membrane, with only a diffuse perinuclear signal evident. This differential membrane association was not only observed in two recent studies characterizing Ras trafficking (Choy *et al.*, 1999; Apolloni *et al.*, 2000), but also in earlier immunofluorescence studies. These early studies utilized full-length GFP-Ras chimeras, truncated GFP-Ras hypervariable domain chimeras and full-length Ras proteins (Hancock *et al.*, 1991a; Thissen *et al.*, 1997). Although these differences were not the focus of those studies, they provided the first evidence that variable Ras lipidation mediated differential membrane associations.

In live COS-7 cells, loss of the palmitate or polybasic second signal within the respective mutant GFP-Ras chimeras prevented plasma membrane localization but retained them on similar intracellular structures. Additionally, the presence of the prenyl moiety excluded these chimeras from the nucleus, a phenomenon previously noted by Hancock and coworkers (1991a). The N-RasC181S, H-RasC181,4S and K-RasKQ chimeras, which lacked the second signal, all associated with reticular endomembranes and accumulated on a distinctive perinuclear structure. In addition, neither the N-RasC181S or H-RasC181,4S chimera produced the punctate structures observed with their wild-type forms. The singly palmitoylated GFP-H-RasC181S and GFP-H-RasC184S mutants exhibited perinuclear and variable plasma membrane association, with the C184S mutant demonstrating more extensive plasma membane association. Interestingly, the H-RasC184S chimera, which is palmitoylated and prenylated on the same cysteine residues as the N-RasWT chimera, did not produce the same prominent punctate structures observed for the latter chimera. These chimeras differ only with respect to the amino acid residues surrounding the lipidated cysteines. This may indicate a role for these intervening amino acids in specific membrane association, although this is unlikely since the punctate-producing H-RasWT chimera is identical to the H-RasC184S chimera except for the presence of an additional palmitate. It is more likely to indicate that both of the palmitate moieties comprising the H-Ras second signal are required for efficient incorporation into the motile, coalescing transport vesicles described by Choy and coworkers (1999), similar to the punctate structures observed in our studies.

Mutation of the N-Ras and H-Ras CaaX cysteine to serine in any of the chimeras

produced unmodified, soluble fusion proteins which did not appear to associate with any subcellular structure. Mutation of the K-Ras CaaX cysteine produced an unprenylated but polybasic chimera which associated with reticular endomembranes, was present in the nucleus, and concentrated within discrete nuclear substructures suggestive of nucleoli. Although other unmodified chimeras concentrated in the nucleus, none were densely concentrated in similar discrete structures. Hancock and coworkers (1990) previously concluded that in the absence of a CaaX motif, the K-Ras4B polybasic domain had no specific nuclear targeting function. The relevance of this nuclear concentration remains obscure, as the identity of these nuclear substructures was not established.

4.4.2 Colocalization Studies Identify Lipidation-dependent Membrane Associations of the Variably Lipidated GFP-Ras Chimeras

The identification of the reticular, perinuclear and punctate structures required colocalization of the GFP-Ras chimeras with various organelle markers. Calreticulin was chosen as the endoplasmic reticulum (ER) marker as it produced distinct perinuclear and peripheral signals. The alternative ER marker calnexin was also examined but the calnexin-c antibody utilized produced a more diffuse, grainy pattern at all dilutions attempted, which was difficult to interpret. The medial Golgi marker giantin was used to identify the perinuclear signal. Mannosidase II was also attempted as a Golgi complex marker, but did not produce a useful signal in COS-7 cells. The endosome marker diI-LDL was chosen as an endosome marker as it produced distinctive peripheral and perinuclear signals in COS-7 cells (McCabe and Berthiaume, 1999).

4.4.2a Calreticulin Colocalization

No significant colocalization between any of the wild-type GFP-Ras chimeras

and the ER marker calreticulin could be demonstrated, consistent with the findings of Apolloni and coworkers (2000). This implies that clearance of wild-type Ras isoforms from the ER is rapid. In contrast, GFP-Ras chimeras lacking the second signal colocalized with calreticulin to varying extents. In our study, the reticular GFP-Ras pattern present in live cells was somewhat dissipated and less distinct in fixed cells, often appearing grainy. Despite this, the loss of palmitoylation (on N-RasC181S and H-RasC181,4S) appeared to mediate a more extensive calreticulin colocalization than loss of a polybasic domain (on K-RasKQ). In addition, the K-RasC185S chimera, which produced a reticular pattern in some live cells, was not colocalized with calreticulin. The weaker ER association of the K-RasKQ second signal mutant may reflect faster ER clearance resulting from the more efficient farnesylation and carboxymethylation of the K-Ras4B CaaX motif as compared with the N-Ras and H-Ras CaaX motifs (James *et al.*, 1995; Choy *et al.*, 1999). Conversely, it may be consistent with the current model of differential Ras isoform trafficking, which proposes that the route of plasma membrane delivery diverges after prenylation at the ER, in a manner dependent on the nature of the second signal.

Trafficking of N-Ras and H-Ras, but not K-Ras4B, appears to follow the secretory pathway, and palmitoylation of these isoforms appears to occur on ER or ER-Golgi intermediate membranes (Apolloni *et al.*, 2000). In contrast, K-Ras4B appears to rapidly traffic directly from the ER to the plasma membrane in a manner not requiring Golgi or vesicular mediation (Choy *et al.*, 1999). Palmitoylation has been postulated to behave as the "exit" signal required for efficient release of the N-Ras and H-Ras isoforms from ER membranes, allowing subsequent association with the Golgi and vesicular delivery to the

plasma membrane (Apolloni *et al.*, 2000). This may explain why the GFP-H-RasC181S and GFP-H-RasC184S single cysteine mutants did not colocalize with calreticulin; the presence of even a single palmitate enabled relatively efficient release from ER membranes.

4.4.2b Giantin Colocalization

While none of the wild-type chimeras colocalized with calreticulin, the GFP-N-RasWT and GFP-H-RasWT chimeras, but not GFP-K-RasWT, were extensively colocalized with the Golgi marker giantin. Similarly, the singly palmitoylated H-RasC181S and H-RasC184S chimeras were extensively colocalized with giantin. These colocalizations were imperfect, however, and additional peri-Golgi GFP-Ras signals were observed, both in the absence and presence of nocodazole.

Although loss of the second signal resulted in the retention of the mutant chimeras on ER membranes, it did not appear to mediate colocalization of any of these chimeras with the medial Golgi marker giantin. The N-RasC181S and H-RasC181,4S second signal mutants showed no giantin colocalization, and often demonstrated a less distinctive perinuclear signal in fixed cells as compared with live cells. The perinuclear signal present in live cells expressing the K-RasKQ second signal mutant was absent in fixed cells. A similar loss of resolution in fixed cells was also observed by Choy and coworkers in a comparable study of Ras localization (Choy *et al.*, 1999). Despite this, the existing perinuclear signals produced by the N-RasC181S and H-RasC181,4S chimeras appeared interposed to the giantin signal, aligning near or around, but not with, the giantin signal. These results contrast those of Choy and coworkers

(1999), who concluded that the perinuclear signal produced by GFP-RasCaaX chimeras, which lacked all sequences upstream of the CaaX motif, represented the Golgi complex. This conclusion was not confirmed with specific colocalization data, but was instead extrapolated from their colocalization of full-length GFP-N-Ras with the medial Golgi marker mannosidase II. Since giantin is a medial Golgi protein (Linstedt and Hauri, 1993), this apparent lack of colocalization between giantin and our N-RasC181S and H-RasC181,4S mutants does not preclude their association with the *cis*-Golgi compartment. However, colocalization of these non-palmitoylated chimeras with a *cis*-Golgi marker would be inconsistent with the aforementioned Ras trafficking model, which proposes that ER-mediated palmitoylation of Ras is required for subsequent Golgi association (Apolloni *et al.*, 2000).

Since K-Ras4B is thought to traffic directly from ER to plasma membrane (Choy *et al.*, 1999; Apolloni *et al.*, 2000), the apparent association of the GFP-K-RasKQ second signal mutant with a polarized perinuclear structure in live cells is unusual. In fact, wild-type K-Ras4B demonstrates a weak perinuclear association in live cells, and is also present in a Golgi-enriched cellular fraction (Choy *et al.*, 1999). It therefore appears that K-Ras4B may associate with the Golgi, albeit transiently. Consequently, the perinuclear localization of GFP-K-RasKQ and the N-Ras and H-Ras second signal mutants in live cells may indicate that after prenylation and putative palmitoylation on the ER, all Ras isoforms are directed to the Golgi, regardless of the second signal. This would amend the emerging model of Ras trafficking such that the Golgi, and not the ER, acts as the sorting center, sensing the presence and combination of signals on the bound isoforms and directing them appropriately. The polybasic K-

Ras4B would be quickly released from Golgi membranes and delivered to the plasma membrane, possibly by binding to and moving along microtubules (Thissen *et al.*, 1997). This transient interaction would explain the diffuse nature of the perinuclear signal produced by full-length and truncated chimeric Ras proteins. Conversely, the palmitoylated N-Ras and H-Ras isoforms would be retained on Golgi membranes until their incorporation onto vesicles for plasma membrane delivery. Figure 4.1 describes the current model of Ras trafficking and the amended model suggested here. Since the dissipation of the perinuclear GFP-K-RasKQ signal in fixed cells prevented identification of that structure, further colocalization or biochemical data for the GFP-K-RasKQ mutant are required to support or amend the current model.

4.4.2c DiI-LDL Colocalization

The punctate structures observed in live and fixed COS-7 cells expressing GFP-N-RasWT and GFP-H-RasWT were suggestive of transport vesicles or endosomes. It was not clear whether these structures were delivering N-RasWT and H-RasWT chimeras to the plasma membrane, or whether they were directing the recycling or degradation of chimeras which had already reached the plasma membrane.

None of the chimeras, whether wild-type, lacking a second signal or completely unmodified, demonstrated any significant colocalization with peripheral or perinuclear diI-LDL in either the absence or presence of nocodazole. In particular, the nocodazole treatment clearly indicated that the dispersed perinuclear GFP-Ras signals do not codistribute with the diI-LDL signal. The fact that none of the GFP-Ras chimeras colocalized with diI-LDL excludes their

presence in only those endosomes incorporating this marker. Various species of endosomes exist, which represent different stages of the endocytic process, and which direct recycling or degradation of the cargo. The diI-LDL is internalized via clathrin-coated pits and is ultimately delivered to lysosomes for hydrolysis (Brown and Goldstein, 1986). As a result, association of N-RasWT and H-RasWT chimeras with other endosomal species cannot be exluded. Furthermore, the peripheral punctate structures may not be endosomes but transport vesicles delivering Ras to the plasma membrane. Choy and coworkers (1999) described the involvement of motile, coalescing vesicles in delivery of full-length GFP-N-Ras to the plasma membrane.

Discerning the identity of these punctate structures will require comprehensive assessment using various markers for intracellular trafficking. The Rab family of proteins may prove useful in this assessment. Different members of this family associate with distinct intracellular membranes of the secretory or endocytic pathways; Rab1 associates with the ER, Rab2 with an ER-Golgi intermediate compartment, Rab6 with medial and *trans*-Golgi, Rab4 and 5 with the plasma membrane and early endosomes, Rab7 with late endosomes and Rab11 with recycling endosomes (Giannakouros and Magee, 1993; Ullrich *et al.*, 1996). Colocalization of the N-RasWT and H-RasWT chimeras with these markers may facilitate identification of both the peripheral punctate structures and those perinuclear signals not colocalizing with giantin. Such colocalization studies provide one approach to the mapping of secretory and/or endocytic pathways utilized by Ras. Another informative approach involved time-course studies which helped establish the sequential subcellular distributions of the variably lipidated Ras isoforms.

4.5 Time Course Studies Indicate Sequential Membrane Associations

Two sets of time-course studies were carried out which compared the sequential subcellular distributions of full-length EGFP-Ras chimeras and truncated RSGFP-Ras hypervariable domain chimeras. The results suggest that it is the Ras hypervariable domains, and their inclusive lipid modifications, which direct the differential membrane associations of the variably lipidated isoforms. A similar conclusion could be derived from both sets of results: the association of N-Ras, H-Ras and K-Ras4B with a perinuclear structure precedes plasma membrane association. This is supported by similar trafficking data from Choy and coworkers (1999). However, three differences between full-length EGFP-Ras and truncated RSGFP-Ras localization were observed. First the RSGFP-K-RasWT chimera consistently produced a weak, diffuse perinuclear signal at all time points. In contrast, EGFP-K-Ras4B produced a sustained, compact perinuclear signal at 24 hours post-transfection and beyond. Second, while all three EGFP-Ras chimeras displayed prominent, apparently coincident plasma membrane association, the plasma membrane association of RSGFP-H-RasWT and RSGFP-K-RasWT preceded that of RSGFP-N-RasWT by at least six hours. Third, RSGFP-N-RasWT and RSGFP-H-RasWT illuminated punctate structures before they associated with the plasma membrane. Conversely, plasma membrane association of EGFP-N-Ras and EGFP-H-Ras did not appear to be preceded by association with punctate structures.

While the subcellular distribution trends of both the full-length and hypervariable domain chimeras are similar, the perinuclear and plasma membrane association of the full-length EGFP-Ras chimeras occurs within earlier time frames. The GFP variant used in each set of time-courses may

exaggerate the differences observed between the EGFP and RSGFP chimeras. The shifted time frames, sustained EGFP-K-Ras4B perinuclear localization and coincident plasma membrane association of the EGFP-Ras chimeras could result from the enhanced expression of EGFP as compared with RSGFP (Haas *et al.*, 1996; Cormack *et al.*, 1996). They may also result from enhanced intrinsic EGFP fluorescence intensity, which may augment the signal derived from conjugated antibody. Control slides in which the secondary antibody was absent indicated that significant GFP fluorescence was maintained despite fixation and permeabilization. The absence of punctate EGFP-N-Ras and EGFP-H-Ras structures in the hours preceding plasma membrane association is unexplained.

The lagging plasma membrane association of truncated RSGFP-N-RasWT may not be fully explained by differences in EGFP versus RSGFP expression or fluorescence intensity. This chimera also demonstrated weaker plasma membrane association than the H-RasWT and K-RasWT chimeras in both live confocal and colocalization studies. This may indicate that plasma membrane delivery of N-Ras is slower than that of K-Ras4B and H-Ras, or that its association is more transient due to more efficient palmitate turnover.

4.6 Conclusions and Future Studies

The concept of novel trafficking pathways for variably lipidated proteins has been in development for several years. Evidence in support of distinct trafficking pathways was suggested by studies which demonstrate that membrane association is slowed when one native N-terminal lipidation sequence is replaced with another (van't Hof and Resh, 1997). Recent novel studies have described the trafficking of specific lipidated proteins. Bijlmakers and Marsh

(1999) described the trafficking of myristoylated, palmitoylated Lck tyrosine kinase to the plasma membrane via the exocytic pathway. Choy and coworkers (1999) and Apolloni and coworkers (2000) proposed models of lipidation-dependent trafficking for the major Ras isoforms.

The work presented here confirms and augments the emerging model of differential, lipidation-dependent Ras isoform trafficking. In addition, the differential membrane associations of our N-Ras, H-Ras and K-Ras4B chimeras were similar to those observed throughout the literature. This suggests that the transposition of the H-Ras and K-Ras4B CaaX motifs in our chimeras did not affect their ultimate subcellular localization, iodopalmitate or prenyl incorporation, or fractionation behaviour.

With respect to the collective literature, our results are unique in the following aspects:

(1) the consistent, equal presence of GFP-N-RasWT in both the S100 and P100 fractions
(2) the differential iodopalmitate incorporation and membrane association of the singly palmitoylated H-RasC181S and H-RasC184S chimeras
(3) the differential association of similarly lipidated N-RasWT and H-RasC184S chimeras with punctate structures
(4) the perinuclear association of the GFP-K-RasKQ chimera in live COS-7 cells
(5) the unusual concentration of the unprenylated but polybasic GFP-K-RasC185S within discrete nuclear substructures suggestive of nucleoli

These results form the basis for future studies of differential, lipidation-

dependent Ras trafficking. The question of variable palmitoylation efficiency based on the position of the modified cysteine relative to the prenylcysteine could also be addressed. This might be accomplished by appending a synthetic 15 amino acid C-terminus to GFP, comprised only of glycine for example, but terminating in an authentic CaaX motif. Cysteine residues may be introduced at various positions upstream of the prenylcysteine, and labelling studies would be carried out to establish both the presence and relative extent of iodopalmitate incorporation. This would not only establish whether cysteine position affects palmitoylation efficiency, but also if the unique combination of amino acids surrounding the lipid-modified amino acids plays a role in palmitoylation efficiency.

Examination of the role of unique hypervariable domain sequences in differential association of N-RasWT and H-RasC184S chimeras with punctate structures would provide additional insight into this domain. Conservative point mutations within the hypervariable domains, followed by fractionation, pulse-chase studies and confocal assessment, may more precisely address the relative roles of lipids and intervening amino acids in the differential membrane associations of all Ras isoforms.

Identification of both the punctate structures and the non-colocalized perinuclear signals produced by the wild-type N-Ras and H-Ras chimeras, the H-Ras single cysteine mutants and all second signal mutants would help resolve the developing model of differential Ras trafficking. In particular, one approach may be to colocalize the second signal mutants with a *cis*-Golgi marker to establish whether, in the absence of the second signal, these mutants truly

interact with Golgi membranes to create the observed perinuclear signal. If so, it would suggest that the Golgi, and not the ER, sorts the Ras isoforms in a modification-dependent manner. This provides an additional element to the emerging model of lipidated-dependent protein trafficking; Figure 4.1 visualizes the current, emerging model of differential Ras isoform trafficking, and our proposed alternative model, which might be resolved by future studies based on the work presented in this thesis (Figure 4.1).

The variably lipidated Ras isoforms provide an especially useful model with which to study lipidated-protein trafficking, since the major isoforms are highly homologous except for their C-termini. Ultimately, studies of Ras lipidation and trafficking may be relevant to similar studies of other lipidated proteins. Such studies will collectively establish whether unique lipidation states direct the differential trafficking and specific membrane associations of certain proteins.

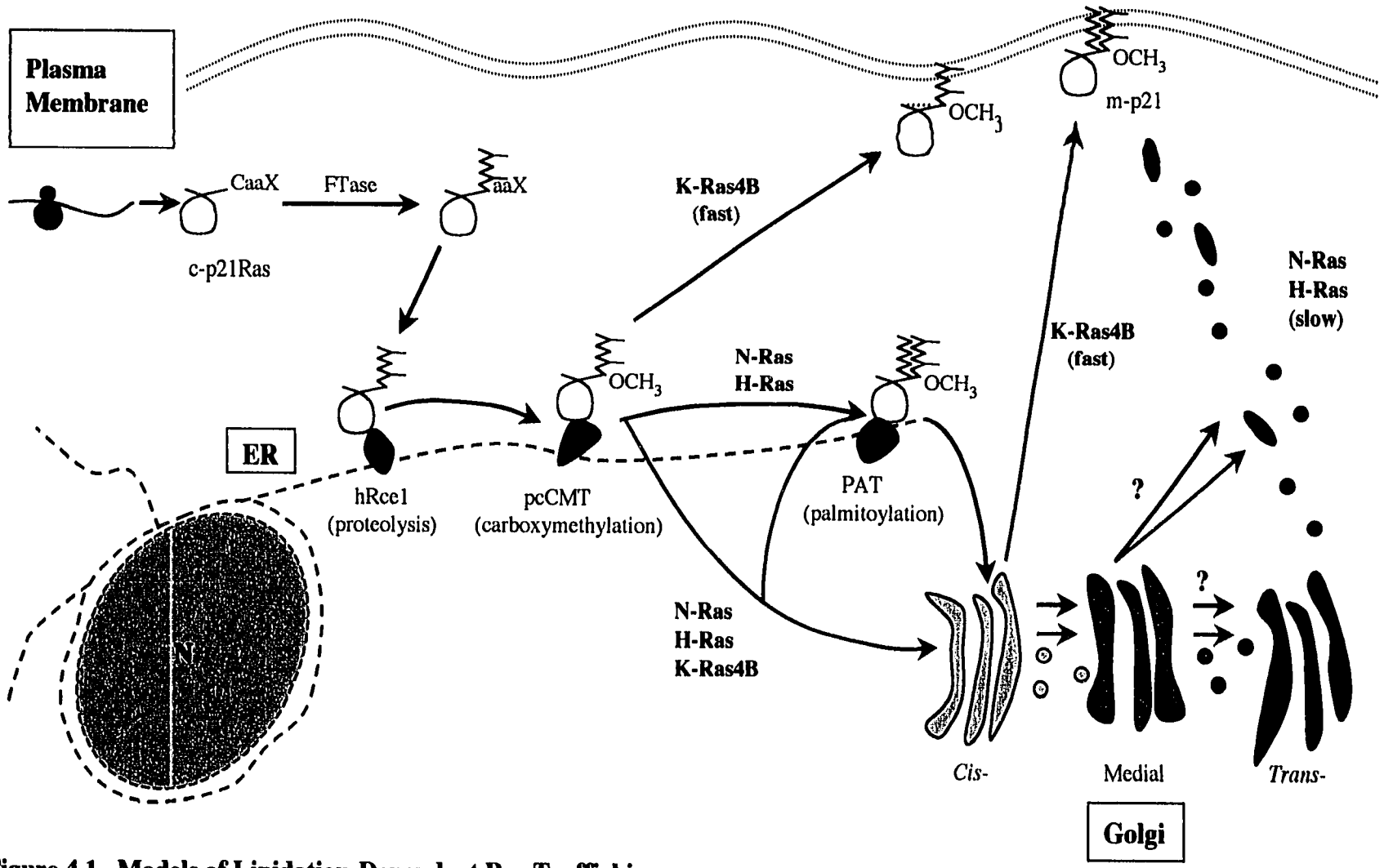


Figure 4.1 Models of Lipidation-Dependent Ras Trafficking.
The current model proposing isoform sorting at the ER is represented by the blue arrows; the amended model proposing isoform sorting at the Golgi is represented by red arrows. Common components of Ras processing are represented by black arrows. N; nucleus, ER; endoplasmic reticulum, hRce1; human Ras converting enzyme, pcCMT; prenyl cysteine carboxylmethyltransferase, PAT; palmitoyl acyltransferase, FTase; farnesyltransferase, c-p21; cytoplasmic p21Ras, m-p21; membrane-associated p21Ras.

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