

**DOCOSAHEXAENOIC ACID DIFFERENTIALLY MODULATES  
PLASMA MEMBRANE TARGETING AND SUBCELLULAR LOCALIZATION  
OF LIPIDATED PROTEINS IN COLONOCYTES**

A Dissertation

by

JEONGMIN SEO

Submitted to the Office of Graduate Studies of  
Texas A&M University  
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2004

Major Subject: Nutrition

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**ABSTRACT**

Docosahexaenoic Acid Differentially Modulates Plasma Membrane Targeting and Subcellular Localization of Lipidated Proteins in Colonocytes. (December 2004)

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Correct localization of lipidated cytosolic proteins to the plasma membrane (PM) is mediated by interactions between lipid anchors of proteins and cell membranes. Previously, dietary fish oil and its major n-3 polyunsaturated fatty acid (PUFA), docosahexaenoic acid (DHA), have been shown to decrease Ras membrane association, concomitantly reducing rat colon tumor incidence and Ras signaling, compared with corn oil and linoleic acid (LA), a highly prevalent vegetable fat and dietary PUFA in the U.S. diet. In order to explore the potential regulatory role of the cellular lipid environment in PM targeting of lipidated proteins, young adult mouse colon (YAMC) cells were treated with 50  $\mu$ M DHA, LA, or oleic acid (OA) 24 h prior to and 36-48 h after transfection with green fluorescent protein (GFP) fusion constructs of various lipidated cytosolic proteins. Relative expression of each GFP fusion protein at the PM and the Golgi in living cells was quantified using z-serial confocal microscopy and digital image processing. DHA differentially altered the subcellular localization of Ras isoforms and Src-related tyrosine kinases in a reversible manner. DHA significantly decreased the PM localization and increased the endomembrane association of H-Ras,

N-Ras, and Lck, which are targeted to the PM via the exocytic pathway, regardless of their functional state. In contrast, the subcellular distribution of K-Ras and Fyn, of which transport is independent of the vesicular transport pathway, was unaffected by DHA. Moreover, DHA selectively inhibited lipidated cytosolic protein targeting since the PM delivery of transmembrane protein cargo was unaffected, indicating that DHA does not alter the bulk flow of secretory vesicular traffic. Overall, the present study presents compelling evidence that select dietary constituents with membrane lipid-modifying properties can differentially modulate subcellular localization of important lipidated signaling proteins depending on their intracellular trafficking route to the PM.

## **DEDICATION**

I would like to dedicate this dissertation to my parents, Tea Young Seo, and Kui Ja Yoon, and to my grandmother, Sun Hee Lee, whose unconditional love and support always inspire me to do my best to be a better person.

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**LIST OF ABBREVIATIONS**

ARF, ADP-ribosylation factor

BSA, bovine serum albumin

DHA, docosahexaenoic acid

DPH, 1,6-diphenylhexatriene

eNOS, endothelial nitric oxide synthase

EPA, eicosapentaenoic acid

ER, endoplasmic reticulum

FBS, fetal bovine serum

FPP, farnesyl pyrophosphate

FTase, farnesyl transferase

GFP, green fluorescent protein

GGTase, geranylgeranyl transferase

GPI, glycosylphosphatidylinositol

HIV-1, human immunodeficiency virus-1

HMG-CoA, hydroxymethylglutaryl CoA

IFN- $\gamma$ , interferon- $\gamma$

ITS, insulin/transferrin/selenium

LA, linoleic acid

MARCKS, myristoylated alanine-rich C kinase substrate

N.A., numerical aperture

NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl

NMR, nuclear magnetic resonance

NMT, N-myristoyl transferase

NRTK, nonreceptor tyrosine kinase

OA, oleic acid

PC, phosphatidylcholine

PE, phosphatidylethanolamine

PLD, phospholipase D

PM, plasma membrane

PS, phosphatidylserine

PUFA, polyunsaturated fatty acid

REP, Rab escort protein

ROI, region of interest

TGN, *trans*-Golgi network

VSVG, vesicular stomatitis virus glycoprotein

YAMC, young adult mouse colon

## CHAPTER I

### INTRODUCTION

Correct subcellular localization of proteins within numerous membrane-bounded intracellular compartments is essential for the fidelity and efficiency of their function. In particular, in the case of specific signaling proteins that transduce the external signals acquired by cell surface receptors to multiple downstream effectors, their correct targeting to the cytoplasmic surface of the plasma membrane (PM) is critical for the ability of cells to communicate with and respond to the external environment (Kholodenko et al., 2000).

Membrane association of otherwise cytosolic soluble proteins is often achieved by regulated processes such as covalent modification of proteins with specific lipid anchors (Casey, 1995). The most common types of cytosolic protein lipidation in eukaryotic cells are fatty acylation with myristate (14:0) or palmitate (16:0) and prenylation with 15-carbon farnesyl or 20-carbon geranylgeranyl moieties (Resh, 1999; Roskoski, 2003). Many cytosolic proteins are specifically targeted to the PM by means of a combination of two different membrane anchors. This two-signal model often features myristate or polyisoprenoid combined with palmitate or a polybasic domain (Casey, 1995; Hancock et al., 1990; Resh, 1994; Resh, 1996), which appear to be functionally interchangeable (Cadwallader et al., 1994; Kwong and Lublin, 1995; Zhou et al., 1994). For example, polyisoprenyl groups must work in concert with palmitate at

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the C-terminus of H-Ras, N-Ras, RhoB, and TC10 for correct PM targeting, while K-Ras4B and members of Rho family including Rac1 use a polybasic stretch of amino acids in conjunction with polyisoprenoids (Cadwallader et al., 1994; Hancock et al., 1990; Michaelson et al., 2001; Williams, 2003). Seven of the nine Src family members including Lck and Fyn and at least five  $\alpha$  subunits of heterotrimeric G proteins are modified by N-terminal dual acylation with myristate and palmitate to achieve PM localization (Koegl et al., 1994; Linder et al., 1993; Parenti et al., 1993; Resh, 1994). A combination of myristate and clusters of basic amino acids can be found in N-terminal targeting regions of Src, myristoylated alanine-rich C kinase substrate (MARCKS), and human immunodeficiency virus-1 (HIV-1) Gag protein (Kim et al., 1994; McLaughlin and Aderem, 1995; Sigal et al., 1994; Zhou et al., 1994).

Recent work has revealed that lipidated cytosolic proteins are transported to the PM via different intracellular trafficking routes. Elegant studies with biochemical, fluorescence and electron microscopic approaches demonstrated that after common processing on the cytosolic surface of the ER, H-Ras and N-Ras traffic to the PM via the classical exocytic pathway while K-Ras4B takes an uncharacterized faster route that is independent of the Golgi-mediated vesicular transport (Apolloni et al., 2000; Choy et al., 1999). Similar to H- and N-Ras, newly synthesized Lck, neuromodulin (also known as GAP-43), and synaptosomal-associated protein of 25 kDa (also known as SNAP-25) have been shown to initially associate with intracellular membranes and subsequently travel through the secretory pathway to the PM (Bijlmakers and Marsh, 1999; Gonzalo and Linder, 1998; Liu et al., 1994). In contrast, Fyn and Moloney murine leukemia virus

Gag protein are targeted rapidly and directly to the PM after biosynthesis on soluble ribosomes (Suomalainen et al., 1996; van't Hof and Resh, 1997), indicating that at least two transport pathways exist for PM targeting of lipidated cytosolic proteins, differing in the dependence on the secretory vesicular transport.

While many PM-associated upstream signaling proteins such as Ras and Src family kinases play essential roles in a variety of cellular functions, their chronic overactivation has contributed to deleterious cellular malfunction including oncogenesis and inflammatory diseases (Bos, 1989; Campbell and Der, 2004; Orchard, 2002; Singer and Koretzky, 2002). Consequently, substantial efforts have been devoted to develop therapeutic agents to inhibit Ras and tyrosine kinase function with some promising progress (Garcia-Echeverria, 2001; Kamens et al., 2001; Kloog and Cox, 2000; Levitzki, 1994; Lodge et al., 1998; McWherter et al., 1997; Wittinghofer and Waldmann, 2000; Wong and Leong, 2004). Co-translational and post-translational modifications responsible for protein PM targeting have been extensively studied and subsequently exploited in the development of farnesyl transferase- and N-myristoyl transferase-directed drugs for inhibition of protein membrane association (Brunner et al., 2003; Bryant and Ratner, 1990; Bryant et al., 1991; Johnson et al., 1994; Prendergast, 2000). However, the potential regulatory role of the cellular lipid environment in intracellular protein trafficking has not been well appreciated to date. Previously, dietary fish oil and its major n-3 polyunsaturated fatty acid (PUFA), docosahexaenoic acid (DHA; 22:6, n-3), have been shown to decrease Ras membrane association compared with corn oil and linoleic acid (LA; 18:2, n-6), which coincided with reduced colon tumor incidence and

Ras-dependent signaling in the carcinogen-injected rat colon and malignant transformed mouse colon cell line, respectively (Collett et al., 2001; Davidson et al., 1999; Singh et al., 1997). However, the exact subcellular distribution of Ras in fish oil- or DHA-treated colon cells as well as the underlying mechanism for the reduced Ras membrane association has not been elucidated. At any given time, a substantially large fraction of Ras is associated with the endomembrane structures such as the endoplasmic reticulum (ER) and the Golgi, in addition to the PM (Apolloni et al., 2000; Choy et al., 1999). Therefore, a reduced membrane association of Ras does not necessarily mean a reduction in Ras PM localization. Using green fluorescent protein (GFP) fusion chimeras and quantitative fluorescence imaging of living cells, the present study demonstrates that DHA differentially alters PM targeting and subcellular localization of Ras isoforms and Src-related tyrosine kinases in colonic epithelial cells in a reversible manner. DHA significantly decreased the PM localization and increased the endomembrane association of H-Ras, N-Ras, and Lck, which are targeted to the PM via the exocytic pathway, regardless of their functional state. In contrast, subcellular distribution of K-Ras-4B and Fyn, of which transport is independent of the vesicular transport pathway, was unaffected by DHA. Moreover, DHA seems to selectively inhibit lipidated cytosolic protein targeting since the PM delivery of transmembrane protein cargo was unaffected. Overall, the present study presents compelling evidence that select dietary constituents with membrane lipid-modifying properties can differentially modulate subcellular localization of important lipidated signaling proteins depending on their intracellular trafficking route to the PM.



## CHAPTER II

### LITERATURE REVIEW

#### **Protein lipidation**

The ability of cells to communicate with and respond to the external environment entails that the external signals must somehow penetrate through the plasma membrane (PM) lipid bilayers and reach the intracellular networks of signaling machinery. In most cases, the external signaling entity does not directly enter the cell but rather transmits its information to specific cell surface receptors. These cell surface receptors subsequently communicate with specific signaling proteins present on the cytoplasmic surface of the PM, which, in turn, relay the information to multiple intracellular downstream effectors. Membrane association of such specific signaling proteins often depends on their covalent modification by specific lipid moieties. This regulated process of protein lipidation can be subdivided into four major categories based on the identity of the lipid attached to the protein: fatty acylation, prenylation, glypiation, and cholesteroylation (Casey, 1995; Mann and Beachy, 2000). Examples of proteins with different lipid modifications are illustrated in Fig. 1.

#### ***Fatty acylation***

The two most common types of protein fatty acylation are modification with palmitate (16:0), a 16-carbon saturated fatty acid, and myristate (14:0), a 14-carbon saturated fatty acid. Whereas the enzymology of myristoylation reaction is extensively

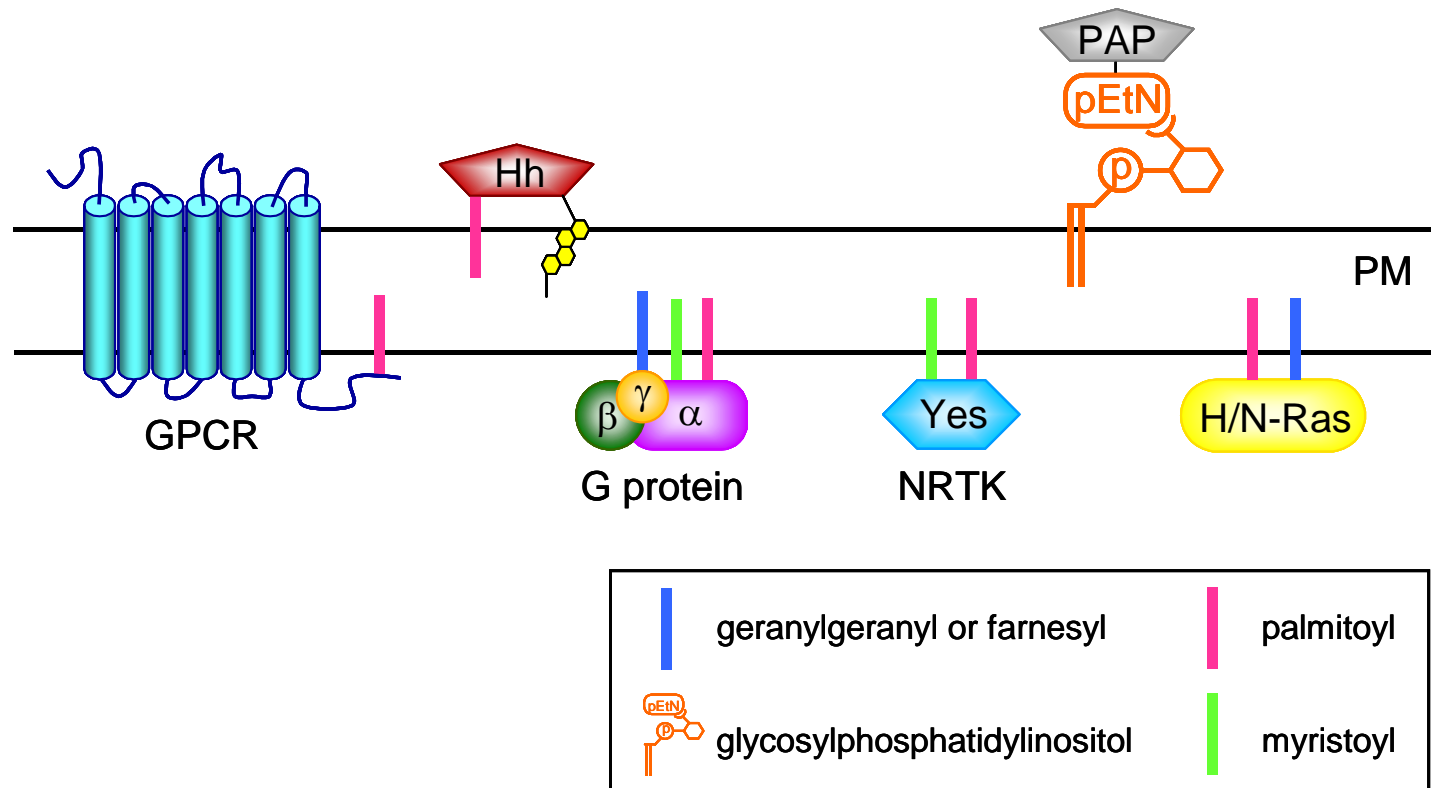


Figure 1. **Examples of lipidated PM signaling proteins.** Palmitoylated proteins are exemplified by G protein-coupled receptor (GPCR), heterotrimeric  $G_{\alpha}$  subunits, Hedgehog (Hh) morphogen, Yes nonreceptor tyrosine kinase (NRTK), and H- and N-Ras small GTPases. Myristoylation can be found in Yes NRTK, and  $G_{\alpha}$  subunits. Prenylation with farnesyl and geranylgeranyl moieties is exemplified by Ras GTPases and  $G_{\gamma}$  subunits, respectively. Glypiation and cholesteroylation is found in placental alkaline phosphatase (PAP) and Hh, respectively, both of which are on the cell exterior. Prenyl, GPI, palmitoyl, and myristoyl groups are shown in blue, orange, pink, and green, respectively. p, phosphate. pEtN, phosphoethanolamine. Adapted from Berthiaume (Berthiaume, 2002).

characterized, the molecular basis of palmitoylation reaction seems more complex and dynamic and is poorly understood to date.

In nearly all palmitoylated proteins, palmitate is attached to the sulfhydryl group of cysteine residues via a labile thioester bond in a post-translational fashion, with the exception of human Sonic Hedgehog proteins, in which palmitate is attached to the amino group of N-terminal cysteine via an amide bond (Pepinsky et al., 1998). Examples of palmitoylated proteins include H-Ras, N-Ras,  $\alpha$  subunits of heterotrimeric G proteins, Src family of nonreceptor tyrosine kinases (NRTKs) and G protein-coupled receptors (Alland et al., 1994; Bouvier et al., 1995; Hancock et al., 1989; Linder et al., 1993). The location of palmitoylated cysteine residues varies such that they can be found at the N- or C-terminal region or near transmembrane domains (Resh, 1996). Although palmitate is the predominant fatty acid linked to the cysteine residue of proteins, other fatty acyl chains can be incorporated in place of palmitate (Bizzozero et al., 1986; Casey et al., 1994; Fujimoto et al., 1993; Hallak et al., 1994; Muszbek and Laposata, 1993; O'Brien et al., 1987). Thus, the term 'S-acylation' may provide a more accurate description of this type of modification (Casey, 1995). The lability of thioester linkage renders the palmitoylation process reversible and dynamic unlike other permanent lipid modifications, presenting a unique potential to be regulated. Indeed, regulation of protein palmitoylation has previously been reported, as exemplified by the agonist-stimulated turnover of palmitate on  $\beta_2$ -adrenergic receptor,  $\alpha$  subunit of  $G_s$  protein, and endothelial nitric oxide synthase (eNOS) (Degtyarev et al., 1993; Mouillac et al., 1992; Mumby et al., 1994; Robinson et al., 1995; Wedegaertner and Bourne,

1994). Moreover, the activation-induced depalmitoylation of  $G_{s\alpha}$  and eNOS was accompanied by cytosolic translocation of these proteins, indicating that the dynamic acylation state of proteins may regulate protein subcellular localization (Robinson et al., 1995; Wedegaertner and Bourne, 1994). Unfortunately, the molecular mechanism of protein palmitoylation has been poorly understood to date and enzymes responsible for protein palmitoylation have defied purification until recently. Partial purification of protein palmitoyl transferase activities towards different proteins and peptides has been reported from several membrane compartments including the ER, the Golgi, the ER-Golgi intermediate compartment, and the PM (Dunphy et al., 1996; Gutierrez and Magee, 1991; McLaughlin and Denny, 1999; Schroeder et al., 1996; Zhao et al., 2002). Two yeast genes (*erf2* and *erf4*) responsible for Ras palmitoylation have recently been cloned and await further characterization (Bartels et al., 1999; Lobo et al., 2002). Two thioesterases have been identified to depalmitoylate Ras and  $G_{\alpha}$  proteins *in vitro* (Camp et al., 1994; Duncan and Gilman, 1998) and it is likely that additional protein palmitoyl thioesterases exist. In addition, non-enzymatic palmitoylation of proteins has been documented (Bano et al., 1998; Duncan and Gilman, 1996), although the physiological relevance of this spontaneous autoacylation mechanism has not yet been resolved. Clearly, further progress in the identification and characterization of protein palmitoyl transferases and thioesterases is needed to better understand the role of palmitoylation in regulating the localization and function of signaling proteins.

Myristoylated proteins contain myristate typically attached to the N-terminal glycine residue via an amide bond in a co-translational manner, as exemplified by Src

family NRTKs,  $G_{\alpha}$  subunits, recoverin, MARCKS, and mammalian retroviral Gag proteins (Resh, 1999). All known myristoylated proteins initiate with the sequence of Met-Gly. The initiating methionine is removed by methionine amino peptidase during translation and the now N-terminal glycine is myristoylated by N-myristoyl transferase (NMT), a 50-60 kDa monomeric enzyme (Gordon et al., 1991). NMT resides predominantly in the cytoplasm although membrane-associated NMT activity has also been documented (McIlhinney, 1995; McIlhinney and McGlone, 1996). The catalytic mechanism and substrate specificities of NMT are extensively characterized and reviewed (Devadas et al., 1992; Johnson et al., 1994; Kishore et al., 1993; Rudnick et al., 1990). NMT exhibits a remarkable specificity to myristate and longer or shorter fatty acyl CoAs are poor substrates for this reaction. However, select retinal proteins, HIV-1 Gag protein, and Fyn have been reported to be heterogeneously acylated via an amide bond with lauric acid (12:0), 5-*cis*-tetradecenoic acid (14:1, n-9), and 5-*cis*,8-*cis*-tetradecadienoic acid (14:2, n-6) in addition to myristate (14:0) *in vivo* (Dizhoor et al., 1992; Kokame et al., 1992; Liang et al., 2001; Lindwasser and Resh, 2002). A subset of myristoylated proteins such as ADP-ribosylation factor (ARF) and recoverin exists in two conformations differing in the orientation of myristate relative to the protein (Goldberg, 1998; Tanaka et al., 1995). In these proteins, myristate is either sequestered within a hydrophobic cleft of the protein or exposed to the protein surface, thereby being able to participate in membrane binding. The transition between these two conformations is regulated by a mechanism known as the 'myristoyl switch' (McLaughlin and Aderem, 1995). The triggers for the switch include ligand binding,

electrostatics, and proteolysis. Myristoyl switch proteins typically exhibit reversible membrane association as illustrated by  $\text{Ca}^{2+}$ -sensitive membrane binding of recoverin (myristoyl-ligand switch) (Ames et al., 1994; Tanaka et al., 1995), GTP-binding-stimulated translocation of ARF from the cytosol to membranes (myristoyl-ligand switch) (Amor et al., 1994; Goldberg, 1998; Haun et al., 1993), phosphorylation-mediated translocation of MARCKS from membranes to the cytosol (myristoyl-electrostatic switch) (Kim et al., 1994; McLaughlin and Aderem, 1995; Thelen et al., 1991), and cleavage-triggered release of HIV-1 Gag from membranes (myristoyl-proteolytic switch) (Hermida-Matsumoto and Resh, 1999). In addition to the role in membrane binding, myristoylation plays a structural role to stabilize three-dimensional conformation as revealed by crystal structures of the poliovirus protein shell and the catalytic subunit of cAMP-dependent kinase (Chow et al., 1987; Zheng et al., 1993).

### ***Prenylation***

Prenylation refers to the covalent attachment of either 15-carbon farnesyl or 20-carbon geranylgeranyl isoprenoids to one or more cysteines at or near the C-terminus of proteins. The branched unsaturated prenyl group is attached via a stable thioether bond in a post-translational process. The lipid substrates for prenylation are farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate, which are built from isoprene units derived from the mevalonate pathway (Goldstein and Brown, 1990). The enzymes responsible for prenylation have been identified and characterized at a molecular level (Casey and Seabra, 1996; Roskoski, 2003). Three distinct protein prenyl transferases can be subdivided into two functional classes. Protein farnesyl transferase (FTase) and

protein geranylgeranyl transferase I (GGTase I) are classified as the CAAX prenyl transferase and Rab geranylgeranyl transferase or protein geranylgeranyl transferase II (GGTase II) constitutes the second class of prenyl transferase. All three prenyl transferases are zinc metalloenzymes, and FTase and GGTase II but not GGTase I require  $Mg^{2+}$  for activity (Reiss et al., 1992; Zhang and Casey, 1996a; Zhang et al., 2000).

FTase and GGTase I are designated as CAAX prenyl transferase since they catalyze the prenylation of the fourth cysteine from the C-terminus of proteins containing a C-terminal tetrapeptide sequence called a CAAX box, where C stands for cysteine, A stands for an aliphatic residue and X typically refers to methionine, serine, alanine, or glutamine for FTase or to leucine for GGTase I (Clarke, 1992; Zhang and Casey, 1996b). Based on biochemical and structural analyses, ‘molecular ruler’ and ‘second site exclusion’ hypotheses have been proposed to explain different isoprenoid substrate specificities of prenyl transferases (Long et al., 1998; Turek-Etienne et al., 2003). Both FTase and GGTase I can recognize short peptides containing appropriate CAAX motifs as substrates in addition to their physiological substrates. The total number of likely substrates for FTase is about 50 and is about a hundred for GGTase I (Cox and Der, 1997). Examples of FTase substrates include Ras, lamin B, several proteins involved in visual signal transduction, and fungal mating factors. Known GGTase I targets include most  $G_{\gamma}$  subunits, and members of Ras-related Rac/Rho family GTPases. In general, following prenylation, most CAAX proteins are directed to a membrane compartment for further processing driven by the CAAX motif. The terminal

three residues (AAX) are sequentially removed by a CAAX endopeptidase and the carboxyl group of the now C-terminal prenylcysteine is methylesterified (Clarke, 1992; Zhang and Casey, 1996b). CAAX prenyl transferases generally exhibit remarkable selectivity for their protein substrates. However, a few exceptions to the above-mentioned substrate specificity for FTase and GGTase I have been observed as in the case of K-Ras4B and RhoB (Armstrong et al., 1995; James et al., 1995). Both FTase and GGTase I are heterodimers that share a common 49 kDa  $\alpha$  subunit but contain distinct  $\beta$  subunits, exhibiting combined molecular masses of 94 and 91 kDa, respectively (Moomaw and Casey, 1992; Reiss et al., 1990; Seabra et al., 1991; Yokoyama and Gelb, 1993).

GGTase II attaches geranylgeranyl groups to two C-terminal cysteines in Rab family (Ypt/Sec4 in lower eukaryotes) proteins that terminate in Cys-Cys or Cys-X-Cys sequences (Glomset and Farnsworth, 1994; Seabra et al., 1992). Rab family small GTPases function as molecular switches regulating membrane traffic (Seabra et al., 2002) and all Rab proteins tested to date are substrates for GGTase II. Rab proteins ending with Cys-X-Cys residues are methylesterified but those with Cys-Cys are not. GGTase II is a heterodimer composed of a 60 kDa  $\alpha$  subunit and a 38 kDa  $\beta$  subunit that are homologous to  $\alpha$  and  $\beta$  subunits of CAAX prenyl transferases (Armstrong et al., 1993; Brown and Goldstein, 1993). Unlike FTase and GGTase I, GGTase II does not recognize peptide substrates because the actual substrate recognized by GGTase II is a complex of Rab and a Rab escort protein (REP). REP binds newly synthesized Rab proteins and presents them to GGTase II for prenylation (Andres et al., 1993; Cremers et



al., 1994). The findings that defects in REP are implicated in a retinal degeneration disease, choroideremia, underscores the important role of prenylation in Rab function (Seabra et al., 1993).

Characterization of the kinetic mechanism and reaction pathway of FTase has rapidly progressed although the understanding of GGTase I and II is not as complete as that of FTase. Metals are required for direct catalytic roles rather than for substrate binding as revealed by metal substitution and X-ray crystallographic studies (Hightower et al., 1998; Huang et al., 1997; Saderholm et al., 2000). The transition state of the associative mechanism of farnesylation reaction involves a  $Zn^{2+}$ -activated thiolate nucleophile ( $RS^-$ ) of cysteine, which attacks the carbocation at C1 of FPP, and a  $Mg^{2+}$ -coordinated pyrophosphate leaving group (Dolence and Poulter, 1995; Huang et al., 2000). Kinetic and structural data indicate that the reaction catalyzed by FTase is sequential, with the release of a farnesylated protein product being the rate-limiting step (Furfine et al., 1995; Long et al., 2002; Tschantz et al., 1997). In this proposed reaction pathway, FPP initially binds to the free enzyme, followed by peptide binding and farnesylation of the peptide. Subsequently, a pyrophosphate is released, yielding the FTase/farnesylpeptide complex. Dissociation of the farnesylpeptide product from the enzyme requires an addition of fresh substrate(s).

### ***Glypiation***

Glypiation refers to the post-translational modification of proteins by a complex glycosylphosphatidylinositol (GPI) structure (Englund, 1993). GPI-anchored proteins are a functionally diverse class of proteins that are attached to the exoplasmic leaflet of

the PM by means of a GPI moiety. This ubiquitous modification was discovered in the late 1970s when phosphatidylinositol-specific phospholipase C was shown to specifically release alkaline phosphatase, acetylcholinesterase, and 5'-nucleotidase from cell membranes (Low and Finean, 1978; Shukla et al., 1980). To date, approximately 100 distinct GPI-anchored proteins have been identified (Low, 1999). The core structure of the GPI anchor consists of a single phospholipid spanning the exoplasmic leaflet of the PM and a complex head group composed of an inositol, a glucosamine, a linear chain of three mannose sugars, and a phosphoethanolamine. The protein is attached to the GPI moiety via an amide bond between the C-terminal residue of the protein and the amino group of the phosphoethanolamine. Variations in the GPI structure occur in both the lipid part and the tetrasaccharide backbone, although the core structure is conserved across all species studied to date (McConville and Ferguson, 1993).

GPI-anchored proteins are initially directed into the ER lumen by means of an N-terminal signal sequence during translation, where they are post-translationally modified by a pre-assembled GPI moiety (Ferguson, 1999). After glypiation, essentially all of those modified proteins are destined for the cell surface through the classical exocytic pathway (Lippincott-Schwartz et al., 2000). GPI biosynthesis initiates on the cytosolic face of the ER and the fully assembled GPI structure is transferred to the protein in the ER lumen (Vidugiriene and Menon, 1994). Therefore, the cytoplasmically located GPI precursors must be flipped across the bilayers into the lumen prior to the attachment to the protein. However, the topology of each enzymatic step of the sequential GPI biosynthesis has not been fully resolved (McConville and Menon, 2000). Attachment of

a GPI anchor involves the cleavage of the C-terminal transmembrane segment of the protein for the exposure of the GPI addition site, followed by a transamidation reaction linking the complete GPI moiety to the cleaved ectodomain of the protein (Udenfriend and Kodukula, 1995). The protein remains membrane-bound with all of its amino acids exposed initially on the luminal side of the ER and eventually on the cell exterior.

In addition to its role as a membrane anchor, the GPI moiety functions as an apical-targeting signal, directing proteins to the apical domain of polarized epithelial cells (Brown et al., 1989; Lisanti et al., 1989). Moreover, the GPI anchor has been shown to act as a sorting determinant at an early step in the exocytic pathway, i.e., exit from the ER (Muniz et al., 2001; Muniz et al., 2000), and in the endocytic pathway (Sabharanjak et al., 2002).

### ***Cholesteroylation***

Cholesteroylation involves the post-translational modification of proteins with cholesterol, as exemplified by the Hedgehog family of tissue patterning factors (Mann and Beachy, 2000). Similar to glypiated proteins, Hedgehog proteins are modified within the secretory pathway, and their final destination is the outer layer of the PM. Upon entry into the secretory pathway followed by cleavage of an N-terminal signal sequence, Hedgehog protein undergoes an autocatalytic processing mediated by its C-terminal processing domain (Lee et al., 1994; Porter et al., 1995). The autoprocessing results in an internal cleavage to release an N-terminal signaling domain. In addition, the C-terminal processing domain functions as a cholesterol transferase via an intramolecular mechanism, facilitating direct nucleophilic attack of cholesterol on the

thioester intermediate to form an ester-linked cholesterol adduct at the C-terminus of the signaling domain (Porter et al., 1996b). Cholesteroylation is crucial for the spatially restricted tissue localization of the Hedgehog signal, preventing an inappropriate influence beyond its expression site (Porter et al., 1996a).

### ***Membrane binding of lipidated cytosolic proteins***

The most common types of lipid modification of cytosolic proteins are fatty acylation and prenylation (Resh, 1999; Roskoski, 2003). Most lipidated cytosolic proteins are membrane-bound in eukaryotic cells and studies with fatty acid probes have shown that fatty acyl groups attached to proteins are embedded within the lipid bilayers (Moench et al., 1994; Vergeres et al., 1995). In general, site-directed mutagenesis of the modified amino acids, i.e., cysteine or glycine, abolishes incorporation of the lipid moiety and markedly reduces membrane association of cytosolic proteins (Hancock et al., 1989; Resh, 1993). Therefore, lipid modification seems to be necessary for the membrane association of certain cytosolic proteins. However, some lipid modifications have been suggested to be insufficient to promote stable membrane association. The unitary Gibbs free energy for binding of a myristoylated peptide to phospholipid bilayers has been estimated as 8 kcal/mol, equivalent to an apparent dissociation constant ( $K_d$ ) of  $10^{-4}$  M (Peitzsch and McLaughlin, 1993) and a similar binding affinity has been reported for farnesylated peptides (Silvius and l'Heureux, 1994). These lipopeptide studies predict that the presence of myristate or farnesyl moiety alone may not be sufficient to stably anchor a protein to cellular membranes. Consistent with this notion, membrane association of myristoylated or farnesylated proteins often involves additional factors

such as the presence of palmitate or a stretch of basic amino acids (Casey, 1995; Hancock et al., 1990; Resh, 1994; Resh, 1996).

### **Post-translational processing and intracellular trafficking of Ras**

One of the best-studied examples of the lipidation-mediated protein targeting mechanisms is the PM targeting of the small GTPase Ras. Ras is a crucial component of numerous signal transduction pathways that link diverse extracellular stimuli to the control of cell growth, differentiation, and survival (Shields et al., 2000). Ras genes (*H-ras*, *N-ras*, and *K-ras*) are the most frequently mutated protooncogenes both in animal and human cancers (Bos, 1989), producing constitutively GTP-bound active forms when mutated. The aberrant Ras signaling caused by either mutational activation of Ras or chronic up-regulation of upstream receptors plays a key role in human malignancies including colon cancer (Campbell and Der, 2004; Houlston, 2001; Janes et al., 1994). The major signaling platform of Ras proteins is the PM and the localization at the inner leaflet of the PM is required for both normal and oncogenic activities of Ras (Der and Cox, 1991; Kato et al., 1992). However, recent studies revealed that Ras can engage different signaling pathways with distinct kinetics in endomembranes including the Golgi, the ER, and endosomes as well as at the PM (Arozarena et al., 2004; Bivona et al., 2003; Caloca et al., 2003; Chiu et al., 2002; Hancock, 2003; Jiang and Sorkin, 2002; Perez de Castro et al., 2004).

*ras* genes encode four Ras isoforms, H-Ras, N-Ras, K-Ras4A, and K-Ras4B, with the latter two being alternative splicing variants of *K-ras* gene. Mammalian cells

ubiquitously express three main isoforms, H-Ras, N-Ras, and K-Ras4B, whereas K-Ras4A is expressed in only a limited number of tissues including small and large intestines (Plowman et al., 2003). Despite the 85% overall sequence homology, Ras isoforms exhibit distinct biological properties as evidenced by substantial differences in the mutation frequency in different types of cancer (Bos, 1989), the role in normal mouse embryo development (Johnson et al., 1997; Koera et al., 1997; Plowman et al., 2003; Umanoff et al., 1995), the activation profile of their common downstream effectors (Voice et al., 1999; Walsh and Bar-Sagi, 2001; Yan et al., 1998), and cellular function (de Castro et al., 2003; Santillo et al., 2001; Wolfman and Wolfman, 2000). The mechanism responsible for the PM targeting of three main Ras isoforms is well understood. The PM targeting domain of each Ras isoform consists of the C-terminal CAAX motif, common to all isoforms, and distinct second signal sequences (Fig. 2). This PM targeting domain contains all the necessary and sufficient information for Ras PM targeting, directing not only the sequential post-translational processing of each Ras isoform but also their distinct intracellular trafficking route to the PM (Apolloni et al., 2000; Choy et al., 1999; Prior et al., 2001). Initially synthesized as cytosolic soluble proteins, Ras undergoes a series of post-translational modifications to form mature lipidated proteins that associate with the PM (Silvius, 2002). This process is initiated by a C-terminal CAAX motif, which is farnesylated in the cytoplasm and then AAX proteolyzed and methylesterified on the cytosolic surface of the ER. Upon completion of the common CAAX processing, H- and N-Ras are further modified with one (N-Ras) or two (H-Ras) palmitates for their second targeting signal and traffic to the PM via the

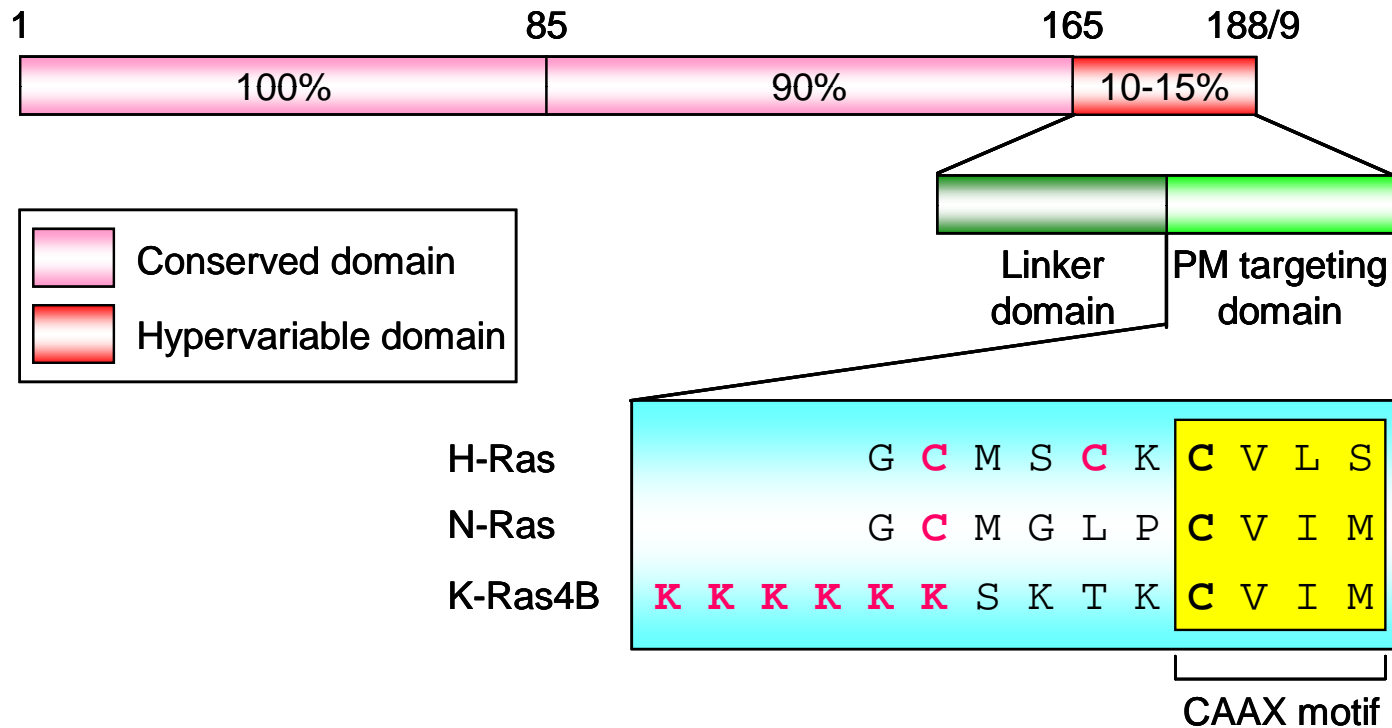


Figure 2. **Domain structure of Ras isoforms.** The N-terminal catalytic domains (amino acids 1-165) harboring all the binding sites for effectors, exchange factors, and a nucleotide are highly conserved (90-100%) in all Ras isoforms. The C-terminal hypervariable region is significantly divergent, exhibiting less than 10-15% sequence identity between any two isoforms. The hypervariable region can be divided into the PM targeting domain and the linker domain. The PM targeting domain consists of the C-terminal CAAX motif, common to all Ras isoforms, and distinct second signal sequences. The second signal (shown in pink) comprises two palmitoylation sites (C181 and C184) in H-Ras, a single palmitoylation site (C181) in N-Ras, or a polybasic domain of six contiguous lysine residues (K175-K180) in K-Ras4B. Adapted from Hancock (Hancock, 2003).

Golgi-mediated classical exocytic pathway (Apolloni et al., 2000; Choy et al., 1999). Therefore, H- and N-Ras are cytoplasmic cargo proteins of the exocytic pathway and their delivery to the PM relies on the membrane traffic. In contrast, K-Ras4B utilizes a polybasic domain of six contiguous lysine residues for its second signal, and takes an uncharacterized Golgi-independent route to the PM. Unlike H- and N-Ras, which are distributed throughout the secretory pathway, K-Ras4B is not detected in the Golgi by immunofluorescence or electron microscopy even when overexpressed and its delivery to the PM is insensitive to the inhibition of the constitutive secretory pathway. Fig. 3 summarizes the post-translational processing and intracellular trafficking route of each Ras isoform.

In addition to dictating the transport pathway to the PM, distinct membrane anchors of Ras isoforms influence the compartmentalization into specialized PM microdomains. In general, palmitoylated peripheral membrane proteins and peptides associate preferentially with the semi-transient, cholesterol-rich microdomains of lipid rafts and caveolae because the saturated palmitate readily packs into the liquid-ordered raft structure, whereas unsaturated, branched prenyl groups do not (Melkonian et al., 1999; Wang et al., 2001). While K-Ras4B predominantly resides in the disordered bulk PM regardless of its activation state, palmitoylated H-Ras is in a dynamic equilibrium between lipid rafts/caveolae and the bulk PM in a GTP loading-regulated manner (Hancock, 2003; Niv et al., 2002; Prior et al., 2001; Prior et al., 2003). Palmitoylated N-Ras seems to be targeted to the lipid rafts, similar to H-Ras (Matallanas et al., 2003). In addition, H-Ras but not K-Ras signaling seems to require access to lipid rafts/caveolae,



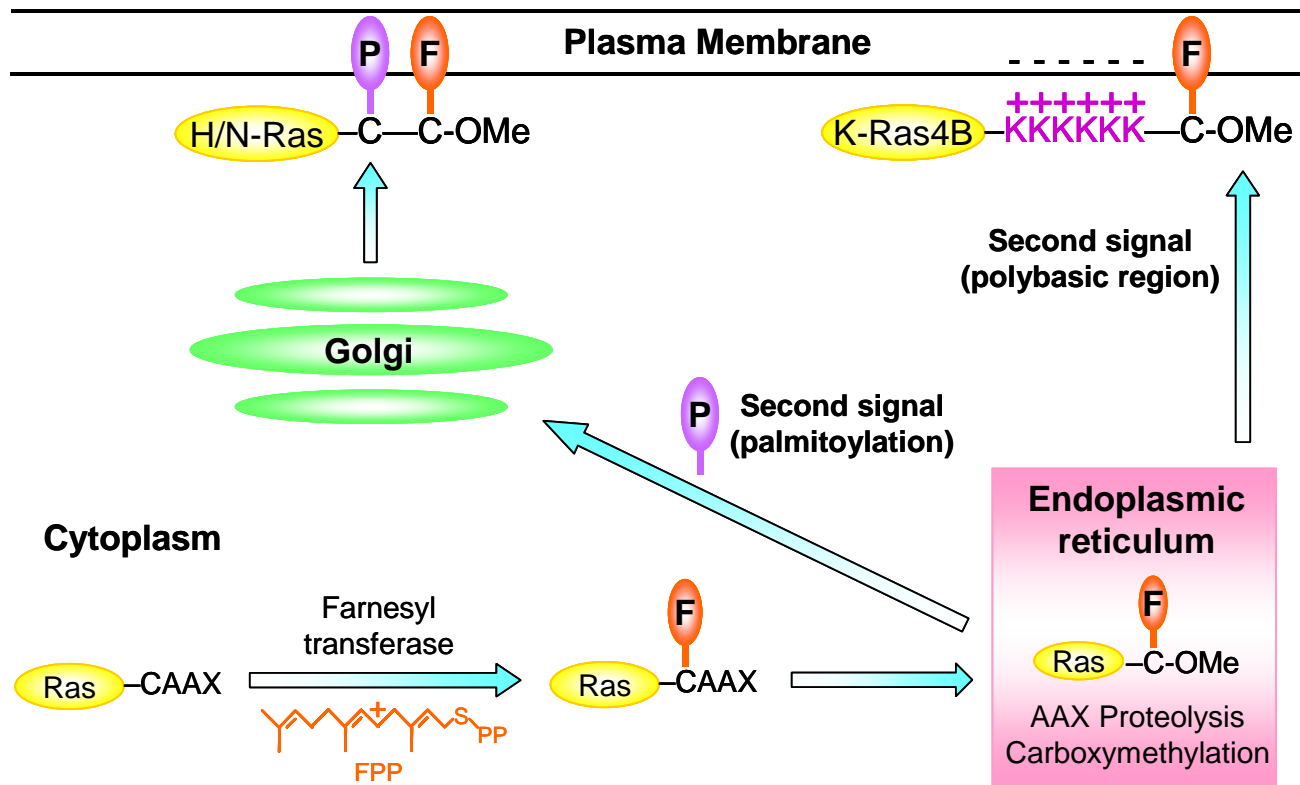


Figure 3. **Post-translational processing and intracellular trafficking of Ras isoforms.** Post-translational processing of Ras is sequential. After biosynthesis of nascent Ras proteins in free ribosomes, CAAX processing is initiated in the cytoplasm by FTase, which transfers a farnesyl group from FPP to the cysteine residue of the CAAX motif. Farnesylated Ras is directed to the cytoplasmic surface of the ER, where the AAX tripeptide is removed and the now C-terminal farnesylcysteine is methylesterified. Upon completion of the common CAAX processing in the ER, K-Ras4B exits the ER and takes an uncharacterized route to the PM, bypassing the Golgi. In contrast, H- and N-Ras are further modified with palmitoylation early in the exocytic pathway, and traffic to the PM via the Golgi-mediated classical secretory pathway as cytoplasmic cargo proteins.

since perturbations of lipid rafts/caveolae by cholesterol depletion or expression of dominant negative caveolin blocks H-Ras but not K-Ras signaling (Carozzi et al., 2002; Roy et al., 1999). Therefore, it has been proposed that the functional differences among Ras isoforms may be explained at least in part by the differential PM microlocalization mediated by distinct membrane anchors.

### **Membrane properties of DHA**

DHA (22:6<sup>Δ4,7,10,13,16,19</sup>, n-3) is the most unsaturated membrane fatty acid commonly found in biological systems and can be obtained by dietary consumption of cold-water fatty fish. Very long chain n-3 PUFAs present in dietary fish oil, such as DHA and eicosapentaenoic acid (EPA; 20:5, n-3), affect diverse physiological processes including cognitive functions, visual acuity, eicosanoid signaling, and whole body glucose and lipid metabolism (Jump, 2002), thereby providing significant protection against a variety of unrelated human afflictions (Hu et al., 2003; Kris-Etherton et al., 2002; Rose and Connolly, 1999; Stulnig, 2003; Terry et al., 2003). DHA is rapidly incorporated into cells, primarily into membrane phospholipids at *sn*-2 position (Anderson and Sperling, 1971), and in general, the cellular DHA level is readily influenced by diet (Katan et al., 1997; Vidgren et al., 1997). The presence of DHA in membrane phospholipids imparts unique physicochemical properties to cellular membranes and DHA-induced alterations in membrane structure and function have been proposed to underlie the pleiotropic positive effects of DHA.

### ***The structure of DHA in phospholipid membranes***

Within the phospholipid bilayer structure, the order of hydrocarbon chains is liquid-like, characterized by rapid isomerization, rotational diffusive motions, and relatively unrestricted lateral diffusion. Unsaturation has a substantial impact on the order and motional properties of lipids in bilayers, by lowering chain order, increasing chain dynamics, and changing the molecular shapes of acyl chains (Eldho et al., 2003; Mitchell and Litman, 1998a; Paddy et al., 1985; Salem et al., 1980; Straume and Litman, 1987; Stubbs et al., 1981). Chain conformations of PUFAs are greatly influenced by the presence of the repeating 1,4-pentadiene structural motifs ( $-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-$ ), in which two double bonds are separated by a methylene group. An earlier view on the PUFA structure favored a rigid arrangement of double bonds in U-shaped (Rich, 1993) or extended conformations such as angle iron or helical (Applegate and Glomset, 1986; Applegate and Glomset, 1991a; Applegate and Glomset, 1991b). Indeed, with six double bonds that are locked in *cis*-configuration, DHA has fewer degrees of freedom than the corresponding saturated hydrocarbon chain, leading to the general perception that polyunsaturated hydrocarbon chains are rigid and bulky. Until recently, the lower order parameters and faster dynamics of unsaturated chains and of labels in polyunsaturated membranes were often attributed to the packing perturbations from the introduction of bulky polyunsaturated chains. However, recent nuclear magnetic resonance (NMR) structural studies and molecular dynamics simulation revealed various degrees of conformational flexibility of the DHA chain (Eldho et al., 2003; Everts and Davis, 2000; Feller et al., 2002; Gawrisch et al., 2003; Huber et al., 2002; Koenig et al.,

1997; Rabinovich and Ripatti, 1991; Saiz and Klein, 2001). DHA is now portrayed as a highly flexible molecule with rapid transitions between large numbers of conformers on the time scale from picoseconds to hundreds of nanoseconds (Gawrisch et al., 2003). Measurements of  $^{13}\text{C}$  spin-lattice relaxation times of the DHA chain in a lipid bilayer have shown that both the amplitude and frequency of motion increase towards the terminal methyl group (motional correlation times in the picosecond range) of DHA, indicating exceptional flexibility of the tail section of DHA (Eldho et al., 2003; Feller et al., 2002). The low energy barriers to rotation about C–C single bonds that link the double bonds with the interrupting methylene carbon are responsible for this unusual flexibility. Quantum mechanical calculation and molecular dynamics simulation estimated the energy barrier to torsional rotation about the C–C bonds of the methylene groups between double bonds as less than 1 kcal/mol (Feller et al., 2002), which is much lower than the height of the rotational barrier in saturated hydrocarbons (more than 3 kcal/mol) (Smith and Jaffe, 1996). Thermal motions within the DHA chain in the fluid phase can easily overcome a barrier of such low height. Molecular dynamics simulation for a 1-stearoyl-2-docosahexaenoyl phosphatidylcholine (18:0-22:6 PC) bilayer, based on the empirical force field parametrized with quantum mechanical calculations of model compounds, demonstrated an excellent quantitative agreement between experiment and theory (Eldho et al., 2003; Feller et al., 2002), confirming the extreme conformational flexibility of DHA chains in membranes. Therefore, the low energy barrier to rotational isomerization seems to more than compensate for the rigidity of

multiple double bonds, allowing the methylene groups between double bonds to act as flexible hinges of the DHA chain.

The presence of DHA at *sn*-2 position in mixed-chain phospholipids also affects the order parameter and molecular shape of the saturated chain in *sn*-1 position. The electron density profiles of the bilayer and simulation results indicate that the DHA chain has a higher volume density near the lipid-water interface whereas the density of the saturated chain is higher in the bilayer center (Eldho et al., 2003). As a result of this redistribution of chain density, the saturated chain has more freedom for movement and lower chain order parameters in the lower half of the chain (Eldho et al., 2003; Holte et al., 1995). Based on these experiments and simulation, Eldho et al. proposed a model of 18:0-22:6 PC in bilayers, in which the saturated and DHA chains adopt wedge and inverted wedge shapes, respectively (Eldho et al., 2003). Notably, the removal of a single double bond at the 19 position from DHA to docosapentaenoic acid (22:5, n-6) equalizes the differences in chain distribution and order parameters seen between saturated and DHA chains, resulting in a more even distribution of chain densities in 18:0-22:5 PC along the bilayer normal (Eldho et al., 2003; Holte et al., 1995). This observation strongly supports the unique properties of DHA-rich membranes.

### ***Phase behavior***

In saturated phospholipid membranes, acyl chains pack together quite uniformly in all-*trans* configuration in the gel state at low temperatures. In membranes containing unsaturated fatty acids, steric restrictions associated with the presence of double bonds distort the packing, thereby reducing intra- and inter-molecular van der Waals

interactions. The consequent decrease in stability results in a substantial decrease in the transition temperature ( $T_m$ ) of the gel-to-liquid crystalline phase transition.

The dependence of  $T_m$  on the number of double bonds in phospholipids has been well documented by differential scanning calorimetry and  $^2\text{H}$ -NMR studies (Coolbear et al., 1983; Holte et al., 1995; Niebylski and Salem, 1994). The relationship between phase behavior and the number and position of acyl chain double bonds does not seem to be straightforward. The common trend is that the introduction of the first double bond into one of the two acyl chains reduces  $T_m$  by  $\sim 60^\circ\text{C}$ , playing the most important role in lowering  $T_m$ . Beyond two double bonds,  $T_m$  is little affected and can even rise slightly. As a result, of a series of PCs containing stearic acid (18:0) at *sn*-1 position, the DHA-containing PC melts at a higher temperature than the  $\alpha$ -linolenic ( $\alpha$ -18:3) or arachidonic (20:4) acid-containing species (Niebylski and Salem, 1994). The endotherms recorded for these species show that the transition enthalpy ( $\Delta H$ ) follows a similar trend.  $\Delta H$  decreases upon introduction of the first two double bonds, reaching a minimum (1.7 kcal/mol) with 18:0-18:2 PC, and then increases with more double bonds to 6.1 kcal/mol for 18:0-22:6 PC. It has been proposed that preferential van der Waals interactions between saturated *sn*-1 chains on neighboring molecules may be responsible for this greater stability of the DHA-containing gel state bilayer, possibly indicating heterogeneous lateral distribution. In addition, the multi-component endotherms for highly unsaturated lipids suggest microclustering within membranes leading to heterogeneous melting.

### ***Fluidity***

Fluidity of membrane bilayers is typically determined by steady-state or time-resolved measurements of fluorescence polarization of extrinsic fluorescent probes incorporated into membranes, which depends on both the rate of motion and the degree of order of the probe. As the addition of acyl chain double bonds generally increases fluidity, membranes rich in DHA are often assumed to be exceptionally fluid.

In studies with model membranes with a well-defined nature, where time-resolved approaches have been applied for more sophisticated analysis, the orientational order of the probe decreases and probe dynamics increases with increasing unsaturation of the *sn*-2 chain. Differences between monounsaturated and highly polyunsaturated (four or six double bonds) phospholipids were much larger than between disaturated and monounsaturated phospholipids (Mitchell and Litman, 1998a; Mitchell and Litman, 1998b). Moreover, lipids with two DHA chains showed a much lower orientational order relative to lipids that were polyunsaturated at *sn*-2 position only. However, results from biological membranes that are compositionally heterogeneous appear quite complex, although membranes isolated from select tissues that are naturally enriched in DHA generally exhibit high fluidity (Harel and Djerassi, 1980; Miljanich et al., 1979; Mitchell et al., 1998; Stubbs and Smith, 1984). Results from dietary studies on whole animals and cultured cells in DHA-supplemented media are contradictory, as both increased fluidity and no change have been reported (Stillwell and Wassall, 2003). The failure to observe a DHA-associated increase in fluidity in many biological membranes has been attributed to the relative subtlety of the changes within membranes that are

already rich in unsaturated fatty acids. The possibility that DHA-associated changes in fluidity may be local rather than global throughout the membranes is another confounding factor. If this is the case, the effects of DHA will be less likely detectable by bulk measurements of membrane fluidity.

### ***Permeability and flip-flop***

Numerous studies using biological and model membranes have reported that DHA increases membrane permeability. DHA-containing membranes exhibited increased permeability to water (Huster et al., 1997),  $H^+$  (Brand et al., 1994; Stillwell et al., 1997),  $^{22}Na^+$  (Hendriks et al., 1976),  $^{51}Cr$  (Jenski et al., 1991; Stillwell et al., 1993), erythritol (Demel et al., 1972; Ehringer et al., 1990; Stillwell et al., 1993), carboxyfluorescein (Stillwell et al., 1993), glucose (Demel et al., 1972), or glycerol (Demel et al., 1972), compared with oleic acid (18:1)- or  $\alpha$ -linolenic acid ( $\alpha$ -18:3)-containing membranes or as a function of DHA content. In addition, dietary DHA or incubation with DHA has been reported to render L1210 leukemia cells grown in animals or in culture more permeable to the anticancer drugs mitoxantrone and doxorubicin (Burns et al., 1988; Burns and Spector, 1990; Spector and Burns, 1987), which are known to cross lipid bilayers by simple diffusion (Burns et al., 1987; Dalmark and Hoffmann, 1983; Decorti et al., 1998).

The polyunsaturated DHA chain is less hydrophobic than saturated and monounsaturated chains, lowering cohesiveness of bilayers. Consistent with the increased water permeability, DHA also increases packing free volume ( $f_v$ ) measured by fluorescence lifetime of membrane probe 1,6-diphenylhexatriene (DPH) (Mitchell and



Litman, 1998a). Increases in  $f_v$  and water permeability both reflect a highly disordered bilayer midplane region with weak chain-chain interactions. The wedge shape of DHA-containing PCs, proposed by Holte et al. based on  $^2\text{H}$  NMR (Holte et al., 1995), predicts that looser lipid packing at the aqueous interface would result in deeper penetration of water and other solutes into the bilayer. As a result, DHA would promote hydration of the head group and interchain region. Indeed, measurements of fluorescence decay of DPH in PC vesicles confirmed that water content in the bilayer hydrocarbon region increases with increasing chain unsaturation, with the greatest reduction in DPH fluorescence lifetime seen in di-22:6 PC (Mitchell and Litman, 1998b). In addition, DHA-induced looser packing, as characterized by the increased  $f_v$ , has been directly correlated with the facilitated MI-to-MII conformational transition of rhodopsin within the lipid bilayer as measured by the equilibrium constant  $K_{\text{eq}}$  (Mitchell et al., 1998). This indicates that polyunsaturated chains provide a flexible environment facilitating structural transitions of membrane-embedded proteins such as rhodopsin.

Changes in lipid packing have been predicted to influence the transbilayer transport of phospholipids (De Kruijff and Wirtz, 1977). Indeed, DHA has been shown to promote transmembrane flip-flop of phospholipids as well as membrane permeability. Armstrong et al. examined the effect of PC acyl chain unsaturation on the transmembrane movement by monitoring dithionite quenching of fluorescent phospholipid probe 7-nitrobenz-2-oxa-1,3-diazol-4-yl phosphatidylethanolamine (NBD-PE) (Armstrong et al., 2003). The flip-flop rate of NBD-PE probes increased as a function of the number of double bonds in PC acyl chains, with a particularly marked

increase in the presence of DHA. The reported flip-flop half-life was 11.5h, 0.29h, and 0.086h for membranes composed of 18:0-18:1 PC, 18:0-22:6 PC, and 22:6-22:6 PC, respectively. Dithionite permeability rate was also enhanced with increasing unsaturation in this study.

### ***Elastic compressibility***

In general, the average cross-sectional molecular area of phospholipid increases with unsaturation and is large in the presence of DHA (Demel et al., 1972; Ghosh and Tinoco, 1972; Ghosh et al., 1973; Smaby et al., 1997). In addition, membranes are more compressible laterally when unsaturation increases, with the effect being maximal with DHA (Smaby et al., 1997). This implies a lower energy requirement to deform unsaturated membranes. Measurements of lateral area compressibility under osmotic stress revealed that 75% of the compressibility of 18:0-22:6 PC membranes is attributed to the DHA chain (Koenig et al., 1997), indicating that the DHA chain is far more compressible laterally than the saturated counterpart. Therefore, DHA allows for larger lateral changes of the lipid matrix without paying a high-energy penalty. The low compressibility coefficient of DHA, which presumably facilitates structural transition of integral proteins, has been proposed to explain the high activity of rhodopsin in DHA-rich membranes. While highly compressible laterally, the chain length and the area per molecule of polyunsaturated membranes is less sensitive to temperature changes than those of saturated membranes (Holte et al., 1995; Separovic and Gawrisch, 1996), indicating that DHA-rich membranes may provide optimal conditions for membrane-embedded proteins over a wider temperature range.

### ***Interaction of DHA and cholesterol***

Cholesterol is a major non-phospholipid component in biological membranes and strongly modulates physical properties of membrane bilayers. It is well established that cholesterol has a strong ordering effect on phospholipid acyl chains in the fluid phase, while disrupting acyl chain packing in the organized gel state (Davis, 1993; Mitchell and Litman, 1998a). This results in condensation of phospholipid monolayers and a reduction in enthalpy of the main gel-to-liquid crystalline phase transition and in general, the transition is eventually eliminated at high cholesterol concentrations (Hernandez-Borrell and Keough, 1993). These effects of cholesterol decrease as acyl chain unsaturation increases, with DHA-containing PCs far less affected than many other PCs (Davis, 1993; Hernandez-Borrell and Keough, 1993; Mitchell and Litman, 1998a). In addition, even at concentrations above 30 mol%, cholesterol has very little effect on the chain packing properties of dipolyunsaturated bilayers. In both di-20:4 PC and di-22:6 PC, cholesterol has almost no effect on the gel-to-liquid crystalline phase transition (Kariel et al., 1991) and causes a minimal change in acyl chain packing (Mitchell and Litman, 1998a) and only a small decrease in the monolayer and bilayer in-plane elasticity (Smaby et al., 1997). In general, the effect of cholesterol was more diminished in di-22:6 PC than in di-20:4 PC.

Cholesterol has been shown to reduce the MI-to-MII structural transition of membrane protein rhodopsin as well as the acyl chain packing in reconstituted model membranes, regardless of phospholipid composition (Litman and Mitchell, 1996; Mitchell et al., 1992; Mitchell et al., 1990). However, DHA-containing species, both di-

22:6 PC and 16:0-22:6 PC, showed the smallest percentage of change upon introduction of 30 mol% of cholesterol. In addition, the MII rhodopsin concentration in the di-22:6 PC/30 mol% cholesterol bilayer was higher than in any of the other cholesterol-free phospholipid bilayers studied. These results demonstrate that the cholesterol effect is highly dependent on phospholipid unsaturation and that DHA-containing membranes are best able to buffer the inhibitory effects of cholesterol on the structural transition of integral proteins.

Accumulating experimental evidence has established preferential affinity of cholesterol for saturated over polyunsaturated chains, providing a basis for the recent hypothesis of a lipid-driven mechanism for lateral segregation into cholesterol-rich and -poor microdomains (Huster et al., 1998; Mitchell and Litman, 1998a; Pasenkiewicz-gierula et al., 1991; Zerouga et al., 1995). Unfavorable interaction between cholesterol and polyunsaturated chains has been clearly demonstrated by the exclusion of cholesterol from dipolyunsaturated PC membranes where it is forced to directly contact with polyunsaturated chains. Solubility of cholesterol is dramatically reduced in di-20:4 PC (~15 mol%) and in di-22:6 PC (~10 mol%), relative to 18:0-20:4 PC (~49 mol%) and 18:0-22:6 PC (~55 mol%), respectively, leading to the precipitation of cholesterol from the bilayer to form cholesterol monohydrate crystals (Brzustowicz et al., 2002a; Brzustowicz et al., 2002b; Brzustowicz et al., 1999; Brzustowicz et al., 2000). Studies with a variety of other techniques including differential scanning calorimetry (Kariel et al., 1991),  $^1\text{H}$  NMR and nuclear Overhauser enhancement spectroscopy with magic angle spinning (Huster et al., 1998), determination of partition coefficients (Niu

and Litman, 2002), measurements of lateral compressibility (Needham and Nunn, 1990), and fluorescence anisotropy (Mitchell and Litman, 1998a; Mitchell and Litman, 1998b) offered a similar conclusion. In general, the interaction of cholesterol with DHA was the weakest of all the acyl chains examined.

Huang and co-workers proposed the umbrella model to describe the solubility and condensing effect of cholesterol within membranes (Huang and Feigenson, 1999). According to this model, phospholipid head groups act as ‘umbrellas’ to prevent the energetically unfavorable contact of the non-polar part of cholesterol with interfacial water. This shielding will be less effective for DHA-containing phospholipids with a large molecular cross-sectional area, facilitating cholesterol precipitation at a lower concentration. In addition, this model allows for speculation that phosphatidylethanolamine (PE) with a smaller head group may enhance the DHA-associated reduction in shielding effects relative to PC. Consistent with this notion, unlike PC bilayers where a marked reduction in cholesterol solubility requires polyunsaturation at both *sn*-1 and *sn*-2 positions, DHA at *sn*-2 position with a saturated *sn*-1 chain is sufficient in PE to trigger cholesterol precipitation (Shaikh et al., 2003). The preferential interaction of cholesterol with 18:0-22:6 PC over 18: 22:6 PE and 18:0-22:6 phosphatidylserine (PS) and the larger increase in cholesterol-induced order in 18:0-22:6 PC in 18:0-22:6 PC/PE/PS/cholesterol mixture may be explained in a similar manner (Huster et al., 1998).

Cholesterol-dependent molecular sorting of membrane lipids based on the differential ordering of saturated versus polyunsaturated chains has provided an

important basis for the mechanism responsible for the heterogeneous lateral organization in membranes. Although support from biological membranes has not been clear, growing evidence from model membrane studies suggest that the energetically less favorable interaction between cholesterol and PUFAs, especially DHA, promotes lateral phase segregation into sterol-poor/PUFA-rich and sterol-rich/saturated fatty acid-rich microdomains (Brzustowicz et al., 2002a; Brzustowicz et al., 2002b; Huster et al., 1998; Mitchell and Litman, 1998a; Niu and Litman, 2002; Pasenkiewicz-Gierula et al., 1990). With regard to the molecular organization inside cholesterol/saturated acyl chain microdomains, Mitchell and Litman (Mitchell and Litman, 1998a) proposed a model of transient, highly dynamic microdomains. They envisaged that cholesterol is surrounded by mixed-chain saturated-polyunsaturated PCs with their saturated *sn*-1 chains pointing toward the tightly packed interior of the domain and the polyunsaturated *sn*-2 chains oriented toward the domain boundary. A similar model was proposed by Huster et al. (Huster et al., 1998). Notably, fluorescence resonance energy transfer experiments have provided evidence of the formation of lateral domains in a mixed PC system composed of di-22:6 PC, di-16:0 PC, cholesterol, and rhodopsin (Polozova and Litman, 2000). In this study, rhodopsin preferentially segregated with di-22:6 PC, while cholesterol with di-16:0 PC. Lateral domain behavior was observed only when both cholesterol and rhodopsin were present in the bilayers. The results of this study predict similar local compositional behavior for other proteins with a preference for a DHA-rich microenvironment.

## CHAPTER III

### MATERIALS AND METHODS

#### **Reagents and plasmids**

RPMEI 1640 was obtained from Mediatech (Herndon, VA). Leibovitz's L-15 was purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Insulin/transferrin/selenium (ITS) was purchased from Collaborative Biomedical Products (Bedford, MA). GlutaMAX-1 and recombinant mouse interferon- $\gamma$  (IFN- $\gamma$ ) were obtained from Gibco BRL (Grand Island, NY). Fatty acid-free bovine serum albumin (BSA) and mycoplasma PCR-based ELISA were from Roche (Indianapolis, IN). Fatty acids were purchased from NuChek Prep (Elysian, MN). FM 4-64, BODIPY-TR ceramide, and Live/Dead Viability/Toxicity kit were from Molecular Probes (Eugene, OR). Effectene Transfection Reagent was purchased from Qiagen (Valencia, CA). LipofectAMINE 2000 was obtained from Life Technologies (Rockville, MD). All other reagents and tissue culture wares were from Fisher Scientific (Fair Lawn, NJ).

An expression vector encoding GFP fused with wild-type N-Ras (GFP-Nras-wt) in pEGFP-C3 (Clontech, Palo Alto, CA) was a kind gift from Dr. Mark Philips (New York University, New York, NY). Expression vectors for GFP-tagged wild-type or oncogenic forms of H-Ras and K-Ras4B (GFP-Hras-wt, GFP-HrasG12V, GFP-Kras-wt, and GFP-KrasG12V, respectively) in pEGFP-C3 were kindly provided by Dr. John Hancock (University of Queensland, Queensland, Australia). Expression vectors

encoding GFP extended with C-terminal 9 amino acids of H-Ras or C-terminal 17 amino acids of K-Ras4B (GFP-tH and GFP-tK, respectively) were also gifts from Dr. John Hancock. GFP fusion constructs of full-length Lck or Fyn (Lck-GFP and Fyn-GFP, respectively) in pEGFP-N1 (Clontech) were generously provided by Dr. Mark Davis (Stanford University, Stanford, CA) and Dr. Marilyn Resh (Memorial Sloan-Kettering Cancer Center, New York, NY), respectively. The GFP fusion construct of the ts045 temperature-sensitive mutant of vesicular stomatitis virus glycoprotein, VSVG (VSVG3-GFP; referred to VSVG-GFP in this manuscript), was a kind gift from Dr. Kai Simons (Max Planck Institute, Dresden, Germany).

### **Cell culture and transfection**

Young adult mouse colon (YAMC) cells were kindly donated by Dr. Robert Whitehead (Ludwig Institute for Cancer Research, Melbourne, Australia). YAMC cells are normal murine colonic epithelial cells derived from the *H-2k<sup>b</sup>-tsA58* transgenic Immortomouse and are conditionally immortalized by a temperature-sensitive mutant of the simian virus 40 large T antigen (tsA58) under the control of IFN- $\gamma$ -inducible *H-2k<sup>b</sup>* promoter (Whitehead et al., 1993). YAMC cells (passages 16-20) were maintained in RPMI 1640 supplemented with 5% FBS, 2 mM GlutaMax-1 (L-alanyl-L-glutamine), ITS (5  $\mu$ g/ml insulin; 5  $\mu$ g/ml transferrin; 5 ng/ml selenious acid), and 5 IU/ml of murine IFN- $\gamma$  under permissive conditions at 33°C in a humidified incubator with 5% CO<sub>2</sub>. When VSVG-GFP was expressed, cells were maintained at the permissive temperature for VSVG



transport, 32°C (Bergmann, 1989). Cultures were routinely confirmed to be mycoplasma free using a PCR-based ELISA.

For transient transfection, YAMC cells were grown on Lab-Tek 2-well glass coverslips (Nalge Nunc, Rochester, NY) and were transfected for 3h using Effectene (0.2 µg DNA per well for GFP-tagged Ras, GFP-tH, and GFP-tK) or LipofectAMINE 2000 (1.5-2.0 µg DNA per well for Lck-GFP, Fyn-GFP, and VSVG-GFP) according to the manufacturers' instructions. All transfection conditions were optimized to minimize the amounts of DNA and lipofection reagent and the length of incubation time in order to prevent any non-specific cytotoxicity.

#### **Preparation of fatty acid-albumin complex**

Free fatty acids in ethanol were stored at -80°C under nitrogen. Fatty acid-BSA complexes were prepared by modification of the method described by Lynch (Lynch, 1980). Briefly, aliquots of fatty acids in ethanol were dried under nitrogen and then dissolved in 50 mM sodium carbonate. Next, fatty acid-free BSA (15%) in RPMI 1640 medium was added to fatty acid sodium salts to give a final fatty acid concentration of 2.5 mM and a fatty acid to albumin molar ratio of 3:1. Aliquots of 0.2 µm filter-sterilized fatty acid-BSA complexes were stored at -20°C and were used within one month after preparation.

### **Confocal microscopy**

Cells were plated onto two-well glass coverslips at a density of  $5\text{-}20 \times 10^3$  cells per well. Next day, fatty acid treatment with  $50 \mu\text{M}$  BSA-bound DHA, LA, or oleic acid (OA) initiated 24h prior to transfection and continued until live cells were examined 36-48h post-transfection using confocal laser scanning microscopy. Where indicated, cells incubated with DHA were washed three times with phosphate-buffered saline 36h after transfection and were subsequently placed in LA- or OA-supplemented medium and imaged 24h later. Medium supplemented with fatty acids was replenished every 24h. Transient transfection was performed in the absence of fatty acids for 3h and cells were subsequently returned to fatty acid-supplemented medium. The experimental time course is summarized in Fig. 4. Images were collected using a Radiance 2000 MP confocal microscope equipped with a LaserSharp 2000 acquisition software (BioRad, Hercules, CA). Cells were stained with a vital fluorescent dye FM 4-64 ( $20 \mu\text{M}$ ) in cold Leibovitz's L-15 medium without phenol red to visualize the PM (Janecki et al., 2000). The low temperature was used to prevent endocytosis of FM 4-64 and to arrest intracellular vesicle traffic during optical scanning (Kuismanen and Saraste, 1989). Immediately after staining, confocal  $z$ -serial images across the entire cell were obtained with a  $0.8 \mu\text{m}$  optical section thickness using a  $60\times$ , numerical aperture (N.A.) 1.2 water immersion objective. For cells expressing GFP-Nras-wt, a  $40\times$ , N.A. 1.3 oil immersion objective and  $0.7 \mu\text{m}$  intervals in the  $z$  axis were used. In general, 9-10 optical sections were required to scan the entire cell, corresponding to approximately  $7\text{-}8 \mu\text{m}$  of cell thickness. GFP and FM 4-64 were excited at  $488 \text{ nm}$  and  $568 \text{ nm}$ , respectively, and

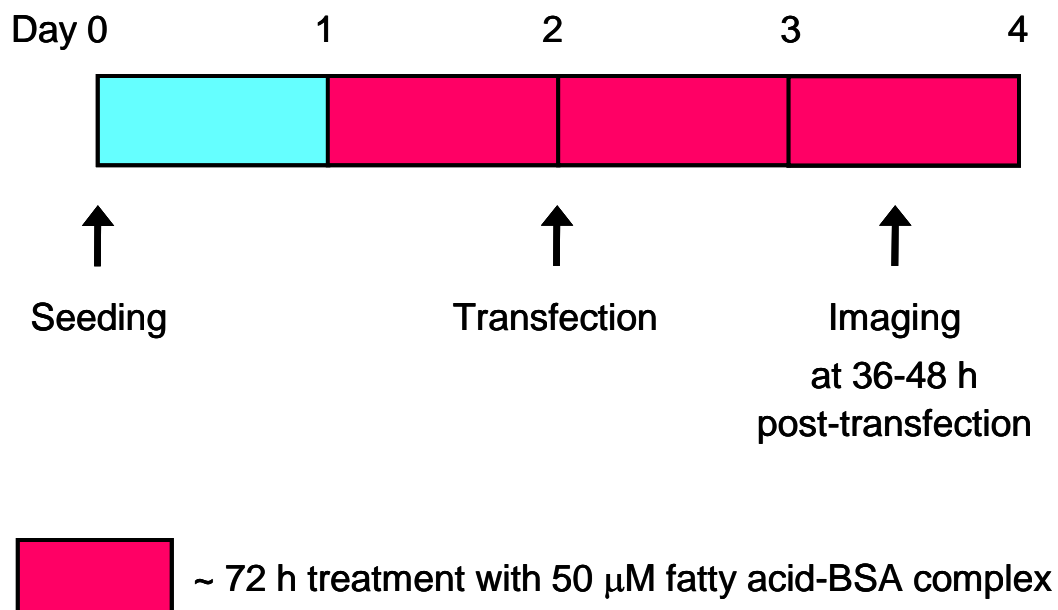


Figure 4. **Summarized experimental time course.** YAMC cells were plated onto glass coverslips and were incubated with 50  $\mu$ M albumin-bound DHA, LA, or OA for 24h prior to and 36-48h after transfection. Live cells transiently expressing GFP-labeled proteins were examined two days post-transfection using confocal microscopy.

emitted fluorescence was collected simultaneously by two separate photomultipliers using  $515 \pm 15$  nm bandpass filter (GFP) and 600 nm longpass filter (FM 4-64). After image acquisition, cell viability was examined using a Live/Dead Viability/Toxicity kit. Cell viability was greater than 90% in all dishes. An example of  $z$ -serial image stacks obtained from a GFP-Hras-wt-expressing YAMC cell with FM 4-64 staining is shown in Fig. 5.

### **Quantification of subcellular localization of GFP-labeled proteins**

The  $z$ -series stack images were digitally processed using MetaMorph software (Universal Imaging Corp., West Chester, PA) to quantify the localization of GFP-labeled proteins at the PM and at the Golgi. The relative amount of GFP-labeled proteins associated with the PM or with the Golgi was expressed as a percentage of total cellular GFP-labeled proteins. Fig. 6 illustrates analytical principles of the quantification of the PM- and Golgi-localized GFP-labeled proteins.

The PM localization of GFP-labeled proteins was quantified using the morphometric method described by Janecki (Janecki et al., 2000) with modifications as follows. First, the optical sections spanning the entire cell were selected from the original image stack for analysis. The optical section corresponding to the bottom of the cell, which contains the basal membrane facing the culture substratum, was identified based on the high intensity GFP and FM 4-64 fluorescence signals homogeneously distributed across the entire cell area. The images of FM 4-64 representing the PM were thresholded, binarized and inverted to generate binary masks of the PM. Next, the

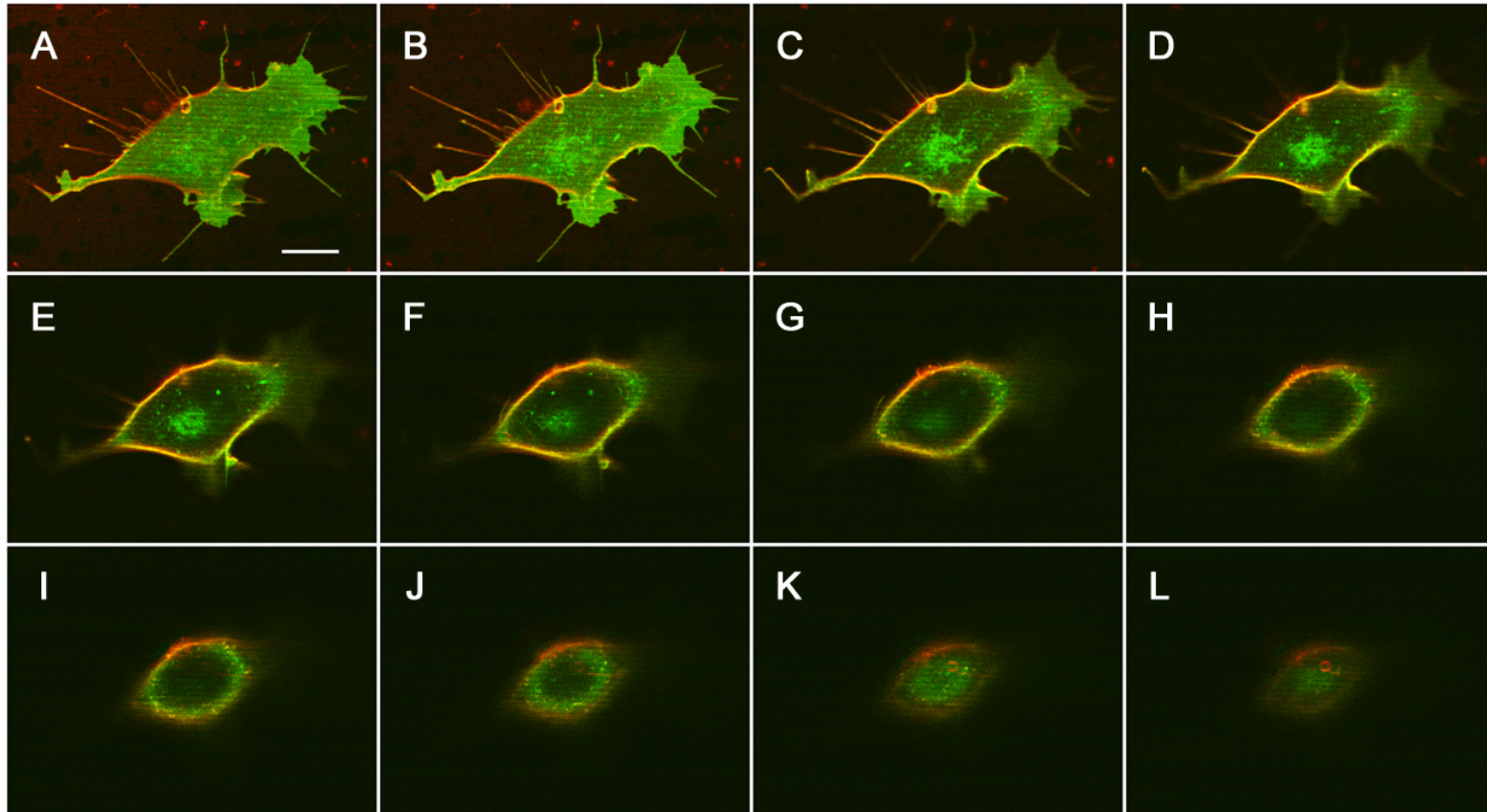


Figure 5. **Confocal serial optical sections of a living YAMC cell stained with FM 4-64.** YAMC cells expressing GFP-labeled proteins were incubated with a vital styryl dye FM 4-64 (20  $\mu\text{M}$ ) on a microscopic stage. Signals from GFP (green) and FM 4-64 (red) were collected by separate photomultipliers. Confocal  $z$ -serial optical sections collected from the bottom towards the top of the cell (A-L) in steps of 0.8  $\mu\text{m}$  are shown as overlay images of green and red fluorescence signals. Yellow or orange regions indicate colocalization of GFP and FM 4-64 signals. Scale bar represents 10  $\mu\text{m}$ .

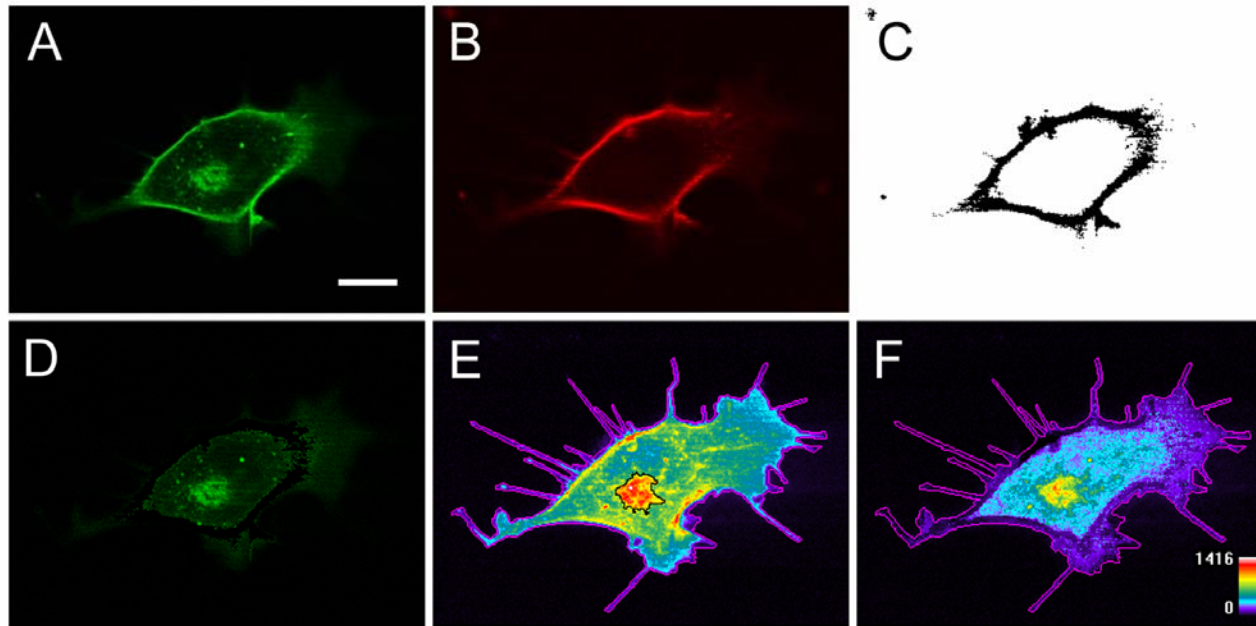


Figure 6. **Analytical procedure to quantify the relative subcellular localization of GFP-labeled proteins.** (A) Background-subtracted GFP image. Fluorescence signals from FM 4-64 illuminating the PM (B) were converted to a binary mask of the PM (C). A logical operation of “AND” was performed on the background-subtracted GFP image (A) and the binary mask of the PM (C), which resulted in a digital subtraction of GFP signals from the PM region (D). This procedure was performed on all optical sections spanning the entire cell. Next, *z*-serial stacks of the background-subtracted original GFP images and the new digitally processed GFP images lacking the signal at the PM were individually summed into single images (E and F, respectively). In E and F, fluorescence intensities are color-coded based on the pseudocolor scale (inset in F). Areas enclosed by pink lines in E and F are the region of interest (ROI) used to define the entire cell area, from which the integrated fluorescence intensities of total cellular ( $F_t$ ) and total intracellular GFP-labeled proteins ( $F_i$ ) were obtained respectively. The black line in E marks the ROI corresponding to the Golgi, from which the integrated fluorescence intensity of Golgi-localized GFP-labeled proteins ( $F_g$ ) was obtained. The percentages of the PM- and Golgi-localized GFP-labeled proteins were calculated using formulae  $[(F_t - F_i)/F_t] \times 100$  and  $(F_g/F_t) \times 100$ , respectively. Scale bar represents 10  $\mu\text{m}$ .

Boolean logical operation “AND” was performed on the pair of the background-subtracted GFP image stack and the binary mask image stack. The result of this operation was a new  $z$ -series of GFP images in which the GFP signal at the PM was digitally subtracted. Then, using “Sum of” stack arithmetic operation, the background-subtracted original and the new processed series of GFP images were individually summed into single images, from which the integrated fluorescence intensities of total cellular ( $F_t$ ) and total intracellular GFP-labeled proteins ( $F_i$ ) were obtained, respectively. The final calculation of the percentage of PM-localized GFP-labeled proteins was conducted using a formula  $[(F_t - F_i)/F_t] \times 100$ .

Alternatively, in an attempt to semi-automate the above-mentioned analytical procedure to quantify the relative PM localization, an algorithm with a graphical interface was developed using the SDC Morphology Toolbox V1.1 for MATLAB Version 6.1.0.450 (R12.1). The numerical values calculated using the algorithm were highly comparable to the manually calculated data using MetaMorph software for the same cell and the difference between the two measurements was statistically insignificant.

The percentage of GFP-Nras-wt, GFP-Hras-wt or GFP-HrasG12V localized at the Golgi was calculated using the single image generated by “Sum of” stack arithmetic operation on the background-subtracted GFP image stack. The region of interest (ROI) corresponding to the Golgi was determined based on the perinuclear cytological positioning and the distinct condensed tubulovesicular morphology well delineated by intense GFP-Ras fluorescence.

Images were processed using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA) for presentation.

### **Statistical analysis**

To examine how the PM localization of GFP-labeled proteins varied with different fatty acid treatment, t- and F-tests under linear mixed-effects models (Pinheiro and Bates, 2000) were performed. Since the measurements were done either manually with MetaMorph software and/or using the automatic algorithm, there was a need to account for the discrepancy between the two measuring methods. Therefore, under the assumption that either a constant shift or a linear model was held between these two types of measurements, a comprehensive mixed effects model was constructed using sub-components to accommodate the general relationships among these two types of measurement methods and different fatty acid treatments. Cell level random effects were used to model the dependency between measurements taken from the same cell by two different measurement methods. Considering that the main interest was on the fatty acid effects, instead of estimating the unknown shift or regression coefficients in the sub-component that linked the two types of measurements and then using them in the estimation of the fatty acid effects under one of the two measuring methods, the unknown parameters were re-parametrized so that a direct estimation of fatty acid effects became feasible. Under this re-parametrization, the best linear unbiased predictions of the average fatty acid effects using both methods with the same sample sizes could be obtained. This direct approach has an advantage of less estimation variation and



consequently a higher power compared to implementing a multi-stage estimation method. Pairwise t-tests accounting for the mixed effects model variation were then carried out to compare the fatty acid treatments.

To determine fatty acid effects on the Golgi localization of GFP-labeled proteins, the statistical significance of differences among means was analyzed using one-way ANOVA and pairwise t-tests.

## CHAPTER IV

### RESULTS

#### **DHA alters PM targeting and subcellular distribution of GFP-Nras-wt**

Ras targeting to the inner surface of the PM, the main Ras signaling platform, remains crucial for its physiological and oncogenic activities, although recent findings revealed that Ras can engage signaling pathways even in the intracellular membrane compartments (Arozarena et al., 2004; Bivona et al., 2003; Caloca et al., 2003; Chiu et al., 2002; Jiang and Sorkin, 2002; Perez de Castro et al., 2004). To determine whether the PM localization of Ras is altered by fatty acid treatment, YAMC cells transiently overexpressing GFP-Nras-wt were incubated with a physiological dose (50  $\mu$ M) (Conquer and Holub, 1998) of DHA (22:6, n-3) or LA (18:2, n-6) and were examined alive using three-dimensional confocal microscopy 36-48h post-transfection. For accurate quantification of PM-associated GFP-Nras-wt, a lipophilic, membrane-impermeable fluorophore, FM 4-64, was used to specifically label the PM (Janecki et al., 2000). The effects of DHA and LA were examined because DHA present in fish oil has previously been shown to decrease Ras membrane association as well as Ras-dependent signaling relative to LA, the major dietary PUFA in the U.S. diet (Collett et al., 2001). Untreated cells were used as a negative control to account for any non-specific effects of fatty acid supplementation on the subcellular distribution of GFP-Nras-wt. Cell viability measured after image acquisition was greater than 90% in all treatment groups,

indicating that neither fatty acid treatment nor fluorescence imaging caused non-specific cellular toxicity.

In agreement with previous reports on GFP-Nras subcellular localization (Choy et al., 1999), GFP-Nras-wt expression was evident at the PM and endomembrane structures in YAMC cells, with the most prominent feature being the intense GFP fluorescence delineating a compact perinuclear structure, presumably the Golgi (Fig. 7). The perinuclear structure was confirmed as the Golgi by colocalization with a Golgi-specific vital dye, BODIPY-TR ceramide (Pagano et al., 1991). As illustrated by representative images in Fig. 7, while untreated and LA-treated cells shared a similar subcellular distribution of GFP-Nras-wt with substantial PM staining, the distribution pattern was strikingly different in DHA-treated cells, displaying very little PM fluorescence and predominant localization of GFP-Nras-wt at the endomembrane compartments with extensive staining at the Golgi. These contrasting distributions were also evident in axial z sections cutting through the nucleus and the Golgi region (Fig. 7 insets). Cells with varying total cellular fluorescence intensities from six repeated experiments showed a very similar distribution pattern of GFP-Nras-wt within the same treatment group, indicating that 1) the expression level of GFP fusion proteins does not affect their intracellular trafficking and localization under the defined experimental conditions, and 2) the predominant association of GFP-Nras-wt with the endomembrane structures in DHA-treated cells is not due to differences in the protein expression level. In order to quantitatively compare the fatty acid effects on the subcellular localization of GFP-Nras-wt in YAMC cells, the percentage of GFP-Nras-wt localized at the PM and

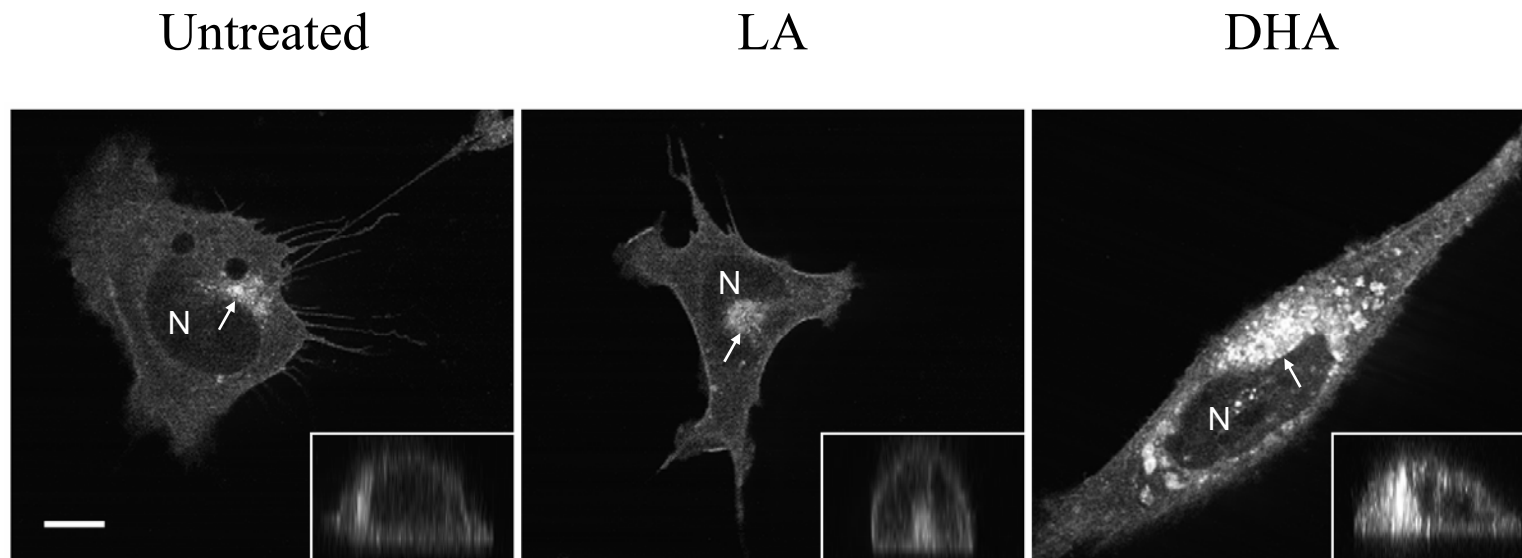


Figure 7. **DHA alters the subcellular distribution of GFP-Nras-wt.** YAMC cells were incubated with 50  $\mu$ M LA, or DHA or left untreated for 24h prior to transfection with pGFP-Nras-wt. Cells were further incubated with fatty acids or left untreated until live cells were examined by confocal microscopy 36-48h after transfection. Representative confocal *enface* sections of untreated and LA- or DHA-treated cells are shown. Insets in each panel depict reconstructed axial *z* sections cutting through the nucleus and the Golgi region of the same cell. Note the prominent GFP illumination of endomembrane structures in DHA-treated cells. Arrows and N mark perinuclear Golgi structures and nuclei, respectively. Scale bar represents 10  $\mu$ m.

the Golgi was quantified using digital image processing (Fig. 8). Unlike the ER, the area corresponding to the Golgi was readily identifiable due to the distinct compact multivesicular morphology and perinuclear positioning illuminated by intense GFP fluorescence. Therefore, meaningful quantification of the Golgi-localized GFP-Nras-wt was feasible within this experimental setup although it should be noted that the measured Golgi fluorescence intensity contained small contributions from fractions of the ER and the PM that overlap the Golgi ROI (Fig. 6). PM localization of GFP-Nras-wt (% of GFP fluorescence at the PM relative to the total cellular GFP fluorescence) in LA-treated cells ( $21.2 \pm 0.8\%$ ; mean  $\pm$  SE) was slightly higher than that of untreated cells ( $16.8 \pm 1.8\%$ ) but there was no significant difference in the Golgi association ( $15.7 \pm 1.6\%$  and  $12.9 \pm 2.4\%$ , respectively). In contrast, DHA treatment markedly reduced PM-localized GFP-Nras-wt ( $9.3 \pm 0.7\%$ ) by 56% and 44% compared with LA and no treatment respectively, while significantly increasing the Golgi localization ( $23.0 \pm 1.6\%$ ) by 55% compared with both LA and no treatment ( $14.9 \pm 1.3\%$ ; pooled from untreated and LA-treated cells), consistent with the qualitative observation of the increased endomembrane association at the expense of PM expression. A total of 50 cells with varying expression levels of GFP-Nras-wt (integrated total cellular fluorescence intensities of  $6.5 \times 10^5$ - $1.5 \times 10^7$  or average pixel intensities of 13-850) were analyzed and no statistical relationship was found between total cellular fluorescence intensities and the relative PM or Golgi localization, confirming that the subcellular targeting of GFP-Nras-wt was largely independent of its protein expression level. In addition, this indicates that the secretory

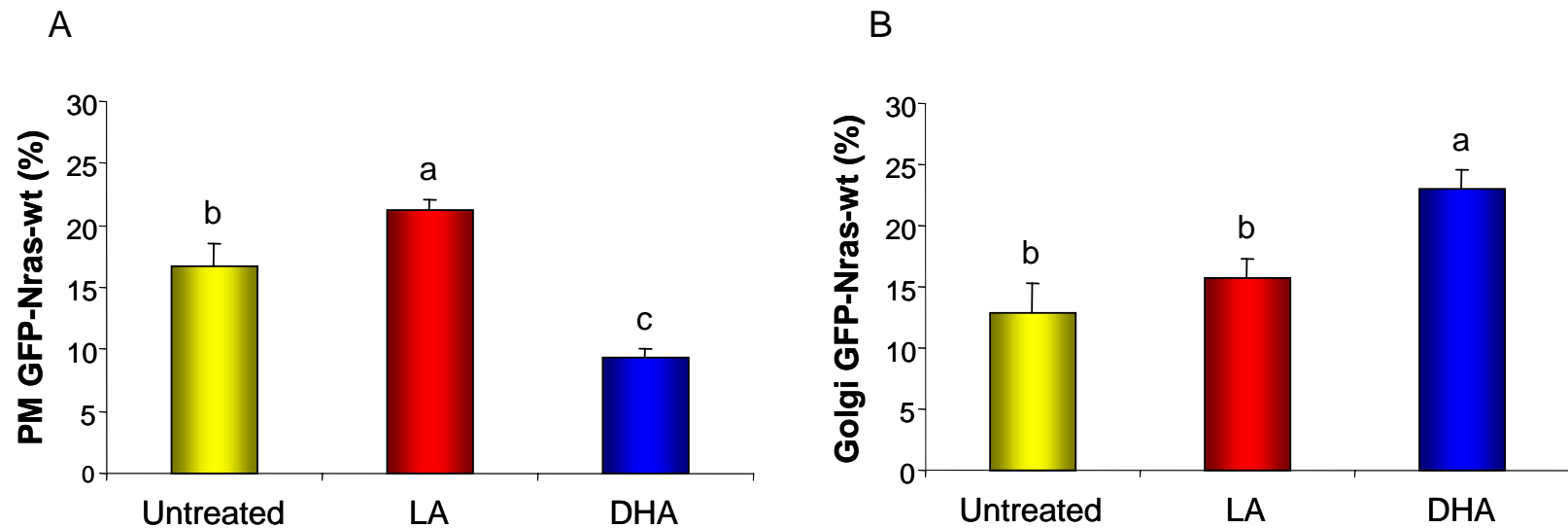


Figure 8. **DHA decreases PM localization and increases Golgi association of GFP-Nras-wt.** YAMC cells were incubated with 50  $\mu$ M LA, or DHA or left untreated for 24h prior to and 36-48h after transfection with pGFP-Nras-wt. Cells were stained with a vital dye FM 4-64 on a microscopic stage and z-series images of living cells expressing GFP-Nras-wt were obtained by confocal microscopy 36-48h post-transfection. The relative amount of GFP-Nras-wt localized at the PM or at the Golgi was quantified as percent of total cellular GFP-Nras-wt as described in Materials and Methods. (A) Relative PM localization of GFP-Nras-wt. (B) Relative Golgi localization of GFP-Nras-wt. A total of 50 cells from 6 independent experiments were analyzed and data are presented as mean  $\pm$  SE. Different letters denote statistical significance at  $p < 0.007$  for (A) and at  $p < 0.003$  for (B), respectively.

transport machinery or rate-limiting steps therein was not saturated by the overexpression of GFP fusion proteins under the defined experimental conditions.

**DHA alters intracellular trafficking of GFP-Hras but not GFP-Kras to the PM regardless of their mutation status**

Ras isoforms, H-Ras, N-Ras, and K-Ras4B, utilize different membrane anchors and trafficking routes to the PM. Upon completion of the common sequential post-translational processing driven by a CAAX motif on the cytoplasmic surface of the ER, Ras isoforms take different trafficking routes to transit to the PM. H- and N-Ras are additionally palmitoylated on one (N-Ras) or two (H-Ras) cysteine residue(s) adjacent to the C-terminal CAAX motif and traffic to the PM along the exocytic pathway via vesicular transport through the Golgi. In contrast, K-Ras4B containing a polybasic region of six contiguous lysine residues takes an as yet undefined pathway to the PM that bypasses the Golgi (Apolloni et al., 2000; Choy et al., 1999). Therefore, it was investigated whether DHA exerts the same effects on the PM targeting of GFP-labeled H-Ras and K-Ras4B as it does on GFP-Nras-wt. Because the similar distribution pattern of GFP-Nras-wt between untreated and LA-treated YAMC cells indicated that fatty acid supplementation *per se* does not cause significant alterations in subcellular targeting of GFP-labeled proteins, the effects of DHA were compared with other common dietary unsaturated fatty acids, LA and OA. LA (18:2, n-6) is the predominant dietary PUFA in Western diets (Baghurst et al., 1988; Mann et al., 1995; Okuyama et al., 1996) and the major fatty acid in most vegetable oils. OA (18:1, n-9) is highly prevalent in the

Mediterranean diet and enriched in olive oil. Dietary consumption of DHA, LA, or OA readily alters cellular lipid environment by incorporation into membrane phospholipids (Glatz et al., 1989; Katan et al., 1997; Pagnan et al., 1989; Vidgren et al., 1997). Illustrated in Fig. 9 are representative images of GFP-Hras-wt-expressing cells incubated with fatty acids. Whereas the overall subcellular localization of GFP-H-Ras-wt was similar to that of GFP-Nras-wt in terms of the PM, Golgi, and ER expression, as previously reported (Choy et al., 1999), some quantitative differences were apparent. GFP-Hras-wt-expressing cells had much more prominent PM fluorescence and less intense Golgi staining compared with GFP-Nras-wt in all fatty acid treatment groups. The subcellular distribution of GFP-Hras-wt was indistinguishable between LA- and OA-treated cells, as confirmed by no difference in either PM- or Golgi-associated fractions (Fig. 10). DHA, however, significantly reduced the PM localization ( $37.7 \pm 1.5\%$ ) of GFP-Hras-wt by 36% and increased the endomembrane association as evidenced by 56% increase in the Golgi localization ( $10.4 \pm 1.3\%$ ), compared with LA and OA ( $59.1 \pm 1.2\%$  at the PM and  $6.6 \pm 0.5\%$  at the Golgi; pooled values from LA- and OA-treated cells). GFP-Kras-wt was expressed at the PM along with a rather diffuse staining over the cytoplasm occasionally in a punctate manner, suggestive of ER association (Fig. 11). As expected, there was no particular intense GFP fluorescence illuminating any distinct perinuclear vesicular structure. Interestingly, unlike palmitoylated H- and N-Ras with the same trafficking route to the PM, the relative PM expression as well as distribution pattern of GFP-Kras-wt was unaffected by fatty acid treatment (Fig. 11 and Fig. 12). The magnitude of PM localization of GFP-Kras-wt



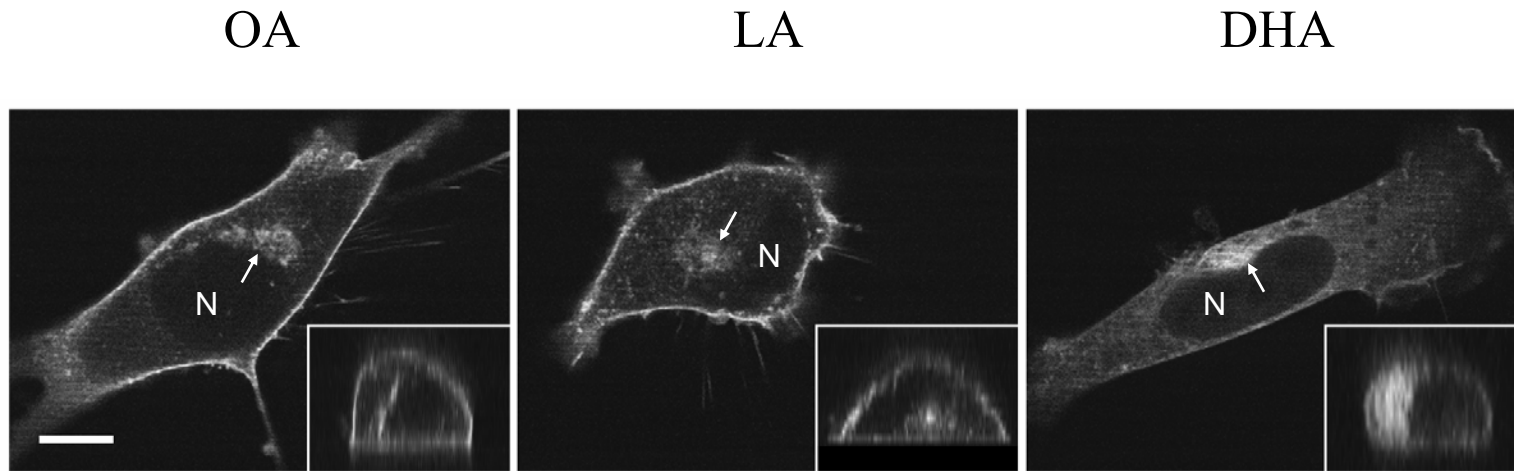


Figure 9. **DHA alters the subcellular distribution of GFP-Hras-wt.** YAMC cells were incubated with 50  $\mu\text{M}$  OA, LA, or DHA for 24h prior to transfection with pGFP-Hras-wt. Cells were further incubated with fatty acids until live cells were examined by confocal microscopy 36-48h after transfection. Representative confocal *enface* sections of OA-, LA-, or DHA-treated cells are shown. Insets in each panel depict reconstructed axial z sections cutting through the nucleus and the Golgi region of the same cell. Note the prominent GFP illumination of endomembrane structures in DHA-treated cells. Arrows and N mark perinuclear Golgi structures and nuclei, respectively. Scale bar represents 10  $\mu\text{m}$ .

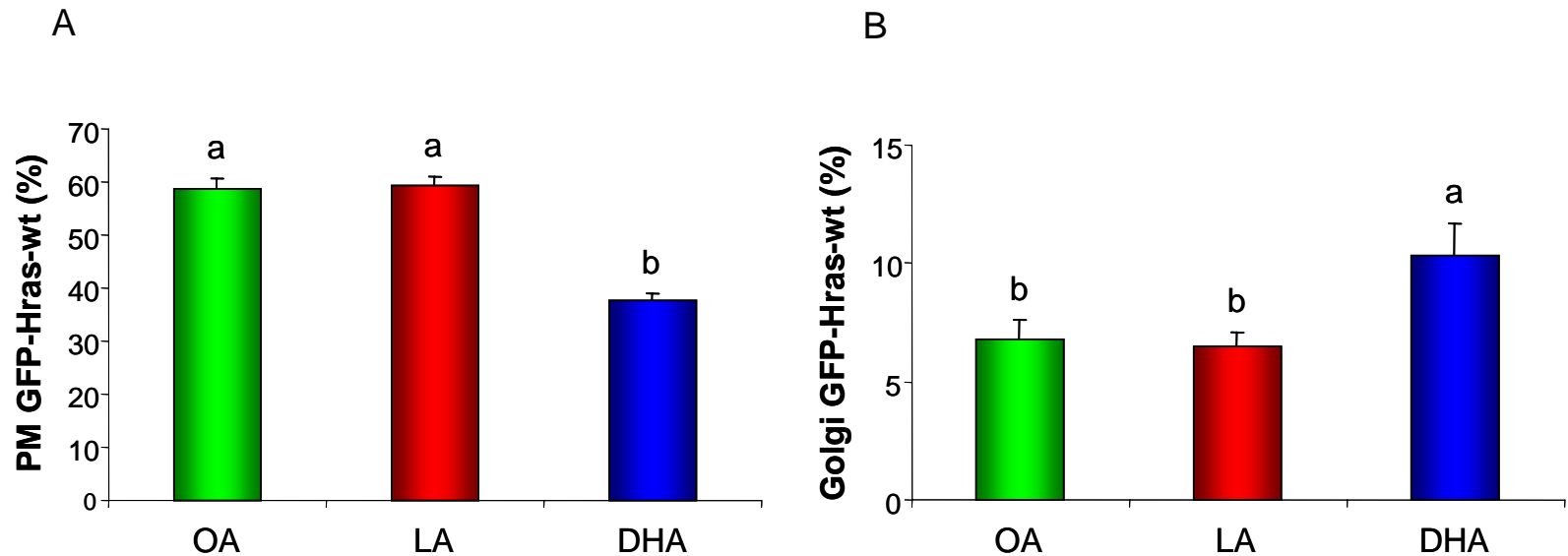


Figure 10. **DHA decreases PM localization and increases Golgi association of GFP-Hras-wt.** YAMC cells were incubated with 50  $\mu$ M OA, LA, or DHA for 24h prior to and 36-48h after transfection with pGFP-Hras-wt. Cells were stained with a vital dye FM 4-64 on a microscopic stage and z-serial images of living cells expressing GFP-Hras-wt were obtained by confocal microscopy 36-48h post-transfection. The relative amount of GFP-Hras-wt localized at the PM or at the Golgi was quantified as percent of total cellular GFP-Hras-wt as described in Materials and Methods. (A) Relative PM localization of GFP-Hras-wt. (B) Relative Golgi localization of GFP-Hras-wt. A total of 33 cells from 2 independent experiments were analyzed and data are presented as mean  $\pm$  SE. Different letters denote statistical significance at  $p < 0.0001$  for (A) and at  $p < 0.02$  for (B), respectively.

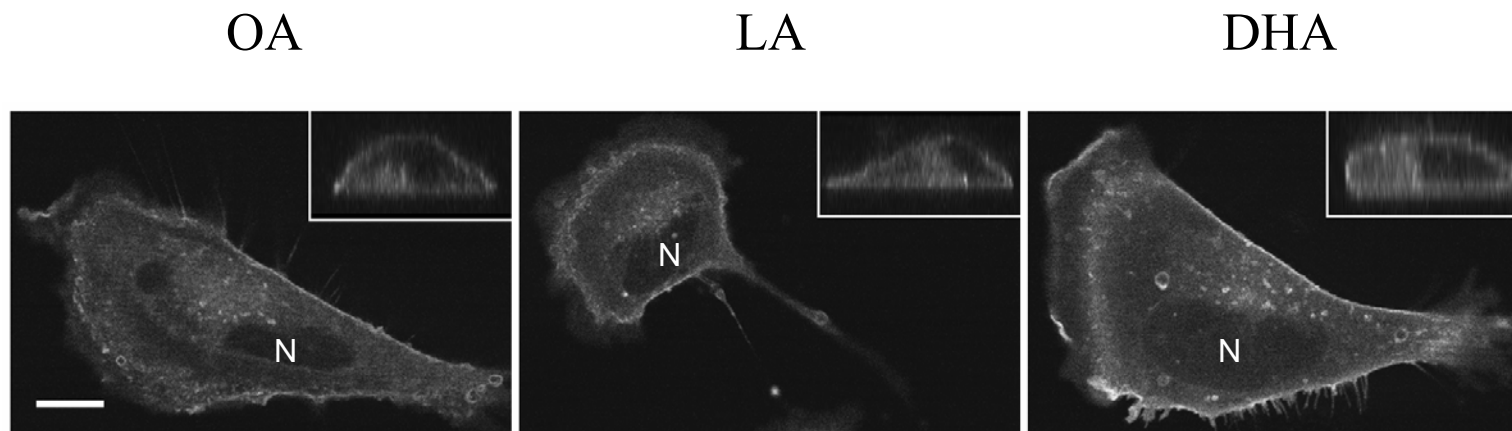


Figure 11. **Subcellular distribution of GFP-Kras-wt is not altered by fatty acid treatment.** YAMC cells were incubated with 50  $\mu$ M OA, LA, or DHA for 24h prior to transfection with pGFP-Kras-wt. Cells were further incubated with fatty acids until live cells were examined by confocal microscopy 36-48h after transfection. Representative confocal *enface* sections of OA-, LA-, or DHA-treated cells are shown. Insets in each panel depict reconstructed axial *z* sections cutting through the nucleus of the same cell. Nuclei are marked with N. Scale bar represents 10  $\mu$ m.

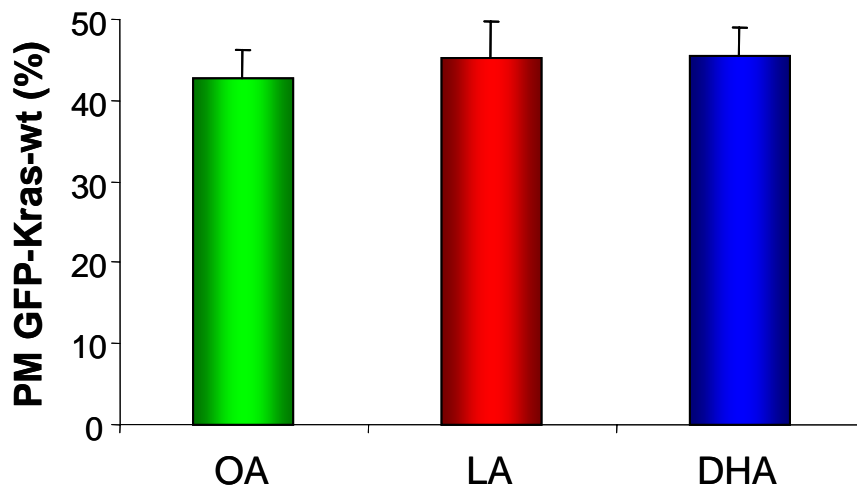


Figure 12. **PM localization of GFP-Kras-wt is not altered by fatty acid treatment.** YAMC cells were incubated with 50  $\mu$ M OA, LA, or DHA for 24h prior to and 36-48h after transfection with pGFP-Kras-wt. Cells were stained with a vital dye FM 4-64 on a microscopic stage and  $z$ -serial images of living cells expressing GFP-Kras-wt were obtained by confocal microscopy 36-48h post-transfection. The relative amount of GFP-Kras-wt localized at the PM was quantified as percent of total cellular GFP-Kras-wt as described in Materials and Methods. A total of 30 cells from 2 independent experiments were analyzed and data are presented as mean  $\pm$  SE. No significant difference was observed among means ( $p > 0.58$ ).

( $44.5 \pm 2.3\%$ ) was less than that of GFP-Hras-wt ( $59.1 \pm 1.2\%$  in LA- and OA-treated cells) but greater than that of N-Ras-wt ( $16.8 \pm 1.8\%$ , and  $21.2 \pm 0.8\%$  in untreated and LA-treated cells, respectively).

As oncogenic Ras proteins carrying a point mutation at either codon 12, 13, or 61 play a major role in numerous human malignancies (Bos, 1989; Campbell and Der, 2004), Ras itself and Ras-dependent signaling pathways have been important molecular targets for anticancer therapies. Among others, inhibition of Ras PM association has been the major approach to develop Ras-directed anticancer agents (Kloog and Cox, 2000; Wittinghofer and Waldmann, 2000). In light of DHA-induced differential alterations in the PM targeting of wild-type Ras isoforms tagged with GFP, the ability of DHA to alter the PM localization of GFP-labeled oncogenic H-RasG12V and K-RasG12V was determined. In parallel with the results from wild-type Ras isoforms, while DHA treatment dramatically altered the subcellular localization of GFP-HrasG12V compared with LA and OA (Fig. 13 and Fig. 14), there was no difference among fatty acid-treated cells with regard to PM localization and distribution of GFP-KrasG12V (Fig. 15 and Fig. 16). Quantitatively, the extent of the DHA effect on GFP-HrasG12V was similar to its wild-type version in that DHA caused suppression of GFP-HrasG12V localization at the PM ( $41.9 \pm 2.4\%$ ) by 32%, concomitant with 85% increase in the Golgi fraction ( $14.9 \pm 3.1\%$ ) reflecting an increased endomembrane association, compared with LA and OA ( $62.0 \pm 1.8\%$  at the PM and  $8.1 \pm 0.7\%$  at the Golgi; pooled values from LA- and OA-treated cells) (Fig. 14). However, in GFP-HrasG12V-expressing cells treated with DHA, the overall endomembrane staining often seemed

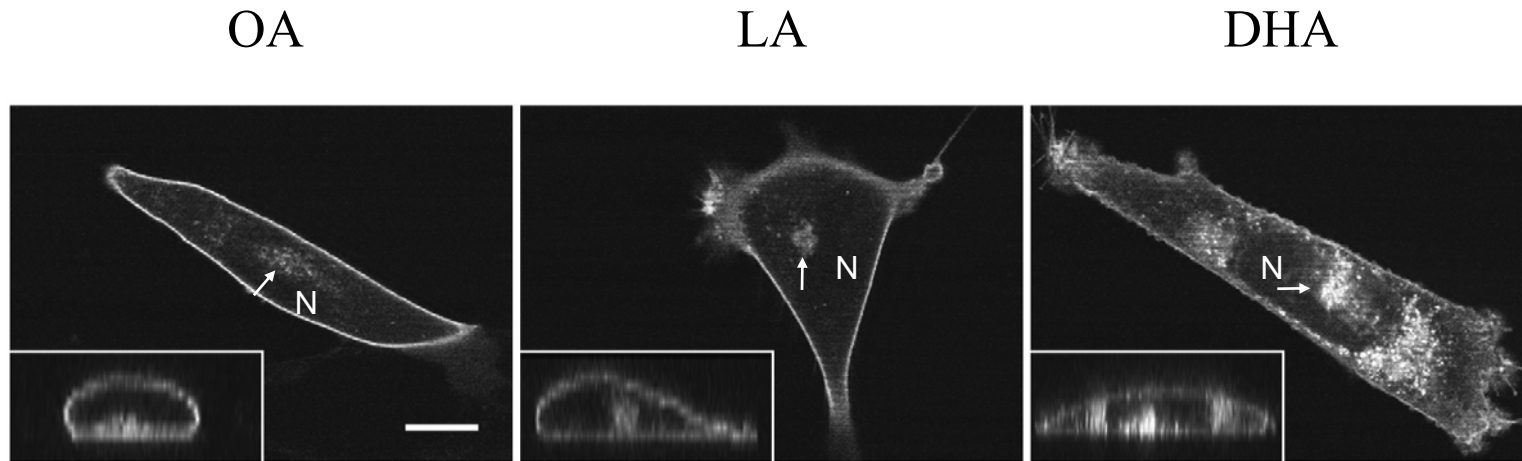


Figure 13. **DHA alters the subcellular distribution of GFP-HrasG12V.** YAMC cells were incubated with 50  $\mu$ M OA, LA, or DHA for 24h prior to transfection with pGFP-HrasG12V. Cells were further incubated with fatty acids until live cells were examined by confocal microscopy 36-48h after transfection. Representative confocal *enface* sections of OA-, LA-, or DHA-treated cells are shown. Insets in each panel depict reconstructed axial *z* sections cutting through the nucleus and the Golgi region of the same cell. Note the prominent GFP illumination of endomembrane structures in DHA-treated cells. Arrows and N mark perinuclear Golgi structures and nuclei, respectively. Scale bar represents 10  $\mu$ m.

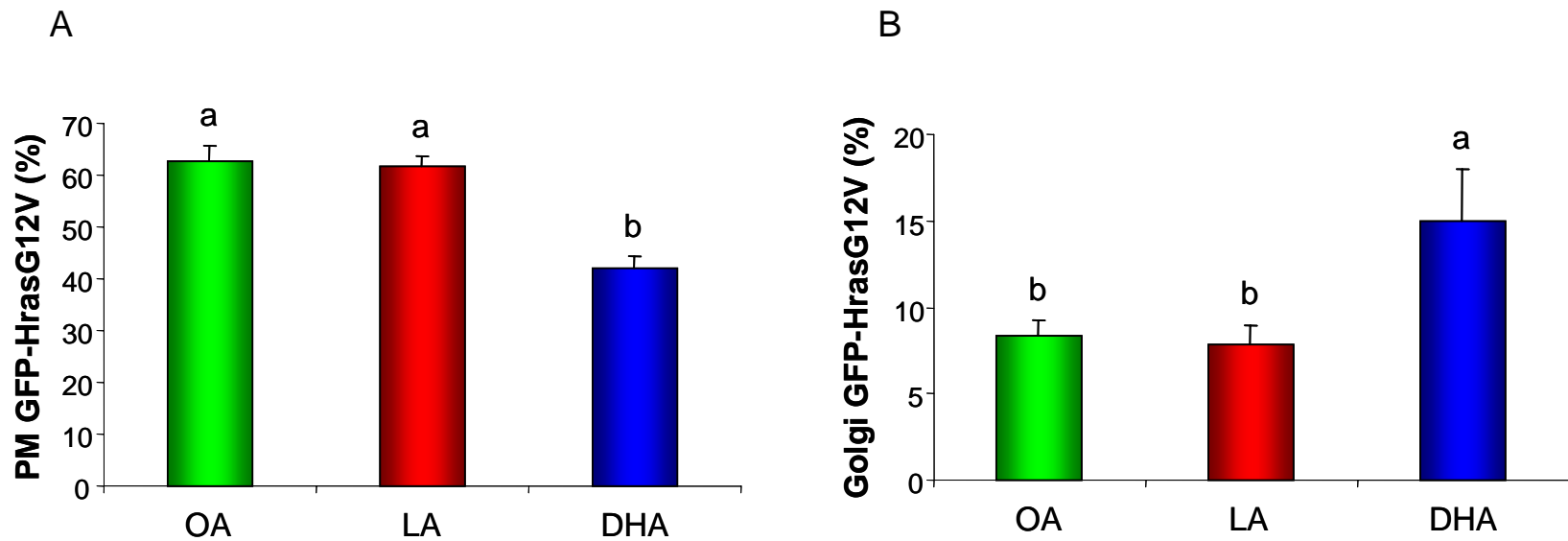


Figure 14. **DHA decreases PM localization and increases Golgi association of GFP-HrasG12V.** YAMC cells were incubated with 50  $\mu$ M OA, LA, or DHA for 24h prior to and 36-48h after transfection with pGFP-HrasG12V. Cells were stained with a vital dye FM 4-64 on a microscopic stage and  $z$ -serial images of living cells expressing GFP-Hras-G12V were obtained by confocal microscopy 36-48h post-transfection. The relative amount of GFP-HrasG12V localized at the PM or at the Golgi was quantified as percent of total cellular GFP-HrasG12V as described in Materials and Methods. (A) Relative PM localization of GFP-HrasG12V. (B) Relative Golgi localization of GFP-HrasG12V. A total of 35 cells from 2 independent experiments were analyzed and data are presented as mean  $\pm$  SE. Different letters denote statistical significance at  $p < 0.0001$  for (A) and at  $p < 0.04$  for (B), respectively.

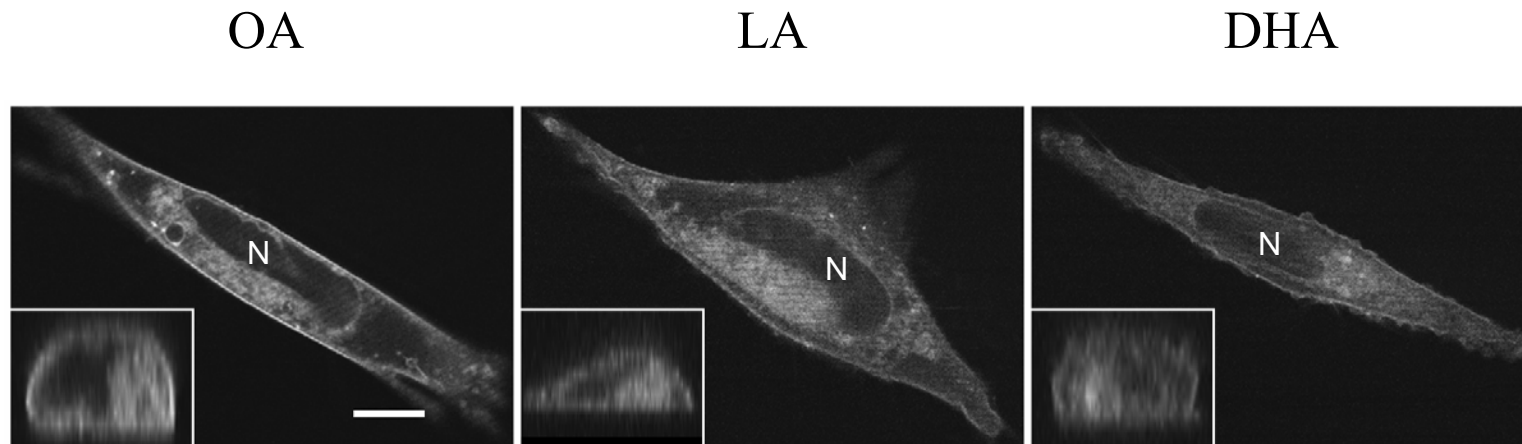


Figure 15. **Subcellular distribution of GFP-KrasG12V is not altered by fatty acid treatment.** YAMC cells were incubated with 50  $\mu$ M OA, LA, or DHA for 24h prior to transfection with pGFP-KrasG12V. Cells were further incubated with fatty acids until live cells were examined by confocal microscopy 36-48h after transfection. Representative confocal *enface* sections of OA-, LA-, or DHA-treated cells are shown. Insets in each panel depict reconstructed axial *z* sections cutting through the nucleus of the same cell. Nuclei are marked with N. Scale bar represents 10  $\mu$ m.



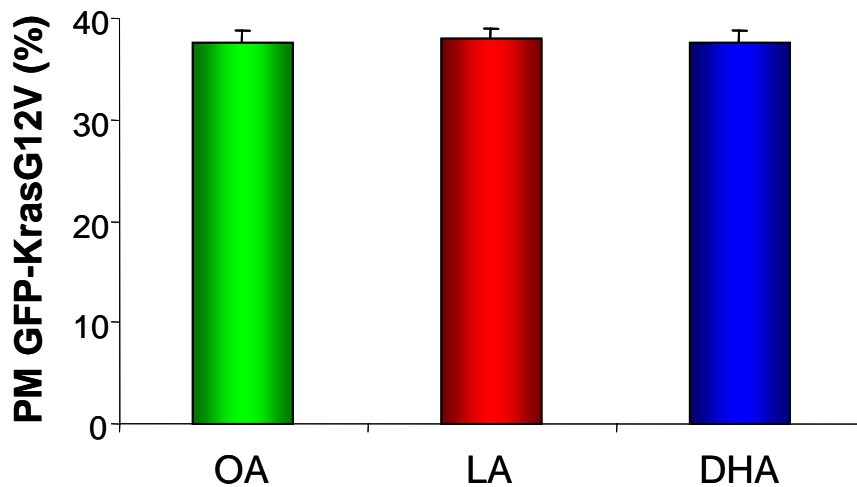


Figure 16. **PM localization of GFP-KrasG12V is not altered by fatty acid treatment.** YAMC cells were incubated with 50  $\mu$ M OA, LA, or DHA for 24h prior to and 36-48h after transfection with pGFP-KrasG12V. Cells were stained with a vital dye FM 4-64 on a microscopic stage and z-serial images of living cells expressing GFP-KrasG12V were obtained by confocal microscopy 36-48h post-transfection. The relative amount of GFP-KrasG12V localized at the PM was quantified as percent of total cellular GFP-KrasG12V as described in Materials and Methods. A total of 57 cells from 6 independent experiments were analyzed and data are presented as mean  $\pm$  SE. No significant difference was observed among means ( $p > 0.83$ ).

more concentrated around the juxtannuclear region rather than the cell periphery (Fig. 13) compared with the more spread and diffuse endomembrane illumination by GFP-labeled wild-type N-Ras and H-Ras in DHA-treated cells (Fig. 7 and Fig. 9). Similarly, GFP fluorescence reported a more prominent and concentrated endomembrane association of oncogenic K-Ras relative to wild-type K-Ras in all fatty acid-treated cells (Fig. 11 and Fig. 15), although intense GFP fluorescence signifying the compact multivesicular Golgi structure was absent in both. These observations suggest that subtle differences exist in the subcellular localization between wild-type and oncogenic Ras proteins.

Taken together, these findings clearly demonstrate that DHA differentially alters the subcellular localization of Ras isoforms depending on their trafficking routes and/or membrane anchors, thereby decreasing PM localization and increasing endomembrane association of GFP-labeled N-Ras and H-Ras but not K-Ras, regardless of their mutation status.

### **DHA-induced alterations in Ras trafficking are independent of Ras signaling**

Ras downstream signaling pathways can affect membrane traffic directly and indirectly by modulating regulators of vesicular traffic and cytoskeleton dynamics (Babia et al., 1999; Lanzetti et al., 2001; Ridley, 2001; Tall et al., 2001; Xu et al., 2003). GFP-labeled full-length Ras isoforms are biologically functional. They recruit and activate downstream effectors upon upstream stimulation and oncogenic forms retain transforming activity (Niv et al., 1999; Niv et al., 2002; Perez de Castro et al., 2004). Therefore, DHA-induced differential alterations in PM targeting of Ras isoforms may be

attributed to the interference with the isoform-specific signaling functions of overexpressed Ras. To test this hypothesis, the effect of DHA on the ability of isolated H- and K-Ras targeting sequences to direct GFP to the PM was examined. GFP-tH and GFP-tK are biologically inert GFP chimeras, which are extended at the C-terminus with the last 9 amino acids of H-Ras and the last 17 amino acids of K-Ras4B, respectively. These extensively characterized GFP fusion proteins retain the trafficking characteristics and localization pattern of their cognate full-length Ras isoforms, due to the presence of Ras C-terminal sequences containing all the necessary and sufficient information for PM targeting (Apolloni et al., 2000; Choy et al., 1999; Prior et al., 2001).

As shown in Fig. 17 and Fig. 18, DHA treatment, compared with LA and OA, significantly reduced PM localization by 40% and increased the endomembrane pool of GFP-tH. The endomembrane staining pattern of GFP-tH in DHA-treated cells was very similar to that of GFP-labeled wild-type H-Ras (Fig. 9) rather than oncogenic H-Ras in DHA-treated cells (Fig. 13). When the effect of DHA on GFP-tH PM targeting was compared with GFP-labeled full-length wild-type and oncogenic H-Ras, it became apparent that the relative PM localization in DHA-treated cells ( $36.1 \pm 1.7\%$ ,  $37.7 \pm 1.5\%$ , and  $41.9 \pm 2.4\%$ , respectively) as well as the extent of DHA-induced decrease in PM targeting (by 40%, 36%, and 32%, respectively, relative to values pooled from LA- and OA-treated cells) was remarkably similar among all three constructs, indicating that DHA-induced alterations in Ras PM targeting do not involve Ras-dependent signaling. In addition, the localization pattern of GFP-tH in LA- and OA-treated cells was essentially equivalent to those of GFP-labeled wild-type and oncogenic H-Ras, all

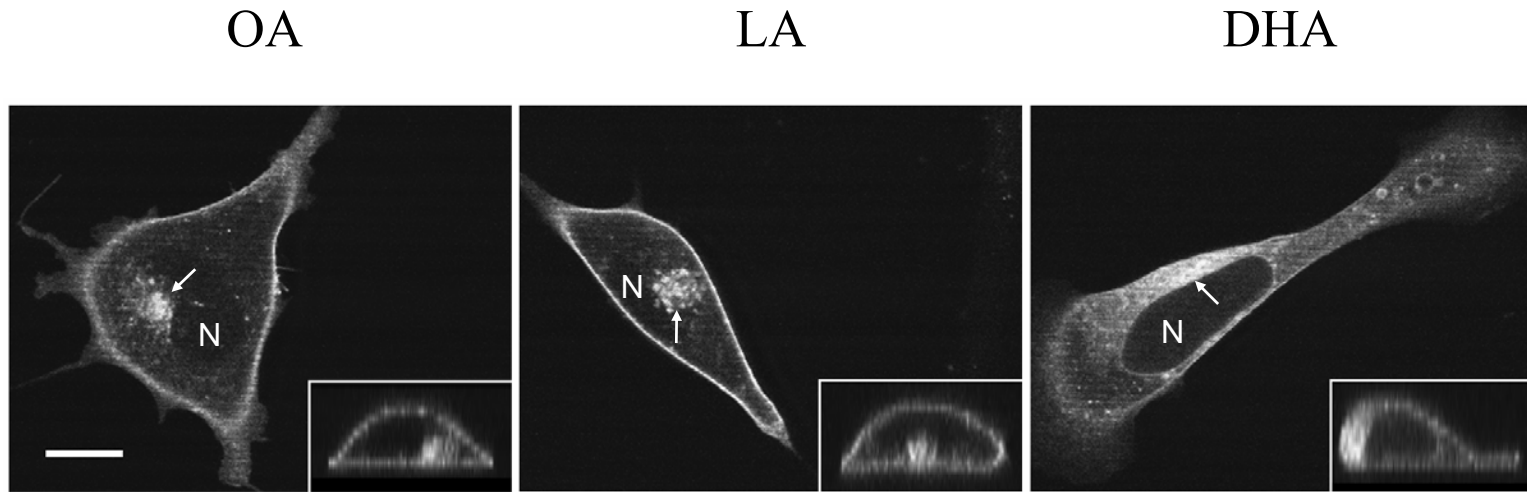


Figure 17. **DHA alters the subcellular distribution of GFP-tH.** YAMC cells were incubated with 50  $\mu$ M OA, LA, or DHA for 24h prior to transfection with pGFP-tH. Cells were further incubated with fatty acids until live cells were examined by confocal microscopy 36-48h after transfection. Representative confocal *enface* sections of OA-, LA-, or DHA-treated cells are shown. Insets in each panel depict reconstructed axial *z* sections cutting through the nucleus and the Golgi region of the same cell. Note the prominent GFP illumination of endomembrane structures in DHA-treated cells. Arrows and N mark perinuclear Golgi structures and nuclei, respectively. Scale bar represents 10  $\mu$ m.

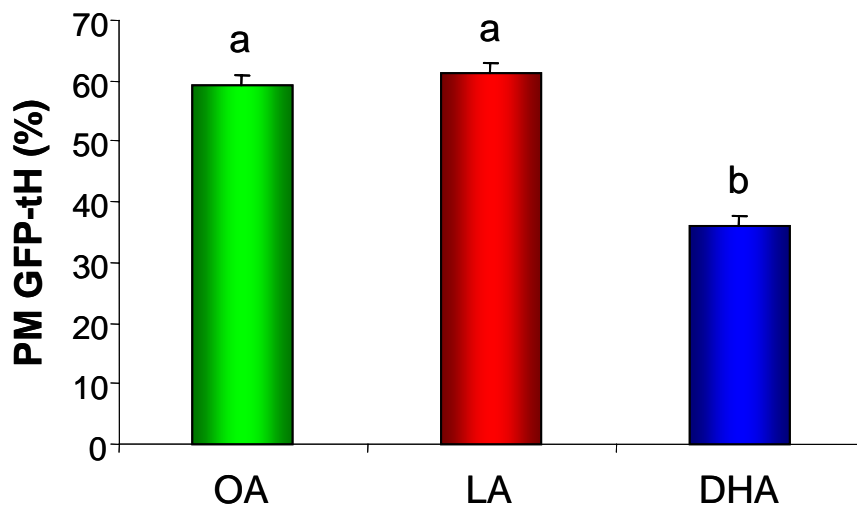


Figure 18. **DHA decreases PM localization of GFP-tH.** YAMC cells were incubated with 50  $\mu$ M OA, LA, or DHA for 24h prior to and 36-48h after transfection with pGFP-tH. Cells were stained with a vital dye FM 4-64 on a microscopic stage and z-serial images of living cells expressing GFP-tH were obtained by confocal microscopy 36-48h post-transfection. The relative amount of GFP-tH localized at the PM was quantified as percent of total cellular GFP-tH as described in Materials and Methods. A total of 31 cells from 2 independent experiments were analyzed and data are presented as mean  $\pm$  SE. Different letters denote statistical significance at  $p < 0.0001$ .

exhibiting ~60% relative PM localization. This further demonstrates that biologically inert GFP-tH ideally reproduces the intracellular trafficking of its full-length counterparts. In marked contrast, there was no difference whatsoever among fatty acid treatments with respect to GFP-tK subcellular localization and relative PM expression (Fig. 19 and Fig. 20), similar to GFP-labeled full-length wild-type and oncogenic K-Ras. However, differences in subcellular distribution were evident between GFP-tK and GFP-labeled full-length K-Ras. GFP-tK predominantly localized to the PM to a much greater extent ( $69.0 \pm 0.8\%$ ) than GFP-Kras-wt ( $44.5 \pm 2.3\%$ ) and GFP-KrasG12V ( $37.8 \pm 0.7\%$ ), and had negligible expression at the endomembrane compartments unlike the apparent endomembrane association of full-length K-Ras (Fig. 11, Fig. 15, and Fig. 19). These data suggest that there may be differences in the efficiency and kinetics of PM targeting and/or post-translational processing between full-length K-Ras GFP fusion proteins and GFP-tK.

In conclusion, the identical trends in fatty acid effects observed in cells expressing GFP chimeras of either wild-type, constitutively active mutants, or biologically non-functional targeting motifs of Ras clearly indicate that the DHA-induced alterations in PM targeting of Ras isoforms are independent of Ras signaling.

### **DHA effects on intracellular Ras trafficking to the PM are reversible**

Next, experiments were designed to investigate whether DHA-induced changes in protein subcellular localization are reversible. It was reasoned that if DHA enrichment in cellular membranes is involved as an underlying mechanism of action, the DHA-

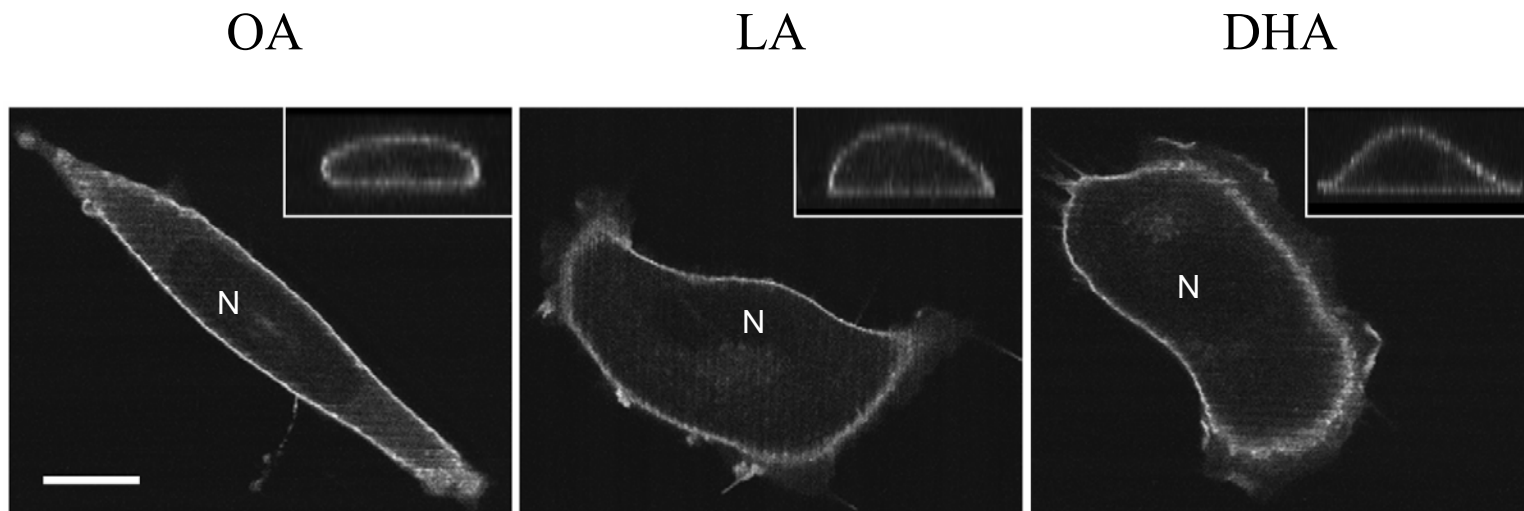


Figure 19. **Subcellular distribution of GFP-tK is not altered by fatty acid treatment.** YAMC cells were incubated with 50  $\mu\text{M}$  OA, LA, or DHA for 24h prior to transfection with pGFP-tK. Cells were further incubated with fatty acids until live cells were examined by confocal microscopy 36-48h after transfection. Representative confocal *enface* sections of OA-, LA-, or DHA-treated cells are shown. Insets in each panel depict reconstructed axial  $z$  sections cutting through the nucleus of the same cell. Nuclei are marked with N. Scale bar represents 10  $\mu\text{m}$ .

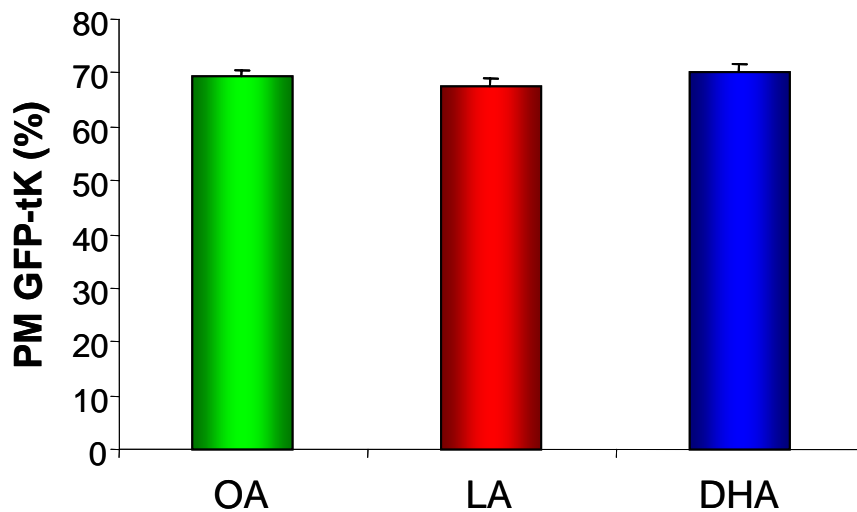


Figure 20. **PM localization of GFP-tK is not altered by fatty acid treatment.** YAMC cells were incubated with 50  $\mu$ M OA, LA, or DHA for 24h prior to and 36-48h after transfection with pGFP-tK. Cells were stained with a vital dye FM 4-64 on a microscopic stage and *z*-serial images of living cells expressing GFP-tK were obtained by confocal microscopy 36-48h post-transfection. The relative amount of GFP-tK localized at the PM was quantified as percent of total cellular GFP-tK as described in Materials and Methods. A total of 30 cells from 2 independent experiments were analyzed and data are presented as mean  $\pm$  SE. No significant difference was observed among means ( $p > 0.16$ ).



induced decrease in protein PM targeting should be reversible upon changing the cellular lipid environment. To test this hypothesis, GFP-tH-expressing cells were initially treated with DHA for 2.5 days and were subsequently washed and further incubated with LA or OA starting at 36h post-transfection. Upon placing DHA-pretreated cells in LA- or OA-supplemented medium for 24h, the DHA-induced unique subcellular distribution of GFP-tH, characterized by substantial endomembrane localization, was changed to a more prominent PM staining pattern similar to LA- and OA-treated cells (Fig. 21). This shift in localization pattern was particularly evident in daughter cells of the originally transfected cells, in which membrane biogenesis and hence membrane incorporation of LA or OA may have actively occurred, resulting in a decrease in the membrane DHA content. After 48h incubation with LA or OA, GFP-tH subcellular distribution in DHA-pretreated cells was indistinguishable from those of LA- or OA-treated cells.

### **DHA suppresses PM localization of Lck but not Fyn**

To further extend the observations on GFP-labeled Ras, it was investigated whether the selective modulation of PM targeting by DHA is specific to Ras isoforms or more broadly effective on other lipidated proteins. Of the lipid-modified proteins localized at the PM, only a few have been studied with respect to their trafficking routes and transport mechanisms. Lck and Fyn are members of Src family NRTKs and play key roles in T cell-mediated immune responses (Lowell, 2004). Both Lck and Fyn are dually acylated at the N-terminus with myristate and palmitate for PM association. Similar to H- and N-Ras, newly synthesized Lck initially associates with intracellular membranes

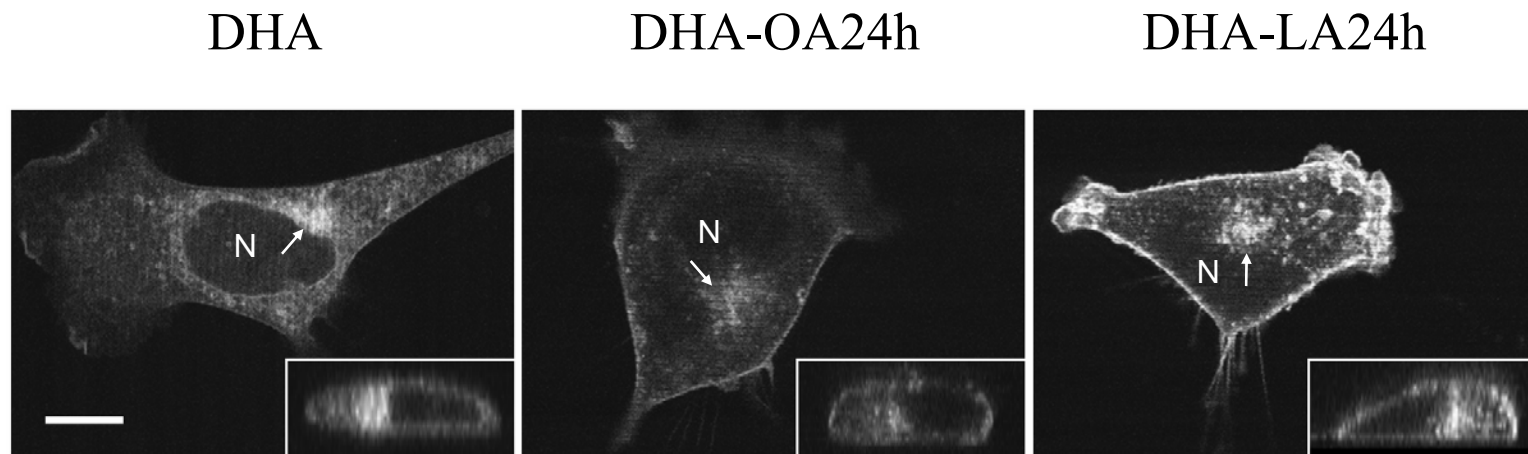


Figure 21. **DHA-induced alterations in GFP-tH subcellular localization are reversible.** YAMC cells incubated with 50  $\mu\text{M}$  DHA for 24h prior to and 36h after transfection with pGFP-tH were washed and further incubated with 50  $\mu\text{M}$  OA or LA until live cells were examined by confocal microscopy 24h later. Representative confocal *enface* sections of DHA-treated cells before wash (DHA) and after further incubation with OA or LA for 24h (DHA-OA24h and DHA-LA24h, respectively) are shown. Insets in each panel depict reconstructed axial *z* sections cutting through the nucleus and the Golgi region of the same cell. Note the predominant GFP signals at the endomembrane structures before wash and the more prominent PM staining after 24h incubation with OA or LA. Arrows and N mark perinuclear Golgi structures and nuclei, respectively. Scale bar represents 10  $\mu\text{m}$ .

where palmitoylation occurs, and subsequently traffics to the PM via the exocytic pathway (Bijlmakers and Marsh, 1999). In contrast, Fyn is targeted rapidly (within 2-4 min) and directly to the PM after biosynthesis on soluble ribosomes (van't Hof and Resh, 1997). In addition, PM targeting of newly synthesized Fyn was insensitive to inhibitors of vesicular transport such as brefeldin A and monensin as well as disruption of microtubule and actin network, somewhat analogous to K-Ras trafficking (Choy et al., 1999; van't Hof and Resh, 1997). Endogenous Lck and Lck-GFP in human T cells have been shown to localize at the PM and pericentrosomal vesicular structures, which colocalized with markers of late endosomes and/or the *trans*-Golgi network (TGN) (Ehrlich et al., 2002; Ley et al., 1994). In accord with these reports, Lck-GFP was detected in the PM and perinuclear structures in YAMC cells (Fig. 22). When Lck-GFP-expressing cells were incubated with fatty acids, the fluorescence pattern was indistinguishable between LA- and OA-treated cells, with the majority of Lck-GFP ( $60.8 \pm 1.0\%$ ) localized at the PM (Fig. 22 and Fig. 23). In contrast, DHA significantly decreased the PM localization ( $44.0 \pm 1.4\%$ ) by 28% and increased the intracellular pool of Lck-GFP markedly at the perinuclear vesicular structure as well as the cytoplasm, compared with both LA and OA. Fyn-GFP has been shown to localize primarily at the PM in COS-1, COS-7, and MDCK cells (Choy et al., 1999; Webb et al., 2000). In addition, a close association of endogenous Fyn with the centrosome has been observed in human T lymphocytes (Ley et al., 1994). In YAMC cells, Fyn-GFP was predominantly expressed at the PM with very little perinuclear staining in all fatty acid-treated cells, somewhat similar to GFP-tK (Fig. 19), although apparent perinuclear

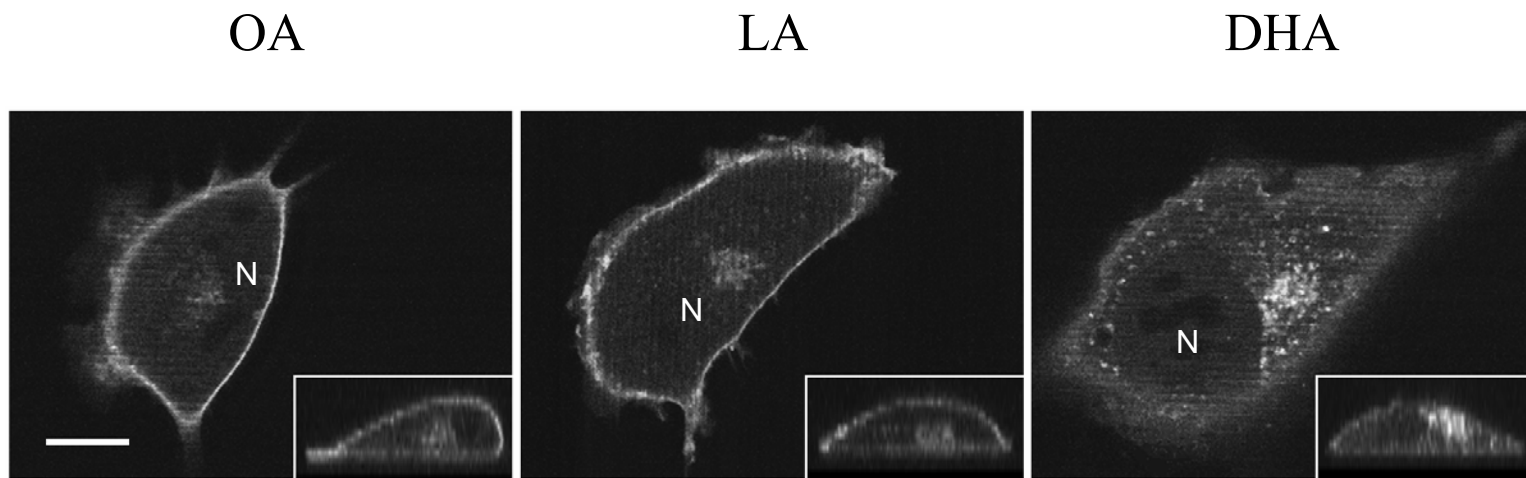


Figure 22. **DHA alters the subcellular distribution of Lck-GFP.** YAMC cells were incubated with 50  $\mu$ M OA, LA, or DHA for 24h prior to transfection with pLck-GFP. Cells were further incubated with fatty acids until live cells were examined by confocal microscopy 36-48h after transfection. Representative confocal *enface* sections of OA-, LA-, or DHA-treated cells are shown. Insets in each panel depict reconstructed axial *z* sections cutting through the nucleus and the perinuclear vesicular structure of the same cell. Note the prominent GFP illumination of endomembrane structures in DHA-treated cells. Nuclei are marked with N. Scale bar represents 10  $\mu$ m.

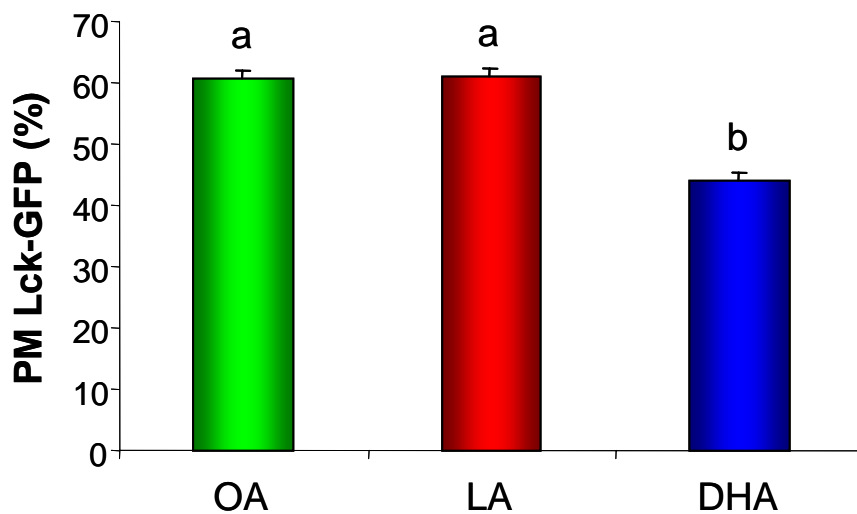


Figure 23. **DHA decreases PM localization of Lck-GFP.** YAMC cells were incubated with 50  $\mu$ M OA, LA, or DHA for 24h prior to and 36-48h after transfection with pLck-GFP. Cells were stained with a vital dye FM 4-64 on a microscopic stage and *z*-serial images of living cells expressing Lck-GFP were obtained by confocal microscopy 36-48h post-transfection. The relative amount of Lck-GFP localized at the PM was quantified as percent of total cellular Lck-GFP as described in Materials and Methods. A total of 30 cells from 2 independent experiments were analyzed and data are presented as mean  $\pm$  SE. Different letters denote statistical significance at  $p < 0.0001$ .

fluorescence was occasionally observed (Fig. 24). Similar to K-Ras-derived GFP fusion constructs, subcellular localization of Fyn-GFP in DHA-treated cells was essentially identical with those of LA- and OA-treated cells, with the majority of fluorescence ( $63.4 \pm 1.3\%$ ) emanating from the PM (Fig. 25). Interestingly, similar observations were obtained for the subcellular distribution of Lck and Fyn by other investigators using dietary fatty acids. Stulnig et al. reported that incubation of Jurkat T cells with 25  $\mu\text{M}$  EPA (20:5, n-3), a very long-chain n-3 PUFA found in fish oil along with DHA (22:6, n-3), resulted in a rather diffuse distribution of endogenous Lck with a marked decrease in the PM localization, compared with untreated and stearic acid (18:0)-treated cells, although quantification of this result was not attempted (Stulnig et al., 1998). In contrast, PUFA treatment with 50  $\mu\text{M}$  arachidonic acid (20:4, n-6), EPA (20:5, n-3), or DHA (22:6, n-3) did not affect Fyn localization to the PM fraction in Cos-1 cells (Webb et al., 2000), consistent with the results in this study.

These data suggest that the differential effects of DHA on protein PM targeting via vesicular transport-dependent versus independent pathways may be universal to lipidated proteins, regardless of the orientation (C- versus N-terminal modifications) and composition of membrane anchors used as targeting signals.

### **DHA does not alter intracellular trafficking of VSVG-GFP to the PM**

To test whether DHA also suppresses the PM delivery of conventional exocytic cargo, the subcellular distribution of VSVG-GFP was examined at 36-48h post-transfection in YAMC cells treated with fatty acids at the permissive temperature, 32°C. As

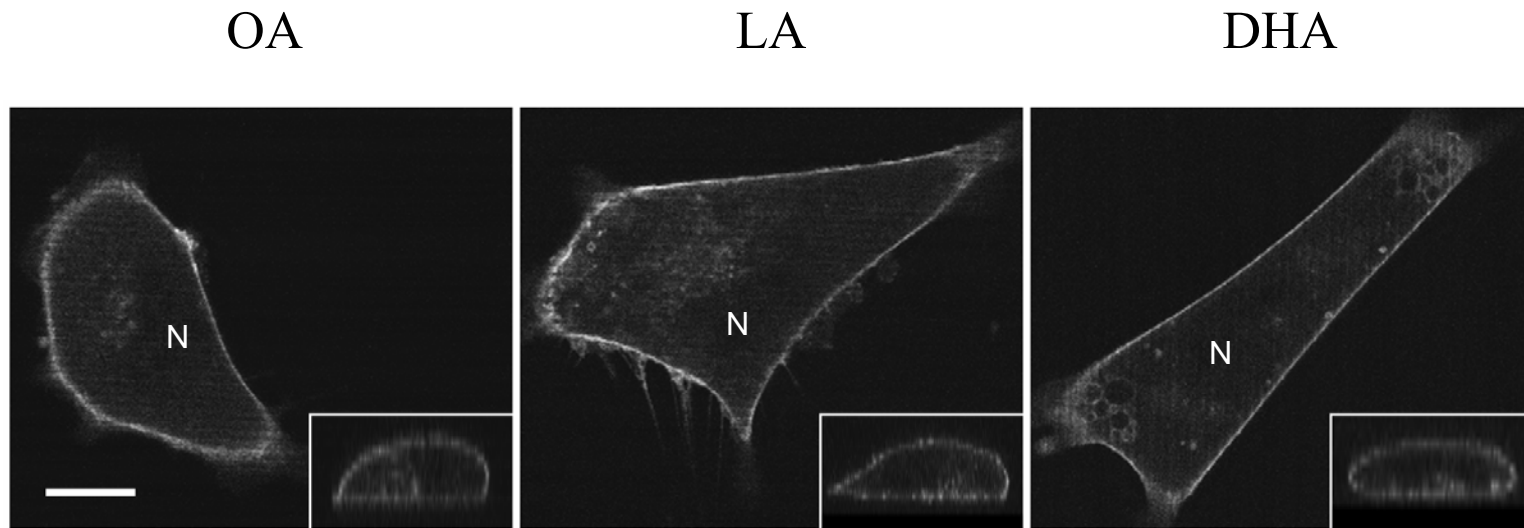


Figure 24. **Subcellular distribution of Fyn-GFP is not altered by fatty acid treatment.** YAMC cells were incubated with 50  $\mu$ M OA, LA, or DHA for 24h prior to transfection with pFyn-GFP. Cells were further incubated with fatty acids until live cells were examined by confocal microscopy 36-48h after transfection. Representative confocal *enface* sections of OA-, LA-, or DHA-treated cells are shown. Insets in each panel depict reconstructed axial *z* sections cutting through the nucleus of the same cell. Nuclei are marked with N. Scale bar represents 10  $\mu$ m.

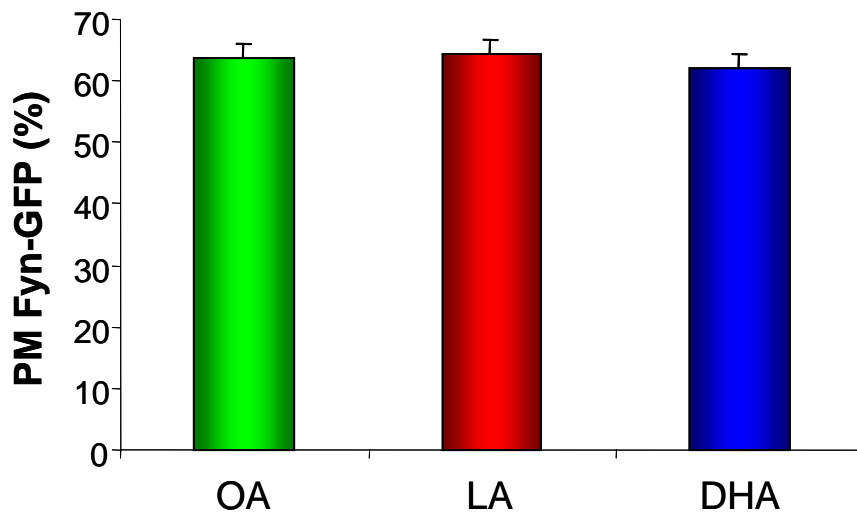


Figure 25. **PM localization of Fyn-GFP is not altered by fatty acid treatment.** YAMC cells were incubated with 50  $\mu$ M OA, LA, or DHA for 24h prior to and 36-48h after transfection with pFyn-GFP. Cells were stained with a vital dye FM 4-64 on a microscopic stage and *z*-serial images of living cells expressing Fyn-GFP were obtained by confocal microscopy 36-48h post-transfection. The relative amount of Fyn-GFP localized at the PM was quantified as percent of total cellular Fyn-GFP as described in Materials and Methods. A total of 30 cells from 3 independent experiments were analyzed and data are presented as mean  $\pm$  SE. No significant difference was observed among means ( $p > 0.48$ ).



transmembrane protein cargo in the exocytic pathway, the thermoreversible folding mutant ts045 VSVG protein, and its GFP chimeras have been widely used to study secretory membrane transport (Hirschberg et al., 1998; Presley et al., 1997; Scales et al., 1997; Toomre et al., 1999). As shown in Fig. 26, VSVG-GFP expression was evident at the PM and the cellular processes extending from the cell surface as well as at the well-delineated perinuclear Golgi and numerous intracellular vesicular-like structures in all fatty acid-treated YAMC cells. There was no difference among fatty acid-treated cells with respect to the relative PM expression ( $63.9 \pm 1.4\%$ ) and overall fluorescence distribution (Fig. 27). These data suggest that DHA selectively modulates the PM targeting of lipidated cytoplasmic cargo rather than compromising the integrity of exocytic pathway and the bulk flow of general vesicular traffic.

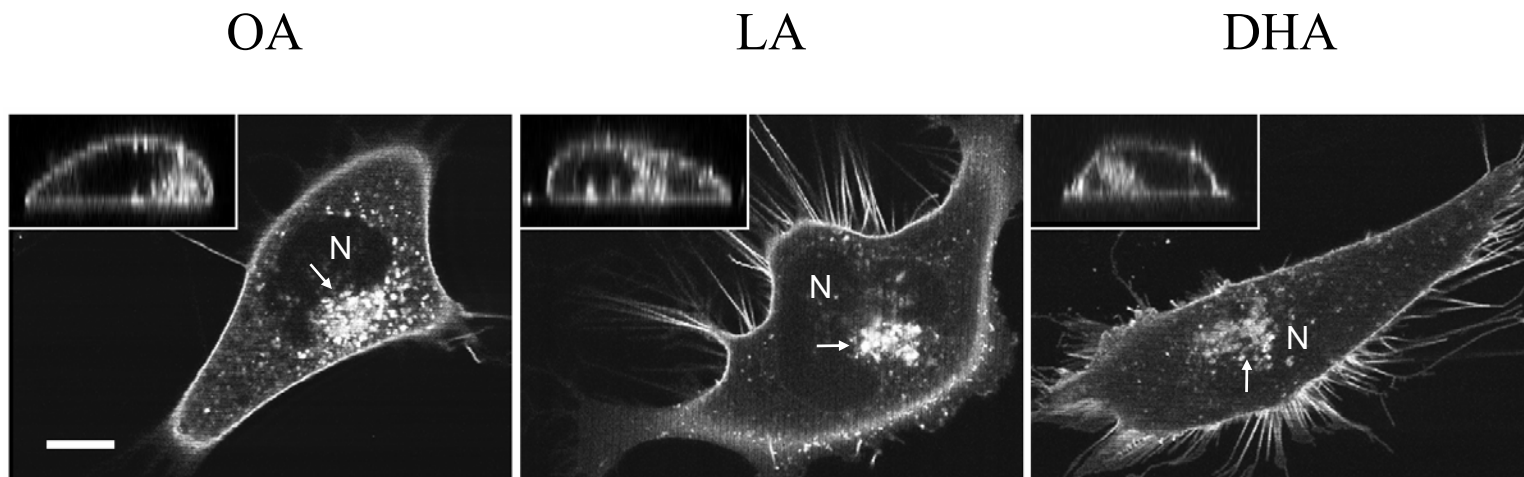


Figure 26. **Subcellular distribution of VSVG-GFP is not altered by fatty acid treatment.** YAMC cells were incubated with 50  $\mu$ M OA, LA, or DHA for 24h prior to transfection with pVSVG-GFP. Cells were further incubated with fatty acids until live cells were examined by confocal microscopy 36-48h after transfection. Representative confocal *enface* sections of OA-, LA-, or DHA-treated cells are shown. Insets in each panel depict reconstructed axial *z* sections cutting through the nucleus and the Golgi region of the same cell. Arrows and N mark perinuclear Golgi structures and nuclei, respectively. Scale bar represents 10  $\mu$ m.

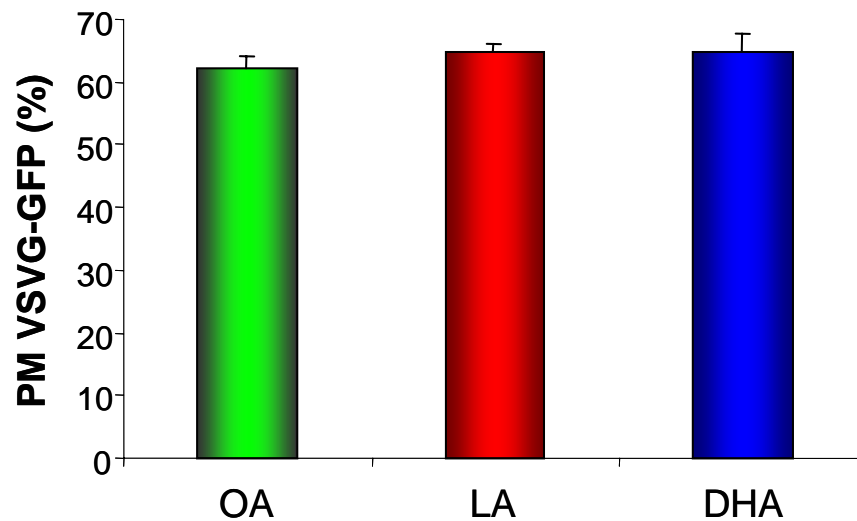


Figure 27. **PM localization of VSVG-GFP is not altered by fatty acid treatment.** YAMC cells were incubated with 50  $\mu$ M OA, LA, or DHA for 24h prior to and 36-48h after transfection with pVSVG-GFP. Cells were stained with a vital dye FM 4-64 on a microscopic stage and  $z$ -serial images of living cells expressing VSVG-GFP were obtained by confocal microscopy 36-48h post-transfection. The relative amount of VSVG-GFP localized at the PM was quantified as percent of total cellular VSVG-GFP as described in Materials and Methods. A total of 22 cells from 2 independent experiments were analyzed and data are presented as mean  $\pm$  SE. No significant difference was observed among means ( $p > 0.44$ ).

## CHAPTER V

### DISCUSSION

Correct localization of lipidated cytosolic proteins to the PM is mediated by interactions between lipid anchors of proteins and cell membranes. While the importance of post-translational protein lipidation to provide membrane anchors and targeting signals is well established and our understanding of protein transport mechanisms and machineries has substantially progressed, the potential regulatory role of the cellular lipid environment in the subcellular targeting of lipidated proteins has not been well appreciated. The present study, using GFP fusion chimeras and quantitative fluorescence imaging of living cells, provides evidence that a membrane lipid-modifying dietary constituent can differentially modulate the intracellular trafficking of lipidated proteins to the PM in a reversible manner. DHA, a dietary n-3 PUFA enriched in fish oil, selectively decreased the PM targeting of cytoplasmic lipidated protein cargo of the exocytic pathway as opposed to the vesicular transport-independent protein trafficking in colonic epithelial cells. This DHA effect seems to be only dependent on the protein trafficking route, irrespective of the types of membrane anchors as well as the functional status of lipidated proteins. In addition, only the PM delivery of lipidated cytosolic proteins, but not transmembrane protein cargo, seems to be affected by DHA, suggesting that the DHA-induced suppression of lipidated protein PM targeting is not due to alterations in the bulk flow of secretory vesicular traffic.

Table 1. Effects of Dietary Fatty Acids on Protein PM Targeting

GFP construct	Membrane anchor	Dependence on exocytic pathway	Relative PM localization		Relative Golgi localization		Effects of DHA (versus LA and OA)
			treatment (n)	%; mean $\pm$ SE*	treatment (n)	%; mean $\pm$ SE*	
GFP-Nras-wt	palmitate + farnesyl	yes	Untreated (9)	16.8 $\pm$ 1.8 <sup>b</sup>	Untreated (8)	12.9 $\pm$ 2.4 <sup>b</sup>	↓ PM ↑ endomembranes
			LA (21)	21.2 $\pm$ 0.8 <sup>a</sup>	LA (19)	15.7 $\pm$ 1.6 <sup>b</sup>	
			DHA (20)	9.3 $\pm$ 0.7 <sup>c</sup>	DHA (19)	23.0 $\pm$ 1.6 <sup>a</sup>	
GFP-Hras-wt	palmitate + farnesyl	yes	OA (11)	58.8 $\pm$ 1.9 <sup>a</sup>	OA (11)	6.8 $\pm$ 0.8 <sup>b</sup>	↓ PM ↑ endomembranes
			LA (11)	59.5 $\pm$ 1.7 <sup>a</sup>	LA (11)	6.5 $\pm$ 0.6 <sup>b</sup>	
			DHA (11)	37.7 $\pm$ 1.5 <sup>b</sup>	DHA (11)	10.4 $\pm$ 1.3 <sup>a</sup>	
GFP-Kras-wt	polybasic region + farnesyl	no	OA (10)	42.8 $\pm$ 3.5			—
			LA (11)	45.2 $\pm$ 4.6			
			DHA (9)	45.5 $\pm$ 3.5			
GFP-HrasG12V	palmitate + farnesyl	yes	OA (10)	62.7 $\pm$ 2.9 <sup>a</sup>	OA (10)	8.4 $\pm$ 0.9 <sup>b</sup>	↓ PM ↑ endomembranes
			LA (13)	61.5 $\pm$ 2.3 <sup>a</sup>	LA (13)	7.9 $\pm$ 1.0 <sup>b</sup>	
			DHA (12)	41.9 $\pm$ 2.4 <sup>b</sup>	DHA (12)	14.9 $\pm$ 3.1 <sup>a</sup>	
GFP-KrasG12V	polybasic region + farnesyl	no	OA (19)	37.7 $\pm$ 1.0			—
			LA (18)	38.0 $\pm$ 1.1			
			DHA (20)	37.7 $\pm$ 1.0			
GFP-tH	palmitate + farnesyl	yes	OA (11)	59.2 $\pm$ 1.7 <sup>a</sup>			↓ PM ↑ endomembranes
			LA (10)	61.1 $\pm$ 1.8 <sup>a</sup>			
			DHA (10)	36.1 $\pm$ 1.7 <sup>b</sup>			
GFP-tK	polybasic region + farnesyl	no	OA (10)	69.3 $\pm$ 1.4			—
			LA (10)	67.4 $\pm$ 1.4			
			DHA (10)	70.3 $\pm$ 1.4			
Lck-GFP	myristate + palmitate	yes	OA (10)	60.7 $\pm$ 1.4 <sup>a</sup>			↓ PM ↑ endomembranes & cytoplasm
			LA (10)	60.9 $\pm$ 1.4 <sup>a</sup>			
			DHA (10)	44.0 $\pm$ 1.4 <sup>b</sup>			
Fyn-GFP	myristate + palmitate	no	OA (10)	63.8 $\pm$ 2.2			—
			LA (10)	64.3 $\pm$ 2.2			
			DHA (10)	62.1 $\pm$ 2.2			
VSVG-GFP	transmembrane domain	yes	OA (7)	62.1 $\pm$ 2.2			—
			LA (7)	64.8 $\pm$ 1.3			
			DHA (8)	64.6 $\pm$ 3.2			

\* different letters denote statistical significance at  $p < 0.05$

The advent of GFP and techniques to fluorescently probe proteins of interest has revolutionized studies of protein localization and trafficking, which traditionally relied on genetic and biochemical approaches. The advantages provided by the use of GFP tagging over the traditional approaches are remarkable, allowing for real-time monitoring of protein expression, intracellular trafficking path and kinetics, subcellular localization and changes in these parameters in response to cellular regulation while preserving the biological function and distinct stability (half-life) of the cognate protein (Corish and Tyler-Smith, 1999; Hampton et al., 1996; Hirschberg et al., 1998; Piston et al., 1999). More importantly, the remarkable photostability of GFP and the well-defined stoichiometry between the probe and the protein of interest render GFP fusion chimeras particularly suitable for quantitative studies, compared with immunohistochemical methods with a largely qualitative nature (Patterson et al., 1997). Due to inevitable cross-contamination, the conventional approaches of subcellular fractionation cannot provide adequate resolution among different membranous subcellular organelles, which is a critical element of the present study. Moreover, the massive dilution of cellular components following cell disruption predisposes the subcellular fractionation assays to systematically underestimate the extent of *in vivo* membrane association of proteins, as previously pointed out (Roy et al., 2000). This is particularly true for the partially processed proteins with relatively low to moderate membrane affinities as exemplified with studies with palmitoylation-defective mutants of Ras and Src-related tyrosine kinases. Specifically, while predominant endomembrane association was evident for farnesylated Ras mutants and myristoylated N-terminal domains of Fyn and Yes in live

cell images, these palmitoylation-deficient proteins, upon subcellular fractionation, primarily partitioned into the soluble cytosolic fraction or only partially associated with the membrane fraction, leading to misinterpretation of *in vivo* subcellular localization (Cadwallader et al., 1994; Choy et al., 1999; Hancock et al., 1989; Hancock et al., 1990; McCabe and Berthiaume, 1999). In the present study, the application of GFP as a real-time fluorescent reporter in quantitative imaging allowed for detailed observation as well as accurate and sensitive quantification of changes in subcellular protein localization, which cannot be accomplished by using the conventional approaches.

Modulation of lipidated protein localization by different dietary sources of fat has previously been reported by two groups, focusing on the oncogene product Ras and its implication in colon cancer (Collett et al., 2001; Davidson et al., 1999; Singh et al., 1997). In these studies using a rat colon tumor model and malignant transformed mouse colonocytes, dietary fish oil and its major n-3 PUFA, DHA (22:6, n-3), significantly decreased the membrane to cytosol ratio of Ras proteins, relative to corn oil and LA (18:2, n-6), a highly prevalent vegetable fat and dietary PUFA in the U.S. diet. The authors correlated this with decreases in colon tumor incidence and Ras-dependent signaling. In retrospect, it can be reasoned based on the findings from the present study that DHA-induced decreases in H- and N-Ras PM localization may have contributed to the previously observed reduction in Ras membrane association, which was detected from total cellular membrane fractions by using pan-Ras antibodies with broad reactivity to all Ras isoforms. Reddy and co-workers proposed interference with post-translational processing of Ras proteins as the underlying mechanism for the reduced Ras membrane

association, based on the decrease in FTase expression in fish oil-fed rat colon (Singh et al., 1998). However, animal and cell line studies conducted by Chapkin and co-workers demonstrated that farnesylation state of Ras as well as FTase activity and the expression of hydroxymethylglutaryl CoA (HMG-CoA) reductase, the rate-limiting enzyme in farnesylpyrophosphate biosynthesis, is unaffected in colonic epithelial cells following fish oil feeding or DHA treatment (Collett et al., 2001; Davidson et al., 1999). The subcellular distribution of GFP-labeled H- and N-Ras in DHA-treated cells in the present study is consistent with the latter case since DHA increased the endomembrane association of Ras rather than the cytoplasm, which would not be the case for unfarnesylated hence soluble Ras proteins.

The exact mechanism(s) whereby DHA suppresses the lipidated protein PM targeting via the exocytic pathway remains elusive. Although this DHA effect seems universal to lipidated proteins traveling through the secretory pathway, it is unlikely that DHA alters the general secretory vesicular traffic since the PM delivery of the conventional transmembrane protein cargo remained unaffected by fatty acid treatment. However, possible explanations can be raised based on the previous and present findings and are discussed below.

The role of palmitate in protein PM targeting seems to be more than just increasing membrane binding affinity but rather operating as an actual targeting signal directing proteins to the PM, as illustrated by studies with palmitoylation-defective mutants and inhibition of protein palmitoylation by drugs. Farnesylated but not palmitoylated H- and N-Ras proteins remain stably associated with endomembranes but



are not targeted to the cell surface, indicating that palmitoylation is required for correct PM targeting (Cadwallader et al., 1994; Choy et al., 1999; Hancock et al., 1989; Hancock et al., 1990; Michaelson et al., 2001) and essentially identical observations have been reported for members of Rho family GTPases (RhoB and TC10) (Michaelson et al., 2001). Similarly, removal of palmitoylation signals or inhibition of palmitoylation of N-terminal targeting domains of Src-related tyrosine kinases Fyn and Yes resulted in accumulation of myristoylated but not palmitoylated proteins at the endomembranes (McCabe and Berthiaume, 1999; Webb et al., 2000; Wolven et al., 1997). Similar observations have also been reported for palmitoylated transmembrane proteins such as G protein-coupled receptors, further supporting the notion that protein palmitoylation may act as a bona fide PM targeting signal (Blanpain et al., 2001; Tanaka et al., 1998; Zhu et al., 1995). Therefore, there is a possibility that the increased endomembrane association of GFP-labeled H-Ras, N-Ras, and Lck concomitant with the reduced PM localization in DHA-treated YAMC cells may be due to the inhibition of protein palmitoylation by DHA. However, three lines of evidence strongly argue against this possibility. First, Ras palmitoylation in DHA-treated YAMC cells overexpressing v-H-Ras has been shown to be as efficient as in untreated and LA-treated cells (Collett et al., 2001). Given that palmitoylation is the last step of Ras post-translational modification, requiring the prior processing of the CAAX motif including farnesylation, this further indicates that DHA does not compromise the overall post-translational processing of Ras proteins, as also evident from the lack of a DHA effect on FTase activity, HMG-CoA reductase expression and Ras farnesylation state (Collett et al., 2001; Davidson et al.,

1999). Second, Stulnig and co-workers demonstrated that treatment with EPA (20:5, n-3), an n-3 PUFA also found in fish oil, does not alter Lck palmitoylation in Jurkat T cells although EPA did change the subcellular distribution of endogenous Lck, similar to the DHA (22:6, n-3) effect on Lck-GFP in the present study (Stulnig et al., 1998; Stulnig et al., 2001). Third, although H-Ras, N-Ras, Lck, and Fyn all require palmitoylation for PM targeting, PM localization and subcellular distribution of Fyn-GFP was unaffected by DHA, unlike GFP-Nras, GFP-Hras, and Lck-GFP, indicating that the differential effects of DHA on the lipidated protein localization cannot be explained by the inhibition of protein palmitoylation. However, there is evidence that dietary PUFAs including DHA may inhibit palmitoylation of certain proteins. In contrast to the insensitivity of Ras and Lck palmitoylation towards PUFA treatment, palmitoylation of Fyn in COS-1 cells has been shown to be substantially inhibited by 55-73% following arachidonic acid (20:4, n-6), EPA (20:5, n-3), or DHA (22:6, n-3) treatment (Webb et al., 2000). The authors postulated that Fyn became dually acylated by N-myristoylation and S-acylation with PUFAs because membrane binding of Fyn remained unaffected in this study despite the fact that myristoylated but not palmitoylated Fyn fractionates to the cytosol, only partially associating with the membrane fraction (Alland et al., 1994; McCabe and Berthiaume, 1999; Webb et al., 2000; Wolven et al., 1997). While newly synthesized H-Ras, N-Ras, and Lck are palmitoylated prior to transit to the PM presumably early in the exocytic pathway, Fyn has been shown to be rapidly and directly targeted to the PM within 5 minutes after biosynthesis and proposed to be palmitoylated at the PM (Apolloni et al., 2000; Bijlmakers and Marsh, 1999; Choy et al., 1999; van't

Hof and Resh, 1997). These observations imply that different protein palmitoyl transferases with distinct subcellular distributions may be responsible for palmitoylation of these proteins. Indeed, several groups of investigators assigned protein palmitoyl transferase activities towards different proteins to the ER, the Golgi, the ER-Golgi intermediate compartment, or the PM (Dunphy et al., 1996; Gutierrez and Magee, 1991; McLaughlin and Denny, 1999; Schroeder et al., 1996; Zhao et al., 2002). Further progress in the purification and characterization of different protein palmitoyl transferases with regard to protein and fatty acid substrate specificity, subcellular localization, and cellular regulation seems necessary to explain the different sensitivities of protein palmitoylation towards PUFAs.

An alternative possibility concerning protein lipidation is that DHA may be directly acylated to proteins in place of palmitate, thereby altering the membrane binding properties and subcellular localization of normally palmitoylated proteins. Although palmitate (16:0) is the predominant fatty acid linked to cysteine residues of proteins, it appears that some proteins can be S-acylated with other acyl chains with different chain lengths and a varying degree of unsaturation, indicative of a somewhat relaxed fatty acid specificity of protein acylation by thioester linkages (Bizzozero et al., 1986; Casey et al., 1994; Fujimoto et al., 1993; Hallak et al., 1994; Muszbek and Laposata, 1993; O'Brien et al., 1987). Moreover, analysis of thioester-linked fatty acids released from human platelet proteins revealed that the profile of protein-bound fatty acids via thioester linkages can be altered by exogenously supplied fatty acids (Muszbek et al., 1999). In view of the heterogeneity of protein acylation, Resh and co-workers proposed that

protein acylation with unsaturated fatty acids may alter membrane association properties of proteins such as partitioning into the liquid-ordered environment of specialized PM microdomains (Liang et al., 2001; Lindwasser and Resh, 2002). Consistent with this notion, model membrane studies using lipopeptides or purified proteins acylated *in vitro* demonstrated that proteins modified with unsaturated fatty acids are not efficiently targeted to the liquid-ordered domains unlike proteins with saturated counterparts (Moffett et al., 2000; Wang et al., 2001). However, the biological significance and *in vivo* consequences of heterogeneous S-acylation of proteins remain under debate (Stulnig et al., 2001). The extent of heterogeneous S-acylation will be ultimately determined by the substrate preference of protein palmitoyl transferase as well as fatty acyl CoA availability in the context of physiologically relevant concentrations. As discussed above, the understanding of enzymology and regulation of protein palmitoyl transferase has been hampered by failure to successfully purify this enzyme. Nevertheless, several observations stand against the potential relevance of protein acylation with DHA to the present study. First, the efficiency of protein acylation with PUFAs seems extremely low relative to palmitate even in the PUFA-enriched environment such as cells incubated with PUFAs (Liang et al., 2001). More relevant to the present study, base hydrolysis of total proteins or purified rhodopsin from bovine retina, in which DHA is naturally enriched in membranes (up to 50 mol%) and the acyl CoA pool, revealed that retinal proteins are predominantly acylated with palmitate (65-95%) while DHA accounts for only less than 2% of the protein-bound fatty acids (DeMar and Anderson, 1997; O'Brien et al., 1987; Stone et al., 1979). Second, the fact

that the palmitoylation state of Ras is unaffected by DHA in YAMC cells indicates that proteins modified with DHA, if any, must constitute only a minor fraction, arguing that neither dramatic alterations in protein localization profile nor the differential effects on palmitoylated proteins (H-Ras, N-Ras, and Lck versus Fyn) observed in the present study can be accounted for by S-acylation of proteins with DHA. More detailed exploration of the potential alterations in protein palmitoylation by DHA, either inhibition or substitution with DHA, will require further advances in the characterization of protein palmitoyl transferase.

Given that alterations in protein modification can hardly provide sufficient explanations for the selectivity and the substantial magnitude of DHA effects, it seems reasonable to conclude that the unique properties of the DHA-enriched cellular lipid environment and lipid-lipid and lipid-protein interactions therein are primarily responsible for the effects of DHA on lipidated protein localization. Accumulation of PM proteins in intracellular membrane compartments can result from increased recycling from the cell surface via endocytosis (Nichols et al., 2001; Roy et al., 2002; Royle and Murrell-Lagnado, 2003). However, this retrograde vesicular transport of proteins from the PM seems incompatible with the selectivity (H-Ras, N-Ras, Lck versus K-Ras, Fyn and VSVG) of DHA effects seen in the present study. Even after considering the presence of diverse endocytic pathways originating from different PM subdomains (Johannes and Lamaze, 2002; Nichols, 2003; Parton and Richards, 2003), distinct compartmentalization of these proteins within the PM does not correlate with their different sensitivities to DHA in terms of subcellular localization. Of those

affected by DHA, while GFP-Nras-wt, GFP-tH and Lck-GFP are preferentially associated with lipid rafts/caveolae, GFP-H-RasG12V predominantly resides in the disordered bulk PM and GFP-H-Ras-wt is equally distributed in both, as established by earlier work using biochemical, fluorescence and electron microscopic techniques (Brown, 1993; Cinek and Horejsi, 1992; Matallanas et al., 2003; Niv et al., 2002; Prior et al., 2001; Prior et al., 2003; Stefanova et al., 1991). Likewise, of those unaffected by DHA, GFP-labeled wild-type and oncogenic K-Ras, GFP-tK, and VSVG predominantly localize in the bulk PM, while Fyn has been shown to be concentrated in lipid rafts (Chen and Resh, 2001; de Vries et al., 1998; Niv et al., 2002; Prior et al., 2001; Prior et al., 2003; Shenoy-Scaria et al., 1992). Therefore, no particular form of endocytic pathway known to date seems to be capable of distinguishing these proteins in a manner that would explain why DHA differentially alters their subcellular localization.

Taking the previous and present findings together, it can be proposed that DHA-enriched membranes do not provide an environment conducive to forward transport of newly synthesized lipidated proteins. It is likely that lipidated cytoplasmic cargos are discriminated against in DHA-enriched secretory apparatus when it comes to sorting into transport vesicles or carriers in anterograde membrane traffic. There may be subtle changes in the profile of heterogeneous transport vesicle/carrier populations in terms of lipid composition, size, curvature, and budding efficiency such that the general bulk flow of membranes and delivery of conventional exocytic cargos are not affected but sorting of lipidated cytoplasmic cargos into transport vesicles/carriers is attenuated in DHA-enriched secretory apparatus. Reduced PM delivery of lipidated proteins but not

transmembrane proteins may result from the facilitated formation of certain types of transport vesicles in which packaging of membrane-embedded cargo is unaffected but lipidated peripheral proteins are not efficiently anchored on the cytoplasmic surface. However, to sustain this speculation, further studies are required to establish 1) differential membrane binding affinities of lipid-anchored proteins as a function of membrane fatty acyl composition, allowing for the preferential binding to more saturated membranes or microdomains over DHA-enriched counterparts, 2) heterogeneous lateral lipid-protein organization in DHA-enriched ER and Golgi/TGN membranes allowing for the formation of discrete microdomains, which will subsequently give rise to vesicles/carriers with different cargo profiles and possibly with different budding efficiencies, and 3) DHA-induced alterations in the transport vesicle/carrier population in relation to lipid composition and lipidated protein partitioning. The unique properties of DHA-enriched membranes have been well studied using both model and biological membranes in view of the broad health benefits of dietary fish oil and DHA (Stillwell and Wassall, 2003). The presence of six *cis*-double bonds enables DHA to assume unique conformations with large cross-sectional areas per molecule and short axial lengths as well as exceptional flexibility of the acyl chain to bend, tilt, and back-fold within membrane bilayers (Feller et al., 2002; Gawrisch et al., 2003). Consequently, DHA-enriched membranes have looser lipid packing characterized by lower acyl chain order, faster acyl chain dynamics, and increased water permeability, relative to saturated membranes (Eldho et al., 2003; Holte et al., 1995; Huster et al., 1997; Mitchell and Litman, 1998a; Mitchell and Litman, 1998b; Paddy et al., 1985; Straume and Litman,

1987). Therefore, it is conceivable that the less cohesive acyl chain packing in DHA-enriched membranes may weaken van der Waals chain-chain interactions between lipid anchors of proteins and membrane phospholipids, thereby lowering binding affinity of lipidated proteins relative to the more tightly packed saturated membranes. In addition, the presence of DHA renders membranes easily deformable elastically with a much lower energy requirement (Koenig et al., 1997; Smaby et al., 1997), prompting speculation that the local concentration of DHA may influence vesicle budding efficiency. Model membrane studies have also provided a conceptual basis for the heterogeneous lateral distribution of DHA, although direct observation in biological membranes has not been reported to date due to technical challenges (Stillwell et al., 2000). The marked steric incompatibility between disordered, bulky polyunsaturated acyl chains, especially DHA, and the rigid steroid moiety of cholesterol, which exhibits higher affinity for saturated acyl chains in membrane bilayers has led to the model of transient, highly dynamic microdomains (Brzustowicz et al., 2002a; Brzustowicz et al., 2002b; Huster et al., 1998; Mitchell and Litman, 1998a; Niu and Litman, 2002; Pasenkiewicz-Gierula et al., 1990). This model envisages lateral phase separation or molecular sorting of membrane lipids into sterol-poor/PUFA-rich and sterol-rich/saturated fatty acid-rich microdomains to minimize the energetically less favorable contact between PUFA and cholesterol.

Several lines of previous observations from biological membranes support the relevance of the hypothesis of attenuated anterograde transport of lipidated proteins in the DHA-enriched cellular lipid environment. First, studies of photoreceptor membrane



biogenesis provide evidence suggesting that there exist different local concentrations of DHA in TGN membranes as well as distinct protein partitioning therein, allowing for active sorting into discrete post-Golgi vesicles *en route* to specific destinations. Polarized DHA distribution in cell membranes has been well documented in certain cell types naturally enriched in DHA, such as retinal rod photoreceptor cells, brain nerve cells, and sperm (Breckenridge et al., 1973; Connor et al., 1998; Cotman et al., 1969; Fleischer and Rouser, 1965; Poulos et al., 1973; Stone et al., 1979). DHA is particularly concentrated (up to 60 mol%) in the specialized and functionally most active regions such as rod outer segments, the PM and synaptic vesicles in brain synaptic endings, and sperm tails relative to the remaining parts of membranes from retinal rod cells, neurons, and sperm heads, respectively. In an attempt to elucidate the mechanisms responsible for the polarized membrane biogenesis, Rodriguez de Turco and co-workers demonstrated that newly synthesized docosahexaenoyl phospholipids are sequestered and co-transported by rhodopsin-bearing post-Golgi vesicles when they are transported from the ER of inner segments, where their biosynthesis takes place, to rod outer segments (Rodriguez de Turco et al., 1997). Most notably, the time course analysis of pulse-chase labeling indicated that segregation and association of newly synthesized docosahexaenoyl phospholipids and rhodopsin occur on the TGN membranes prior to their exit and subsequent vectorial co-transport on post-Golgi vesicles to rod outer segments. Second, in adrenocortical cells, in which docosapentaenoic acid (22:5, n-3) and DHA (22:6, n-3) only account for 6.9 and 0.1 mol% of total cellular lipids, respectively, distinct types of coated vesicles differing in their size, density, and notably

22:5 and 22:6 profile have been isolated, indicating the presence of heterogeneous intracellular vesicles with different DHA distribution (de Paillerets et al., 1987). Although the origin of these coated vesicles remains unclear, the authors suggested that at least one distinct type of isolated coated vesicles originated from the Golgi, based on the similarity in fatty acid composition. Third, analysis of lipid composition and membrane molecular order of isolated small exfoliated vesicles (50-200 nm in diameter) from T27A leukemia cells revealed that DHA enrichment significantly mitigated or reversed the characteristic differences between the parent PM and shed microdomain vesicles (Williams et al., 1998; Williams et al., 1999). DHA treatment increased DHA content by 10-fold in shed vesicles as well as the parent PM while significantly decreasing cholesterol content (by 65%) and molecular order measured by fluorescence anisotropy in exfoliated vesicles, which are normally characterized with elevated cholesterol content and molecular order relative to the parent PM in untreated cells. Given that exfoliated vesicles normally arise from non-random regions of the cell surface (Armstrong et al., 1988; Beaudoin and Grondin, 1991; Black, 1980; Taylor et al., 1988; van Blitterswijk et al., 1979), these data clearly indicate that cellular lipid environment can influence the lipid composition and structure of vesicles formed therein. Fourth, interesting effects of DHA have been observed on the localization and activation of regulators of vesicular traffic, ARF and phospholipase D (PLD), in human peripheral blood mononuclear cells (Diaz et al., 2002). ARF, a Ras-related small GTPase, regulates coated vesicle biogenesis by controlling assembly of coat proteins and by activating its effector, PLD, which also participates in regulation of membrane traffic

and actin remodeling via phosphatidic acid and indirectly via phosphatidylinositol 4,5-bisphosphate (Brown et al., 1993; Cockcroft, 2001; Roth et al., 1999). In this study, DHA enrichment of cell membranes dose-dependently increased ARF membrane translocation and PLD activation. More importantly, these increases only concerned the disordered non-raft membrane fractions, as DHA treatment displaced both ARF and PLD1 from rafts, which were relatively depleted in unsaturated fatty acids. In view of the role of ARF and PLD in vesicle formation, these findings of increased ARF translocation and PLD activation only in specific regions of DHA-enriched membranes bear potential implications for differential vesicle budding efficiency depending on the local lipid composition in relation to DHA content. Fifth, unsaturated fatty acids are known to promote fusion of natural membranes as well as liposomes (Ahkong et al., 1973; Lavoie et al., 1991; Meers et al., 1988) and this has been related to the curvature stress provided to membranes by inverted phase-forming lipids (Ellens et al., 1989). In particular, this fusogenic effect was clearly correlated with the number of double bonds in acyl chains and was much more pronounced with DHA (22:6, n-3) than  $\alpha$ -linolenic acid ( $\alpha$ -18:3, n-3) (Ehringer et al., 1990; Talbot et al., 1997), implying that local DHA concentrations in the donor and/or acceptor membranes may affect fusion rates of transport vesicles. Finally, in view of the preferential association of peripheral proteins anchored by long, saturated acyl chains with the liquid-ordered microdomains in the PM (Melkonian et al., 1999; Simons and Toomre, 2000), it is conceivable that proteins are also heterogeneously distributed on the surface of intracellular secretory apparatus and related vesicles, provided that discrete subdomains exist with different local lipid

arrangement. Although several lines of circumstantial evidence discussed above potentially supports the hypothesis that DHA-enriched membranes selectively attenuate the forward transport of lipidated cytosolic proteins, further work is clearly required to unravel the underlying mechanisms in relation to the unique lipid/protein lateral organization and physicochemical properties of DHA-enriched membranes.

While attempting to quantitatively compare the effects of dietary fatty acids on PM localization of different lipidated proteins, interesting quantitative and qualitative differences in the subcellular localization were observed among Ras isoforms, regardless of fatty acid treatment. GFP-labeled Ras isoforms showed remarkable differences in the magnitude of PM localization; while the majority of GFP-Hras-wt (~60%) localized to the PM at steady-state, only ~45% of GFP-Kras-wt and ~20% of GFP-Nras-wt were found at the PM in YAMC cells. To the best of our knowledge, these results provide the first quantitative comparison of PM targeting of different Ras isoforms in living cells. Siddiqui and co-workers previously estimated the  $K_d$  for Ras binding to the PM in ascending order of H-Ras (27.7 nM), K-Ras (47.9 nM), and N-Ras (61.1 nM), using competition binding assays with fully processed H-, N-, and K-Ras purified from the baculovirus insect cell expression system and the PM-enriched fraction from NIH3T3 cells (Siddiqui et al., 1998). It is interesting to find the parallel order of Ras isoforms in the extent of the actual *in vivo* PM localization and the estimated *in vitro* binding affinities for the PM, even though the efficiency and kinetics of post-translational processing and intracellular trafficking of each isoform are not accounted for in these estimated  $K_d$  values. In addition to the striking difference in the relative PM expression,

the extent of Golgi localization was also quite different between GFP-labeled H-Ras and N-Ras, despite the common trafficking route and use of palmitate as the second targeting signal to the PM. Whereas the H-Ras Golgi pool (~7-8%) was considerably smaller than the PM-localized H-Ras fraction (~60%), the size of N-Ras Golgi pool (~15%) was almost comparable to the PM fraction (~20%). In fact, it has previously been noted that more N-Ras than H-Ras is associated with the Golgi at an equivalent expression level, based on fluorescence images of GFP-labeled Ras isoforms from several groups, although quantification of these observations has not been attempted (Hancock, 2003). In the context of the role of palmitate as a PM targeting signal, it is tempting to correlate the different PM targeting efficiencies between N-Ras and H-Ras with the presence of one (N-Ras) versus two (H-Ras) palmitates or the possible differences in the efficiency of protein palmitoyl transferase to recognize one versus two adjacent cysteine residues. In addition, in light of the recent findings that Ras engages different signaling pathways with distinct kinetics depending on the subcellular localization (Arozarena et al., 2004; Bivona et al., 2003; Chiu et al., 2002; Perez de Castro et al., 2004), one can further speculate that the different distribution profile of H- and N-Ras at the PM and endomembranes may contribute, at least in part, to the basis of their distinct signaling functions (Ayllon and Rebollo, 2000; Perez de Castro et al., 2004; Voice et al., 1999; Wolfman and Wolfman, 2000). Interestingly, functional status also seemed to affect subcellular distribution of GFP-labeled Ras proteins. This was particularly apparent in K-Ras-derived GFP fusion constructs. Constitutively active oncogenic K-RasG12V exhibited a different endomembrane staining pattern and a slightly lower level of PM

localization (~38%) compared with wild-type K-Ras (~45%). Moreover, GFP-tK, which lacks the N-terminal catalytic domain and the linker domain of the C-terminal hypervariable region, showed negligible endomembrane association unlike its full-length counterparts and predominantly localized to the PM to a much greater extent (~69%). In recent work using a series of H-Ras mutants and a combination of quantitative electron microscopy and lateral diffusion measurements in living cells, Hancock and co-workers demonstrated that in addition to the C-terminal membrane anchors, the N-terminal catalytic domain and its GTP loading state as well as the linker domain of the hypervariable region have distinct roles in regulating membrane affinity and PM association of H-Ras (Rotblat et al., 2004). In the same context, the current results from GFP-Kras-wt, GFP-KrasG12V, and GFP-tK may reflect the potential roles of K-Ras N-terminal catalytic domain and its GTP- and GDP-bound conformations in regulating K-Ras subcellular localization and intracellular trafficking.

Although further studies are required to extend the current observations of dietary lipid-induced differential modulation of intracellular protein trafficking, the findings described in the present study carry significant biological implications, especially considering the potential universality towards general lipidated protein trafficking. Many lipidated signaling proteins play important roles and their aberrant function often results in serious dysregulation of cellular function. H-Ras, N-Ras, and Lck, of which subcellular localization was significantly altered by DHA in the present study, are all essential to a variety of cellular function and adaptive responses to external stimuli. However, overactivation of these proteins either by mutation, overexpression,

or chronic up-regulation of their upstream receptors contributes to deleterious cellular malfunction including oncogenesis and inflammatory diseases (Bolen and Veillette, 1989; Bos, 1989; Campbell and Der, 2004; Kamens et al., 2001; Kim et al., 1997; Yu et al., 2003). Therefore, the selective suppression of protein PM targeting by DHA may constitute a novel modality of the mechanisms responsible for the long appreciated chemoprotective and immunomodulatory effects of dietary fish oil and DHA (Rose and Connolly, 1999; Stulnig, 2003; Terry et al., 2003). In addition, the differential effects of DHA on the PM targeting of H-Ras versus K-Ras observed herein provide a compelling explanation for the selective inhibition of H-Ras activation but not K-Ras, previously observed in DHA-treated colon cells and fish oil-fed mouse colon (Collett et al., 2001; Ma et al., 2004).

## CHAPTER VI

### CONCLUSIONS

A growing body of epidemiological, clinical, and experimental evidence has underscored the pharmacological potential beyond the nutritional value of dietary fish oil enriched in very long chain n-3 PUFAs such as DHA (22:6, n-3) and EPA (20:5, n-3). The broad health benefits of very long chain n-3 PUFAs provide significant protection against a variety of unrelated human afflictions including heart disease, cancer, diabetes, autoimmune and inflammatory disorders (Hu et al., 2003; Kris-Etherton et al., 2002; Rose and Connolly, 1999; Stulnig, 2003; Terry et al., 2003). These highly pleiotropic positive effects of dietary fish oil and DHA have been proposed to involve alterations in membrane structure and function, eicosanoid metabolism, gene expression and formation of lipid peroxidation products, although a comprehensive understanding of the mechanisms of action has yet to be elucidated (Jump, 2002; Larsson et al., 2004). The present study demonstrated that DHA selectively modulates the subcellular localization of lipidated signaling proteins depending on their transport pathway, which may be universally applied to other lipidated protein trafficking. These findings may help develop a new paradigm to better understand the complex effects of DHA on signaling networks.

DHA (22:6, n-3) and EPA (20:5; n-3) often exhibit similar physicochemical properties and biological activities on many occasions and it is likely that EPA may exert similar effects on lipidated protein localization (Stulnig et al., 1998). Future



studies are needed to determine whether these modulatory effects are unique to very long chain n-3 PUFAs found in fish oil or are also shared by other dietary PUFAs such as arachidonic acid (20:4, n-6) and  $\alpha$ -linolenic acid ( $\alpha$ -18:3, n-3).

An interesting possibility raised by the current observations is that lipidated proteins may exhibit different subcellular distribution profiles in various tissues, which contain different membrane lipid composition. It will be interesting to compare the subcellular distribution of lipidated proteins in the nerve tissues and the retina, where DHA constitutes up to 50 mol% of total membrane phospholipids (Anderson, 1970; O'Brien and Sampson, 1965) with that of other tissues typically containing less than 10 mol% of DHA (Dyerberg and Bang, 1979; Sanders et al., 1981; Tinoco et al., 1978).

In addition, the current findings clearly indicate that subcellular localization of proteins with a certain trafficking pathway can be subjected to selective regulation even by dietary manipulation. Although the rationale behind the presence of complicated transport mechanisms for lipidated proteins as opposed to the direct targeting to final destinations remains unknown, regulated PM targeting of a select subset of upstream signaling proteins may provide a means to equip cells with the flexibility to coordinate the arrangement of signaling translators on the cell surface. This may allow cells to optimally decode, respond, and adapt to the vagaries of the ever-changing extracellular environment.

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