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The locations and signaling of H-Ras on endosomes and plasma membrane

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The locations and signaling of H-Ras on endosomes and plasma membrane

by

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CHAPTER 1. GENERAL INTRODUCTION

Introduction

Cancer occurs when cells lose control of their growth due to the abnormal activation of signaling cascades that mediate cell proliferation. Ras proteins are one of the key regulators that control cell growth, proliferation, and differentiation [1], and mutations in Ras proteins are found in multiple human tumors [2].

As a monomeric, guanine nucleotide binding protein, Ras switches between its GTP bound, activated form and its GDP bound, inactivated form. Ras proteins are highly homologous to one another and differ only in their C-termini, which serve as membrane anchors. All Ras isoforms are post-translationally modified by addition of the farnesyl group. N-Ras, H-Ras, and K-Ras4A are acylated with one (for N-Ras and K-Ras4A) or two (for H-Ras) palmitoyl groups, whereas K-Ras4B carries a polybasic amino acid sequence in its C-terminus instead [3].

Originally, the plasma membrane (PM) was thought to be the only platform from which Ras signaling occurs. At the plasma membrane, Ras is able to interact with multiple downstream signaling partners, which ultimately leads to cell proliferation and survival. Among the many Ras signal transduction cascades that take place at the plasma membrane, two of the most studied cascades are the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol-kinase (PI3K)-AKT pathway. Cell proliferation is usually associated with activation of the MAPK signaling pathway, whereas cell survival is usually associated with activation of the AKT/PI3K pathway [4, 5].

The plasma membrane can be further compartmentalized into two subdomains, “raft” and “non-raft,” based on size, composition, and formation [6]. A membrane raft is often
described as a membrane domain that is enriched in cholesterol and sphingolipid [6, 7]. Ras proteins are distributed both in the membrane rafts and bulk plasma membrane [8–11]. How Ras proteins initiate signals within these subdomains on the plasma membrane and whether these different domain locations are essential to the Ras downstream signaling and biological functions are still underdetermined [12].

Much of the research data for the past few years suggest that—in addition to the plasma membrane—Golgi, ER, and endosomes also serve as signaling platforms for Ras [13]. Because different signaling output of Ras is dependent on the spatial organization of the Ras proteins and the regulators of Ras within the cell, learning how Ras is targeted to different cellular compartments may give us valuable insights into the activation of Ras mediated signal transduction and, ultimately, to Ras induced tumorigenesis. A few studies have suggested that H-Ras protein may distribute to the intracellular compartments through the de-acylation/re-acylation cycle [14] and K-Ras could be directed there through phosphorylation [15] or calmodulin/glutamate dependent manner in particular cell types [16, 17]. However, the entire mechanism of how Ras proteins are allocated to the endosomes is not known. Moreover, the kind of endosomes to which Ras binds has not been determined.

In this research presented in this dissertation, my colleagues and I utilized several DNA constructs of Ras variants and Arf6 GTPase and expressed the correspondent Ras and Arf6 proteins in mammalian cells. We isolated the cell membranes and characterized the protein localizations by subcellular fractionation, immunoisolation, density gradient centrifugation, and detergent treatment. We detected the Ras proteins and their signaling cascades by Western blots. We also examined the plasma membrane and intracellular localization of Ras proteins by immunofluorescence and studied the biological activity of
cells expressing Ras proteins through microscopy. Through these methods, we characterized a type of endosomes where H-Ras is localized and thereby identified a new clathrin-independent pathway through which membrane bound H-Ras can allocate to the intracellular membranes. We also found that both of the plasma membrane microdomains, raft and non-raft, allow H-Ras signaling and biological activity. These findings not only enrich our current understanding of trafficking, signaling, lipid modifications, membrane locations, and cell functions of Ras, but also suggest new directions for future research.

**Thesis Organization**

This dissertation follows the journal paper format described by the Graduate College and is comprised of four chapters. The first chapter gives an overview of Ras proteins and their signal transduction cascades. The following two chapters are manuscripts resulting from research conducted in the lab of Dr. Janice Buss at Iowa State University and will be submitted for publication. The first manuscript, *Clathrin-independent endosomes associated with Arf6 bind both active and inactive forms of H-Ras*, describes a new mechanism through which H-Ras allocates to endosomal membranes. The second manuscript, *Biological activity and signaling of H-Ras variants designed to be preferentially “in” or “out” of rafts*, presents the results of a study of two H-Ras variants that are preferentially localized on raft or non-raft domains of the plasma membrane. The final chapter is a general discussion of the results and the conclusions drawn from them.

**Author’s Contributions**

I am one of the two first coauthors of the Chapter 2 manuscript, *Clathrin-independent endosomes associated with Arf6 bind both active and inactive forms of H-Ras*. I performed biochemical studies to determine which endocytosis pathway (clathrin-dependent or
clathrin-independent) H-Ras utilizes. In order to do this, I immunoisolated EEA-1 and conducted the immunofluorescence studies to check the plasma membrane and intracellular locations of EEA-1, clathrin, MHC-I, and H-Ras (as seen in Fig. 1 [except Fig. 1E], Fig. 2 [A, A', A", B, B', and B"]). I examined what class of endosomes H-Ras binds by immunoisolation of the Arf6 endosomes (as seen in Fig. 3). I also checked whether one of the H-Ras effectors, Raf-1, is located on the Arf6 endosomes (as appeared in Fig. 5). Additionally, I examined the endocytosis of K-Ras by testing whether K-Ras4B is present on the perinuclear endosomal recycling center (ERC) and whether C-terminal domain of K-Ras4B proteins targets GFP to the Arf6 endocytic pathway (data can be seen from Fig. 7B and Fig. 8B). Moreover, I organized and edited all figures for this manuscript.

I am the first author of the Chapter 3 manuscript, *Biological activity and signaling of two H-Ras variants designed to be preferentially “in” or “out” of rafts*. My major contribution to this paper was characterizing the signaling transduction pathways of two H-Ras variants that are predicted to be localized to the raft and non-raft domains of the plasma membrane (data as seen in FIG. 7–9). I determined the membrane locations of these Ras variant proteins by immunofluorescence (data as shown in FIG. 2, FIG. 5A, and FIG. 6). My colleagues and I also determined the membrane locations of these Ras variant proteins by density gradient (data as shown in FIG. 4A). I organized and interpreted the data from other researchers in our lab and prepared this manuscript.

**Literature Review**

**General Ras proteins and their basic properties**

Ras is a member of small GTPase superfamily proteins. As GTPases, Ras proteins share a principal biochemical activity of GTP binding and hydrolysis. Ras proteins switch
between the active, GTP bound (“on” position) state and the inactive, GDP bound (“off” position) state. Oncogenic mutations of Ras take place in the GTP/GDP binding regions. These mutations impair the GTP hydrolysis activity and stabilize the Ras GTP binding state and thus lead Ras to have a high binding affinity with its downstream effectors [18, 19].

The first Ras gene was identified and characterized as an oncogene in the Harvey and Kirsten strains of acutely transforming retroviruses [20, 21]. Mutationally activated forms of H-Ras, K-Ras, and N-Ras were subsequently isolated from human tumor cells. Overall, an activating mutation of one of the Ras genes has been found in about 20% of all human tumors [22], with high frequencies seen in cancers that have poor survival rates and limited therapeutic options, including cancers of the pancreas, thyroid, and lung [2].

The Ras proteins are highly homologous in the first 85% of their amino acid sequence, including the guanine nucleotide and effector-binding regions [23]. The remaining C-terminal sequence, which consists of residues 166-185, is known as the hypervariable (HVR) domain. The HVR is highly divergent with the exception of the last four amino acids, the CAAX motif, where a cysteine is followed by two aliphatic residues and one serine or methionine [24]. The CAAX motif directs a series of posttranslational modifications including lipidation with polyisoprene for many prenylated proteins [25].

**Post-translational modification of Ras proteins**

After synthesis, Ras proteins are farnesylated in the cytoplasm by the farnesyl transferase that attaches a farnesyl group to the cysteine residue of the CAAX motif [26] (Figure 1). Next, the farnesylated Ras proteins translocate to the endoplasmic reticulum where the AAX tripeptide is removed by an endopeptidase, Ras converting enzyme 1 (Rce1). Following the cleavage of the AAX, the carboxyl-terminal farnesyl cysteine is methylated by
isoprenylcysteine carboxyl methyltransferase (Icmt) [27, 28]. H-Ras, N-Ras, and K-Ras4A further undergo palmitoylation on cysteine residues near the C-terminus and travel to the plasma membrane (PM) via ER and Golgi [29–31]. Newly synthesized H-Ras also utilizes a Golgi-independent mechanism to traffic to the PM [32]. K-Ras4B does not have a palmitoylated cysteine in the hypervariable domain; it contains multiple lysine residues instead that also help it associate with plasma membrane. Unlike H-Ras, K-Ras4B is targeted to the plasma membrane through a Golgi-independent mechanism, which is currently not understood. The trafficking of K-Ras4B to the plasma membrane may be directed by microtubules [33] or operated by the electrostatic gradient [33, 34].

**Ras effectors and signaling cascades**

Activation of Ras proteins leads to activation of Ras downstream effectors. These effectors include, but are not limited to, protein and lipid kinases like Raf and PI3kinase, guanine-nucleotide exchange factors (GEFs) for other Ras superfamily members. Activated Ras proteins can initiate and activate many downstream signaling cascades by recruiting these effectors (Figure 2).

Even though there are many signal transduction cascades that are mediated by Ras, the best understood among these is the mitogen-activated protein kinase (MAPK) pathway. The MAPK pathway contains at least three kinases—Raf, MEK, and ERK—which transmit signals through sequential phosphorylation procedures. The Ras mediated Raf-MEK-ERK activation pathway is a pathway that can cause cell transformation. In order for Ras to initiate the MAPK signal transduction cascade, Ras must bind to the “Raf-like Ras-binding domain” and the nearby cysteine-rich domain of the Ras effectors, the serine/threonine protein kinase Raf-1 (also named c-Raf) or A-Raf and B-Raf to active Raf-1 kinase [35, 36]. Raf-1 kinase
phosphorylates and activates mitogen-activated protein kinases 1 and 2 (MEK1 and MEK2). After MEK1 and MEK2 are activated, they phosphorylate the downstream mitogen-activated protein kinases (MAPKs) ERK1 and ERK2 (extracellular signal-regulated kinases 1 and 2). The activated ERK proteins can translocate to the nucleus, where they phosphorylate their majority of their substrates, transcription factors, and thus lead to a change in gene expressions. Activated ERKs also phosphorylate other substrates that are found in the cytosol and in other cellular organelles [37]. The activation of ERK1 and ERK2 mediates many cellular activities including cell proliferation, differentiation, mitosis, and apoptosis [37, 38].

The Ras-Phosphatidylinositol 3 kinase (PI3K)-AKT pathway is another well-established Ras signaling pathway. In this pathway, activated Ras binds to the p110 catalytic subunit of PI3 kinase, a Ras effector. The interaction between Ras and PI3 kinase increases the production of phosphatidylinositol 3,4,5-triphosphate (PIP_3). PIP_3, which is localized on the cell membrane, binds to the PH domain containing protein kinases such as AKT (also known as PKB), and thereby recruits AKT to the plasma membrane. AKT can then be activated on the PM by phosphorylation. Activated AKT phosphorylates its downstream substrates such as BAD protein, transcription factor CREB, and thereby regulates multiple biological processes including glucose metabolism, apoptosis, and tumorigenesis [39, 40].

Another downstream signaling cascade occurs when activated Ras recruits MEK (MAPK kinase) kinase (MEKK)[41–43], another Ras effector, which can activate the JNK kinase (JNKK) [44–46] . JNKK1 (also called MEK4) and JNKK2 (also called MEK7) activate their downstream c-Jun NH\textsubscript{2} terminal kinase (JNK) by phosphorylating the Thr-Pro-Tyr phosphorylation sites of JNK [47]. Activated JNK in turn phosphorylates its
downstream substrates—such as transcription factors including c-Jun, p53, ATF-2, Elk-1 and the nuclear factor of activated T cells (NFAT)—and thereby regulates the related gene expression that leads to cell growth, differentiation, or apoptosis [38]. JNK2 is required for H-Ras mediated transformation [48].

The Ras guanine-nucleotide exchange factors (GEFs) catalyze the release of GDP and thus promote the loading of GTP and Ras activation (Figure 2). The three main kinds of Ras GEFs are Sos, Ras-GRF, and Ras GRP. They all share a common CDC25 homology and an adjacent Ras exchange motif [49].

Unlike GEFs, p120Gap, a GTPase activating protein (GAP) negatively regulates Ras GTPase (Figure 2). It catalyzes the hydrolysis of bound GTP from Ras by a mechanism involving stabilizing the transition state of GTP hydrolysis through an arginine finger loop and thus negatively regulates the GTPase [50].

The various isoforms of Ras may interact with their effectors and activate their downstream cascades differently, resulting in differences in biological activity. For example, activated H-Ras is more relevant to the activation of the PI3 kinase pathway, whereas activated K-Ras is more relevant to the activation of the Raf-1 pathway than H-Ras [51]. K-Ras is essential for mouse embryogenesis, whereas N-Ras and H-Ras knockout mice show no apparent abnormalities [52, 53]. A recent study also indicates that K-Ras has a unique role in cardiovascular homeostasis [54]. These differences in effector activation and biological activity may be due to the distinct cellular microenvironments in which these different isoforms of Ras proteins can be localized [55–58].
**Ras signaling on endomembranes**

Ras proteins have been shown to be distributed on endomembranes as well as on the plasma membrane. Ras proteins can also signal from endomembranes such as the Golgi apparatus, ER, and mitochondria [59, 60]. Upon EGF stimulation, Ras becomes activated both on the PM and the Golgi in fibroblasts [61]. N-Ras also demonstrates strong activation in Golgi in T cells [62]. Studies have also shown that mitochondria associated phospho-K-Ras 12V181E can induce apoptosis through a pathway that requires Bcl-XL [15].

Different compartmentalization of Ras also moderates different signaling outputs. Recent studies used H-Ras constructs that are fused to different intracellular tethering domains to study the signaling in the defined sublocations [61, 63]. The results demonstrated that activated H-Ras that was tethered to the KDEL receptor showed strong activation of both the AKT and ERK pathways but weak activation of JNK [63]. On the other hand, H-Ras targeted to the ER by the avian bronchitis virus M1 showed robust activation of JNK but weak activation of AKT and ERK [61, 63]. Thus, the subcellular location of Ras seems to influence Ras signaling outputs.

**General endocytosis and endosomal trafficking**

In eukaryotic cells, proteins and lipids are transported along the endocytic pathways. In the endocytic pathways, molecules are internalized from the PM and then directed into sorting endosomes where they can either continue on to the endocytic recycling compartments and then return to the PM or travel to late endosomes and lysosomes for degradation.

Cells can take up nutrients and internalize particles through endocytosis. In mammalian cells, endocytosis has an essential role in delivering receptors and their
associated ligands, membrane domains and proteins, and soluble molecules to the destination organelles. Endocytosis is classified into two broad categories: phagocytosis (cell eating) and pinocytosis (cell drinking) [64]. Phagocytosis, which involves uptake of large particles, is restricted primarily to specialized cells such as macrophages. On the other hand, pinocytosis is found in all cells. There are at least four distinct pinocytosis mechanisms: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin-independent endocytosis [64, 65].

Of the four types of pinocytosis mechanisms, clathrin-dependent endocytosis is the best understood. Molecules such as low-density lipoprotein (LDL), LDL receptors, transferrin (Tfn), and transferrin receptors all use clathrin-mediated endocytosis [66–68]. Formation of clathrin-coated vesicles begins when triskelions assemble into a polygonal lattice at the plasma membrane and form clathrin-coated pits. Next, clathrin-binding adaptors such as adaptor protein-2 (AP2) directly bind to clathrin and initiate the budding and pinching of coated pits from the PM in a dynamin-dependent manner [64]. Other adaptors such as epsins and phospholipids (i.e., phosphatidylinositol-4, 5-biphosphate [PIP$_2$]) are also found in coated pits and facilitate budding of clathrin-coated vesicles [68, 69].

In contrast to the clathrin-coated vesicular mechanism, clathrin-independent endocytosis is not well understood. Major Histocompatibility Complex I (MHCI) [70], Arf6 GTPase [70–72], CD59 [73], E-cadherin [74], integrins [71, 75], and interleukin-2 (IL-2) receptors on lymphocytes [76] are all thought to be internalized through clathrin-independent mechanisms. Although there are multiple categories of non-clathrin endocytosis, this type of endocytosis can be further categorized into dynamin-GTPase dependent and dynamin-GTPase independent.
After being internalized into the cytosol, clathrin-coated vesicles are uncoated before they fuse with the sorting endosomes [69]. The sorting endosomes are rich in phosphatidylinositol-3-phosphate (PI3P) and some unique FYVE-domain proteins, such as EEA-1 and rabenosyn-5, both of which are required for the fusion of the sorting endosomes [77]. After sorting, receptors can be directed to endocytic recycling compartments and then to the cell surface, or they can be directed to the multivesicular elements that resemble the endosomal carrier vesicles/multivesicular bodies (ECV/MVBs) and then to late endosomes and lysosomes for degradation [77]. The non-clathrin pathway can deliver molecules to multiple intracellular compartments such as the Golgi [78, 79], as well as to the classical endocytic endosomal compartments such as the endocytic recycling compartment [80]. The clathrin pathway can also target molecules to the Golgi [81] or from the Golgi to the endosomes [68].

**Endosomal locations and retrograde trafficking of Ras proteins**

The plasma membrane, where Ras proteins undergo repeated cycles of activation/inactivation and signaling, has been considered the final destination for Ras. Traditionally, Ras was thought to be post-translationally modified and trafficked via ER/Golgi to the plasma membrane. Although H-Ras and N-Ras proteins can be found on the Golgi apparatus, the Ras proteins were thought to be just passing through the Golgi on their way to reach their final destination on the PM. However, recent studies have provided direct evidence that the plasma membrane association of different Ras proteins can be dynamic instead of static. H-Ras proteins appear to be endocytosed as part of their normal function in propagating signals from activated cell surface receptors [82]. Ligand activated epidermal growth factor receptors (EGFR) are rapidly internalized by clathrin-coated pits and delivered
to early endosomes where they are sorted to internal endosomal vesicles for degradation or recycled back to the plasma membrane [83, 84]. Upon insulin or NGF stimulation, Ras and a complete MAPK signaling complex can also be found on the intracellular vesicular complex containing endosomes [85–87]. Signaling of H-Ras to Raf-1 is also reported to be dependent on endocytosis [88]. These results indicate that endosomal trafficking of H-Ras and Raf appear to be critical for growth factor stimulated MAPK activation in these cells and that Ras may not reside on the plasma membrane permanently.

A recent study [14] shows that de-palmitoylation/re-palmitoylation of H-Ras and N-Ras may contribute to the redistribution of Ras between the Golgi and the plasma membrane. The de-palmitoylated Ras can dissociate from the plasma membrane and be released to the cytosol, where it can be randomly captured at the Golgi apparatus and re-palmitoylated again (Figure 3) [14]. Thus H-Ras and N-Ras can traffic backwards from the PM to the Golgi by the de-palmitoylation/re-palmitoylation cycle. Jura, Scott-Lavino, Sobczyk, and Bar-Sagi [89] showed that H-Ras but not K-Ras can be ubiquitinated, and this modification promotes H-Ras’ association with endosomes. Recent studies showed that the GTP-bound form of H-Ras can be endocytosed in the cells overexpressing Rab5 proteins [88], and its return to the plasma membrane is dependent on the endosomal-recycling pathway [90]. Thus, H-Ras not only can internalize from the PM but also can recycle back to the PM as well (Figure 3). As for K-Ras, in rat hippocampal neurons upon glutamate stimulation, K-Ras can be released from the plasma membrane through binding with the Ca\(^{2+}\)/calmodulin at the C-terminus of K-Ras [16]. Recently, K-Ras was also found dissociated from the plasma membrane and distributed on the mitochondria through the phosphorylation of serine 181 within the C-terminal polybasic region by protein kinase C.
These findings suggest that partially neutralizing the positive charge of the polybasic sequence will discharge K-Ras from the PM, but whether or how K-Ras is recycled back to PM is undetermined. Moreover, the precise mechanism of how Ras proteins localized on endosomes and the completed machinery of Ras retrograde trafficking remains to be determined.

Membrane rafts and Ras microdomain locations

The plasma membrane of mammalian cells is a heterogeneous mixture comprised of hundreds of proteins and lipid species. Based on the observed data, researchers have proposed hypotheses to define the structure of the plasma membrane more precisely. A widely accepted hypothesis of the membrane structure is the “membrane raft” hypothesis. The most contemporary definition of rafts [91] states that “Membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized with one another to form larger platforms though protein-protein interactions.” Membrane rafts are rich in saturated phospholipids and cholesterol, are tightly packed and ordered, and have less fluidity than the surrounding plasma membrane. In contrast, “non-raft” membranes are poor in cholesterol, rich in unsaturated phospholipids, and have more fluidity [6]. For this reason, membrane rafts are also defined as liquid ordered lipid domains separated from liquid disordered domains [92, 93]. Because of their relatively compact structure, membrane rafts are poorly solubilized by common nonionic detergent such as Triton X-100 at 4°C. The membrane raft fractions isolated according to this biochemical property are found to be rich in cholesterol and sphingolipids but often poor in glycerolphospholipids, and to accumulate glycosylphosphatidylinositol (GPI) anchored proteins, sphingomyelin, and gangliosides as
well as other specific proteins such as acylated proteins; [92, 94]. This specific composition enables membrane rafts to serve as platforms for protein segregation and cellular signaling [95].

The heterogeneity of plasma membranes in turn leads to the nonrandom microorganization of lipid anchor proteins that are located on the PM. With regard to micro-locations of Ras proteins, most of the research has been done by the John Hancock group, who used several methods including electron microscopy (EM) and fluorescent recovery after photo bleaching (FRAP). For the micro-location of H-Ras, their work indicated that the GDP bound form of H-Ras is mainly associated with cholesterol-dependent (rafts) microdomain, while the GTP bound form of H-Ras is associated with the cholesterol-independent (non-raft) microdomain [55, 96]. They suggested that the hypervariable region (HVR) and the different position of palmitoylated residues of the H-Ras C-terminus contribute to the lateral segregation of H-Ras on raft and non-raft domains [11, 55, 97]. Up to now, the data indicating how the HVR of N-Ras affects the microdomain location of N-Ras is not available. N-Ras, however, is found to be located on the non-raft domain, but localized to rafts when it is activated [11]. Both K-Ras-GDP and K-Ras-GTP are found to be located in the non-raft domains of the plasma membrane [11]. The farnesyl motif seems to play a major role in distributing K-Ras into the non-raft membrane and many farnesylated proteins are sequestered in non-raft membrane microdomains [98–101]. The polybasic region of K-Ras also plays an important function of localizing K-Ras to the plasma membrane through the interaction with acidic lipids, for example, phosphatidylinositol-1, 4, 5-triphosphate (PIP₃), and phosphatidylinositol-4, 5-biphosphate (PIP₂) on the plasma membrane [102].
Recent membrane studies by a fluorescence resonance energy transfer (FRET) examination indicated that GPI anchored proteins, which are raft protein markers, are organized in nanoscale (<5 nm), as nanoclusters on the plasma membrane [103]. This nano-domain is enriched with proteins and is also dynamic. FRAP studies from Kenworthy group demonstrate that both K-Ras and H-Ras diffuse quickly on the plasma membrane, indicating that either these microdomains are quite mobile or the associations of Ras proteins with these domains are very dynamic [104]. Using single fluorophore video tracking (SFVT), Murakoshi et al. [105] showed that during mitogen activation, the diffusion of the H- and K-Ras proteins on the PM decreased and the fractions of immobilized Ras proteins increased. Moreover, the immobility of Ras proteins is not affected by cholesterol depletion, which is a characteristic of non-raft membrane. New experimental data and computer modeling showed that K-Ras nanoclusters operate as a “sensitive digital switch,” a graded system which responds to varied doses of EGF stimulation for MAPK signaling [106]. These studies advance our understanding of how Ras proteins and their associated microdomains are organized with regard to signaling.

**Summary**

Ras traffics and signals from different intracellular locations including the PM, ER, Golgi apparatus, and endosomes. Ras is localized on the cellular membrane and can also be distributed on the different microdomain locations of that membrane. The different trafficking routes and sub-cellular locations of the Ras may influence the Ras signaling ability, specificity, and the biological function of the cell in which Ras is expressed. Therefore, it is important to know where, when, and how Ras signaling is initiated and regulated. Enhanced knowledge of the complexity of Ras signaling and the regulation
mechanisms might allow further understanding of carcinogenesis and provide clues for cancer therapies to be designed to inhibit specific signaling pathways activated by Ras proteins.

References


Figure Legends

Figure 1. Processing of Ras proteins. The Ras processor proteins are first farnesylated in the cytosol by farnesyl transferase (FTase). Processing is completed in the ER by the enzyme Rce1 which removes the –AAX residues followed by carboxyl-methylation on the farnesylated cysteine residue by enzyme Icmt. H-Ras and N-Ras further undergo palmitoylation by the palmitoyl transferase (PAT) and transport to the plasma membrane (PM) via Golgi. H-Ras also utilizes a Golgi-independent mechanism to traffic to the PM. K-Ras, which cannot be palmitoylated, is targeted to the plasma membrane through an unclear, Golgi-independent mechanism.

Figure 2. Major Ras signaling cascades. Illustration of three major signaling cascades of Ras proteins. Guanine nucleotide exchange factors (GEFs) stimulate displacement of GDP, resulting in increased level of Ras-GTP. GTP bound Ras can interact productively with more than 20 effectors including Raf, phosphatidylinositol 3-kinase (PI3K), and MEKK to regulate various cellular responses such as proliferation, survival, and gene expression. Ras signaling is terminated by
hydrolysis to Ras-GDP, and this reaction is catalyzed by the GTPase-activating proteins (GAPs).

**Figure 3. Endocytosis and trafficking map of Ras.** Ras signaling is widely shown on the plasma membrane. Ras proteins have reported to be distributed and signals from internal membranes such as ER and Golgi. Ras proteins are also found on vesicles with all the characteristics of endosomes. Plasma membrane bound H-Ras and N-Ras can traffic from the PM to the Golgi through the de-palmitoylation/re-palmitoylation cycle.

**Figures**
Figure 2

PM

GAPs

Ras-GTP

GEFs

Ras-GDP

PI3K → Raf → MEK1/2 → ERK1/2

MEKK → MEK4/7 → JNK1-3

Survival, Proliferation, Gene expression
CHAPTER 2. CLATHRIN-INDEPENDENT ENDOSONMES ASSOCIATED WITH ARF6 BIND BOTH ACTIVE AND INACTIVE FORMS OF H-RAS

A paper to be submitted to the Experimental Cell Research

Jodi McKay\textsuperscript{1,*}, Xing Wang\textsuperscript{1,*}, Linda Ambrosio\textsuperscript{1}, and Janice E. Buss\textsuperscript{1,2,§}

Abstract

H-Ras that becomes de-palmitoylated at the plasma membrane can relocate through a cytosolic intermediate to internal sites. H-Ras has also been sighted on endocytic vesicles and recently on recycling endosomes, but the class of endosomes to which it bound has remained uncharacterized. The aims of this study were to identify those endosome(s), to examine whether inactive or active forms of H-Ras bound different endosomes, and to determine which endosomes could bind active H-Ras-GTP or the Ras effector, Raf-1. The results showed that oncogenic H-Ras\textsuperscript{61L}, or normal H-Ras (in either basal or EGF-stimulated NIH 3T3 cells), were absent from endosomes of the clathrin-associated pathway. Instead, both forms of H-Ras were present on endosomes of the clathrin-independent Arf6-associated

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pathway and in the endosomal-recycling center. In contrast, Raf-1 was distributed primarily on separate EEA1-containing endosomes of the clathrin-dependent path. Arf6-associated vesicles could also bind K-Ras4B-12V or chimeric green fluorescent proteins with either H-Ras or K-Ras4B C-termini. However, neither K-Ras4B nor the chimeric GFP-K-tail proteins were present in the endosomal-recycling center. These results identify Arf6-associated endosomes as a specific class of constitutive endosomal vesicles that can bind both active and inactive H-Ras proteins and that provide a second platform, in addition to Golgi membranes, for membrane-bound H-Ras to localize on intracellular membranes.

Key words: protein trafficking, perinuclear recycling center, endocytosis, endosome, Arf6, clathrin, EEA1, Raf-1, Ras

Introduction

Although the site of Ras signaling is widely depicted as the plasma membrane, Ras proteins have now been shown to be distributed on, and to produce significant signals from, internal membranes [1–3]. The means by which Ras proteins localize to these internal sites are varied and not fully characterized. In neurons, calcium signals trigger Ca\(^{++}\)/calmodulin binding to K-Ras4B and cause the release of the K-Ras/calmodulin complex from the plasma membrane [4]. This transient, cytosolic complex is subsequently targeted to Golgi and recycling endosomes [5]. For H-Ras, a small portion [6] of the newly synthesized protein is delivered to Golgi membranes, and from there boards outward-bound exocytic vesicles [7, 8]. Recent work indicates that if palmitoylated H-Ras that is already at the plasma membrane becomes de-palmitoylated, it is released into the cytosol, and then can be re-palmitoylated and thus captured on internal membranes from which it can then rejoin the
biosynthetic pathway [9]. However, H-Ras has repeatedly been found on vesicles with all the characteristics of endosomes [10–12], including new observations of H-Ras on membranes of the pericentriolar endocytic recycling compartment [13]. A small portion of H-Ras can also become ubiquitinated, a modification that stabilizes its association with endosomes but that seems to decrease its association with Golgi membranes [14]. It is unclear whether H-Ras that reaches the plasma membrane must await depalmitoylation before it can re-enter the cell and bind to internal membranes or whether a membrane-bound form of H-Ras can traverse the cytosol and serve as a source for membrane-based intracellular signaling.

A clear identity for the endosomes to which H-Ras binds has not been reported. There is even the possibility that more than one type of endosomal pathway might be utilized, as the distribution of H-Ras in the plasma membrane is reported to vary. The inactive form of H-Ras is included in raft-like lipid microdomains, while GTP-bound forms inhabit the more general lipid bilayer [15]. Endocytic pathways also show preferences for one type of domain or the other [16]. A previous study has shown that a Rab5- and dynamin-regulated pathway of endocytosis and recycling is required for signaling by the Ras effector, Raf-1 [17]. Additional studies report that both Rab5 and Rab11 regulate H-Ras trafficking to the pericentriolar recycling center [13]. However, Rab5 is found on endosomal membranes and sorting endosomes that are derived from both clathrin-mediated and clathrin-independent pathways [13, 18], and thus does not define a specific class of endosomes. Expression of the constitutively active Rab5-Q79L protein causes some H-Ras to accumulate on enlarged endosomes, but, notably, not Raf-1 [19]. Thus, the portion of H-Ras that binds those endosomes appears to disengage from this effector. Raf-1 itself has also been observed on endosomes, co-localizing with EEA1, an early endosomal marker for the clathrin-mediated
pathway [11]. Other studies report that blocking clathrin-mediated endocytosis with a dominant, mutant form of epsin or AP180 does not prevent accumulation of H-Ras on intracellular membranes [2, 9]. In addition, in earlier electron microscopy studies, H-Ras was notably absent from clathrin-coated pits or flat arrays on the plasma membrane [15, 20, 21]. Although these studies suggested that H-Ras could leave the plasma membrane and enter an endocytic pathway, they did not provide a clear identity for that pathway or answer whether activated H-Ras could maintain its GTP-bound state while on such endosomes.

Several pathways for clathrin-independent endosomal entry of proteins have been described [16, 18, 22–25, 56]. One of these is a pathway that serves as an entry route for the class I major histocompatibility complex protein (MHC-I), the IL-2 receptor subunit, the beta-1 subunit of integrins, and the Src tyrosine kinase [26–28]. This pathway is identifiable by the presence of Arf6. Arf6 is a member of the Arf and Arl subfamily of small GTPases that participate in vesicular trafficking [29]. Although many Arf GTPases localize primarily on internal membranes, the Arf6 protein is located on the plasma membrane, endosomes, and tubular-vesicular recycling membranes [30]. Several studies suggest that Arf6-associated vesicles converge on the endosomal recycling center (ERC) [31], where they mingle among other endosomes that are derived, independently, from clathrin-mediated endocytosis [27, 32]. Arf6-associated vesicles also participate in a recycling pathway that returns proteins to the plasma membrane [33, 34].

The studies reported here examined what type(s) of endosomes could bind H-Ras and its effector, Raf-1. The results build a new mechanistic link between Arf6 and H-Ras that reaches from the plasma membrane to the perinuclear endosomal recycling center, and complete a framework for a new itinerary of H-Ras movements within the cell.
Material and Methods

Plasmids, cell culture and DNA transfection

The H-Ras and Ext-Ras DNAs in the pcDNA3 vector were described previously [35]. GFP-H-Ras-61Q, GFP-K-Ras4B-G12V, GFP-Ext-Ras61L, GFP-Ext-Ras-61Q, and GFP-H-Ras-61L were constructed using the pcDNA3 versions as PCR templates and transferred to the pEGFP-C3 vector (Clontech Labs). GFP-RBD contains the “Ras binding domain” residues 51–131 of Raf-1 [2] that were transferred to pEGFP-C3 from a cDNA for a GST-RBD that was obtained from the laboratory of Dr. C. Der (University of North Carolina). GFP-H-tail was constructed by attaching the bases encoding the 10 C-terminal amino acids of H-Ras (GCMSCKCVLS), a termination codon, and a Bam H1 restriction enzyme site to pEGFP-C3 by PCR. Arf6-67Q-GFP (Arf6-WT, wild type) and Arf6-Q67L-GFP are in the pEGFP-N1 vector; Arf6-67Q-HA and Arf6-Q67L-HA each with a C-terminal hemagglutinin (HA) epitope tag, are in pXS vectors. Dr. J. G. Donaldson, National Institutes of Health, generously provided all the Arf6 constructs and the GFP-K-tail plasmid in which the last 20 residues of K-Ras4B are attached to the C-terminus of EGFP.

NIH 3T3 cells were cultured at 37°C and 10% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum plus penicillin, streptomycin, glutamine, and sodium pyruvate. For immunofluorescence and immuno-isolation, NIH 3T3 cells were transfected with Effectene (Qiagen) according to the manufacturer’s instructions and used after 24 hours or as mentioned in the results. The H-Ras61L or H-RasWT (HRas cellular form, wild-type) stable cell lines have the H-Ras61L or H-RasWT DNAs expressed from the pZIPneo vector; the GFP-H-Ras61L stable cell line has the GFP-H-Ras61L DNA expressed from the pEGFP-C3 vector. Cells treated with EGF (Gibco BRL) were cultured in the same...
media described above and incubated with 5 or 10 ng/ml of EGF at 37°C for the time as indicated, followed by washing with phosphate-buffered saline and fixation.

**Antibodies**

Antibodies used for immunoblotting included mouse monoclonal anti-Ras (BD Bioscience 610010), mouse monoclonal anti-c-Raf (BD Bioscience 61051), rabbit polyclonal anti-hemagglutinin (Covance PRB-101P), mouse monoclonal anti-GFP (Roche 1814460), and rabbit polyclonal anti-EEA1 (ABR P1-063). For immunofluorescence, rat monoclonal anti-Ras (sc-34), rabbit polyclonal anti-c-Raf-1 (sc-227), and rabbit polyclonal anti-clathrin heavy chain (sc-9069) were from Santa Cruz Biotechnology. Mouse monoclonal antibody (W6/32) to human MHC-I protein was from Leinco Technologies (#H263). Antibody to the cis-Golgi protein, giantin was from Covance. For the experiment in Fig. 4, primary antibody is mouse monoclonal anti-Ras (BD Bioscience 610010). The same EEA1 antibody or mouse anti-hemagglutinin (Covance PRB-101R) were used for both immuno-isolation and immunofluorescence. Murine monoclonal anti-Ras (Quality Biotech LA069) and the above rat monoclonal anti-Ras antibody are also used for immuno-isolation. Secondary antibodies from Molecular Probes included goat anti-mouse IgG coupled to Alexa Fluor 350, goat anti-rat IgG coupled to Alexa Fluor 488, goat anti-mouse IgG coupled to Alexa Fluor 594, goat anti-rabbit IgG coupled to Alexa Fluor 594, donkey anti-rat IgG coupled to Alexa Fluor 594, and transferrin from human serum coupled to Alexa Fluor 594.

**Live cell labeling with transferrin or MHC-I antibody**

To mark early endosomes of the clathrin-dependent pathway, cells were transfected and incubated for 18 hours, then cycloheximide (10 µg/ml) was added for 5.25 hours to allow time for previously synthesized protein to vacate Golgi membranes. Cells were then chilled
for 45 minutes at 4°C in a cold room in medium containing 50 mM HEPES, pH 7.4 buffer, cycloheximide (10 µg/ml), and AlexaFluor-transferrin (Molecular Probes, 10 µg/ml). Finally, the cold medium was removed and prewarmed (37°C) medium containing 50 mM HEPES, pH 7.4 was added and cells were incubated at 37°C in a water bath for one minute before fixation with cold 4% paraformaldehyde and processing for immunofluorescence.

For marking the endosomal recycling center (ERC), cells were transfected with various cDNAs, incubated for 18 hours, and then treated for 5.25 hours with cycloheximide (10 µg/ml). AlexaFluor-transferrin (10 g/ml) was then added for 45 minutes. Cells were washed, fixed, and processed for immunofluorescence.

To monitor MHC-I internalization, HeLa cells were transfected and incubated for 18 hours, then cycloheximide (10 µg/ml) was added for 5.5 hours to allow time for newly synthesized H-Ras proteins to vacate Golgi membranes. Cycloheximide-containing media with MHC-I antibody (200 µg/ml) was equilibrated to 37°C and then added to the above cells for 30 min at 37°C. At the end of incubation, cell surface-associated antibodies were removed by rinsing the cells in low pH solution (0.5% acetic acid, 0.5M NaCl, pH 3.0) for 30 seconds [27]. Cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 10 minutes, permeabilized, and stained in blocking buffer (2% horse serum, 0.4% bovine serum albumin, and 0.2% saponin in PBS, pH 7.4) [27, 36]. The internalized antibodies were visualized with the appropriate fluorochrome-labeled secondary antibody in the above blocking buffer.

Immuno-isolation of membranes

Twenty-four hours after transfection, cells were scraped into 300 µl homogenization buffer (3 mM imidazole, pH 7.4, 1 mM Na₃VO₄, 5 mM MgCl₂, 1% Protease Inhibitor
Cocktail (Sigma), and 5% sucrose) and homogenized with 15 strokes in a Dounce homogenizer followed by 20 passages through a 26-gauge needle. The nuclei and unbroken cells were removed by centrifugation at 1300 $\times$ g (4 °C) for 10 minutes. This post-nuclear supernatant (PNS) was pre-cleared by incubation for one hour at 4°C with protein G- or protein A-agarose beads (Invitrogen). Beads were removed, and the supernatant was then incubated with mouse monoclonal anti-HA antibody or a mixture of mouse and rat monoclonal anti-H-Ras antibodies or rabbit EEA1 antibody overnight. The following day fresh protein G agarose beads (or protein A beads for rabbit EEA1 antibody) were added for two hours at 4°C. The beads were washed three times in cold homogenization buffer and boiled in SDS-PAGE sample buffer. Proteins present in the membranes were separated by SDS-PAGE and detected on immunoblots with the indicated antibodies. Then 95% of the sample was loaded in the IP lane and 5% in the PNS lane.

**Immunoblotting**

Samples collected by immuno-isolation were separated by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Pall Life Science) and then incubated in blocking buffer (Vector Labs 10x casein diluted to 1x ) in TTBS (0.15 mol/L NaCl, 0.05 M Tris-HCl, 0.05% Tween-20, pH 7.4) for 20 minutes at room temperature. PVDF membranes were washed in TTBS and incubated with primary antibodies overnight at 4°C. The proteins of interest were detected using horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies and an enhanced chemiluminescence kit from Pierce.
**Immunofluorescence and preparation of sonicated membrane fragments**

NIH 3T3 cells were plated on coverslips coated with 100 g/ml poly-L-lysine (Sigma) and 500 g/ml fibronectin (Sigma) and transfected with DNA. After 24 hours (or as mentioned in the results), cells were fixed with fresh 4% formaldehyde in phosphate-buffered saline, pH 7.4, at room temperature for one hour then permeabilized with -20°C methanol and quenched with 50 mM ammonium chloride in phosphate-buffered saline. The fixed cells were incubated with blocking buffer (2% horse serum and 0.4% bovine serum albumin in phosphate-buffered saline, pH 7.4), then sequentially with primary and secondary antibodies diluted in blocking buffer. For the experiment shown in Fig. 4D, cells were cotransfected with cDNAs for H-Ras61L and Arf6-Q67L-GFP. Both proteins were expressed in >90% of the transfected cells (data not shown). Cells were fixed and stained with mouse anti-Ras (BD Bioscience 610010) and Raf antibody. The Ras antibody was detected with a goat anti-mouse IgG secondary antibody coupled to AlexaFluor350 (blue), and its co-expression in each cell that expressed the Arf6 protein was visually confirmed, but blue images were not captured.

Experiments to produce adherent plasma membrane fragments were carried out exactly as described previously [37]. The slips were mounted in Vectashield mounting medium (Vector Laboratories). For experiments imaging adherent membrane fragments, cells were analyzed using a 60x oil immersion lens on a Nikon Eclipse E800 microscope with Spot RT digital camera. All the confocal images were taken with a Leica TCS-NT confocal system with a 63x oil immersion lens with an aperture adjustable from 1.32 to 0.6, using an argon laser (488 nm excitation) and a krypton laser (568 nm excitation). Other images were taken with Leica DMIRE2 microscope using a 100x oil immersion lens with an aperture adjustable from 1.3 to 0.6 and Q Imaging Retiga 1300 camera. Images were
exported to Adobe Photoshop 7.0; the images taken with the Leica DMIRE2 microscope were taken in black and white, processed in Openlab3.5.1, displayed in colors, and then exported to Adobe Photoshop 7.0 to equalize image intensity prior to merging. For some experiments, as indicated in the figure legends, a series of z-stack images were acquired using a motorized stage on the Leica DMIRE2 microscope. These images were processed using Openlab’s nearest neighbor deconvolution module and processed as above.

**Results**

**H-Ras does not bind endosomes of the clathrin-dependent pathway**

The previous report of the dynamin sensitivity of H-Ras-triggered Raf-1 signaling [17] suggested that H-Ras might be found on endosomes of the dynamin-sensitive, clathrin-dependent pathway. To inspect specifically only plasma membranes for possible H-Ras and clathrin co-localization, adherent basal plasma membranes of transfected NIH 3T3 cells were prepared by sonication, fixed, and examined by indirect immunofluorescence [37]. However, neither the activated H-Ras61L (Fig. 1A) nor the transfected cellular form of H-Ras (H-Ras-WT; data not shown) overlapped with clathrin heavy chain.

Another marker of a later step in the clathrin-dependent pathway—the early endosome antigen-1 protein (EEA1)—was used to determine if H-Ras was present in early endosomal vesicles that had already lost their clathrin coat. Upon examination of EEA1-positive vesicles in intact cells, no overlap of H-Ras-WT and EEA1 was detected (Fig. 1B). On adherent fragments of basal membranes from sonicated cells, the population of EEA1-positive vesicles was more clearly resolved, but again no H-Ras (this time the activated form, H-Ras61L) was present in these vesicles (Fig. 1C). Because EEA1 marks early endosomal vesicles specifically, these results indicated that neither an oncogenic nor
the normal form of H-Ras were present on early endosomes derived from the clathrin-dependent pathway.

Because the experiments with H-Ras61L used a permanently activated, mutant form of H-Ras, we also tested if H-Ras-WT that became transiently GTP-bound after EGF stimulation now joined the clathrin-dependent pathway. This was a relevant question, as the serum-activated form of H-Ras is reported to shift from raft-like lipid microdomains, to a more general membrane distribution from which clathrin-mediated internalization might occur [15]. However, after stimulation of cells for 10 minutes with a moderate, 10 ng/ml dose of EGF [38], no co-alignment of the EEA1 marker of early endosomes and H-Ras-WT was detected (Fig. 1D).

As an additional test, a fluorescent derivative of transferrin was added to live cells to enable tracking of the first minutes of clathrin-mediated endocytosis. For these experiments, a cycloheximide pretreatment was included to avoid imaging newly synthesized H-Ras on Golgi-derived vesicles that might mingle with the endocytic vesicles. Cells were then chilled and incubated with transferrin, so as to label, but prevent internalization of, transferrin receptors. Cells were warmed briefly (one minute) to allow internalization to restart. Although H-Ras-positive vesicles were present in the rewarmed cells, the transferrin-containing early endosomes did not overlap with GFP-H-Ras61L (Fig. 1E). Thus, although H-Ras was present on vesicles, it was notably absent from endosomes associated with these characteristic steps of the clathrin-dependent endocytic route.

To supplement the previous imaging results, we used a biochemical approach to examine if H-Ras in its GTP-bound form might be present on EEA1-containing endosomes. Using an antibody to EEA1 and a detergent-free protocol for immuno-isolation of membrane
fragments and vesicles [11], the presence of H-Ras and endogenous Raf-1 on
EEA1-containing endosomes was assessed. In agreement with the imaging results, neither
cellular H-Ras nor activated H-Ras61L (Fig. 1F) could be detected on the captured EEA1-
containing membranes.

Thus, by multiple techniques, we found no evidence of H-Ras on endocytic vesicles
that were derived from the clathrin-dependent pathway.

In spite of this absence, endogenous Raf-1 was easily observed in these EEA1
antibody-captured endosomes (Fig. 1F). This ability to capture a protein that did associate
with EEA1-containing membranes verified that the membrane isolation protocol was
successful, and thus validated the absence of H-Ras on those membranes. This result
confirmed previous work that Raf-1 could be found on EEA1-containing early endosomes
[11]. All these studies indicated that endosomes to which H-Ras was bound had not entered
the cells via a clathrin-mediated pathway.

**Endosomes associated with Arf6 bind H-Ras**

A prominent class of non-clathrin plasma membrane structures is comprised of
caveolae. However, the ability of caveolae to internalize (as “caveosomes”) is generally
thought to be restricted to special situations such as viral infection [39]. H-Ras, although
present on membranes with a low buoyant density similar to that of caveolae, is largely
absent from these structures [15, 37]. Thus another class of non-caveolar,
clathrin-independent endosomes for binding H-Ras was sought.

An additional type of clathrin-independent endosomes is those which arise from the
constitutive pathway by which the endogenous major histocompatibility complex I (MHC-I)
is internalized [31]. To examine this pathway, endosomal vesicles containing MHC-I were
identified by applying an antibody that recognizes an external epitope of human MHC-I to live HeLa cells. In addition, to further diminish the signal from endogenous MHC-I and transfected GFP-H-Ras that were within the biosynthetic pathway, cycloheximide was added to the transfected cells for the final six hours before fixation. Anti-MHC-I antibody was added to the culture medium for the last 30 minutes to label MHC-I at the cell surface. Cells were then briefly washed with mild acid to remove MHC-I antibody that had not entered the cell, were fixed, and then were stained with secondary antibody for the internalized MHC-I antibody [36, 40]. The resulting images showed multiple vesicular structures that held both GFP-H-Ras (Fig. 2A and A”) and endogenous MHC-I protein (Fig. 2A’ and A”), as well as other vesicles that held only a single protein.

MHC-I is reported to traffic through a pathway that is associated with the Arf6 small GTPase [41, 42]. The extent of co-localization of externally labeled MHC-I with an expressed Arf6-Q67L tagged with GFP was therefore examined. Similar to the study with H-Ras and MHC-I, there were multiple vesicles that contained both Arf6-Q67L-GFP (Fig. 2B and B”) and MHC-I (Fig. 2B’ and B”), and others that held only a single protein. These results implied that H-Ras should be present on vesicles on which Arf6 was located. To test this prediction, NIH 3T3 cells were co-transfected with H-Ras-WT and a GFP-tagged version of the cellular form of Arf6 (Arf-WT-GFP), and were examined by fluorescence microscopy. H-Ras-WT (Fig. 2C) was observed on the plasma membrane, as expected, and also on both small endosomes and larger, apparently fluid-filled, pinosomes that were also populated by Arf6-WT-GFP (Fig. 2C’). Arf6 is often associated with pinosome structures [43]. At high resolution (in a separate cell), the Arf6 and H-Ras proteins appeared to inhabit adjacent but distinct domains of many of the larger endosomes, producing a beaded appearance in cross-
section (Fig. 2D). After acute expression, constitutively active H-Ras61L was also present on Arf6-associated endosomes (data not shown). These results agree with the previous finding that endogenous Ras proteins can be found on endosomes regardless of their activation state [11]. Long-term expression of activated H-Ras61L causes oncogenic transformation and also stimulates pinocytosis [10]. To learn if the complex cellular changes that accompany transformation might alter H-Ras61L binding to Arf6-associated endosomes, a cell line that stably expressed H-Ras61L was transfected with cDNA encoding Arf6. In these transformed cells, endosomes marked by the Arf6 protein still carried the oncogenic H-Ras61L (data not shown).

These results indicated that endosomes that contained Arf6 could bind H-Ras in both the GDP- and GTP-bound states. This data provided a complement for the earlier biochemical experiments that indicated that neither H-Ras-WT nor H-Ras61L nor EGF-stimulated H-Ras-GTP bound clathrin-dependent endosomes.

To examine if Arf6 activation changed the ability of these endosomes to bind H-Ras, an Arf6 protein that favors the GTP-bound form (Arf6-Q67L) was used. The Arf6-Q67L protein supports the early steps of endosome formation in a normal fashion, but the inability of the protein to hydrolyze its bound GTP prevents the nascent endosomes from further progress, leading to an endocytic cul-de-sac and formation of multiple, enlarged endosomes [30]. In cells that co-expressed Arf6-Q67L-GFP and H-Ras61L, the distinctive groups of large, Arf6-Q67L-GFP endosomes (Fig. 2E and E”) were brightly outlined by the H-Ras61L protein (Fig. 2E’ and E”). Thus, endosomes in which the Arf6 protein was GTP-bound could also bind H-Ras.
To further characterize these endosomes, cells were transfected with a hemagglutinin (HA)-tagged version of Arf6-Q67L-HA along with GFP-H-Ras61L so that Arf6-containing membranes could be immuno-isolated using HA antibody. On these isolated, detergent-free fragments of Arf6-HA-containing membranes, GFP-H-Ras61L was present (Fig. 3A). A reverse experiment, using H-Ras-directed antibody capture, showed that Arf6-Q67L-GFP could also be detected on H-Ras-containing membrane fragments (Fig. 3B). In addition, a control experiment, in which anti-HA antibody-directed membrane isolation was performed using cells that expressed GFP-H-Ras but that had no Arf6-HA to capture, showed that no GFP-H-Ras was present in the HA-antibody-captured sample (Fig. 3C). This indicated that the presence of H-Ras in the sample required Arf6, and was not the result of incomplete membrane washing or non-specific binding of H-Ras to the HA antibody.

**EGF stimulates GTP binding to H-Ras on Arf6 endosomes**

Given the constitutive nature of endocytosis via the Arf6-associated pathway, an important issue was whether binding to these endosomes would result in rapid down regulation of H-Ras GTP binding. This question had not been answered by the previous experiment with H-Ras61L, because H-Ras61L GTP hydrolysis is impaired. This experiment required a cell to simultaneously express 3 proteins: Arf6, H-Ras-WT, and GFP-RBD (a GFP-tagged version of the Ras binding domain of Raf-1 that would bind to and report the location of the GTP-bound form of H-Ras). To accomplish this with only two color filters, the activated Arf6-Q67L was used so that its presence could be detected by the distinctive clustering of endosomes it produced, rather than by staining. The triply transfected cells were then either left untreated or were exposed to a low concentration of EGF for varying times before fixation and imaging.
In untreated cells, the H-Ras-WT protein, in its basal, GDP-bound state, outlined the plasma membrane and the clustered, Arf6 endosomes (Fig. 4A), as before, while the GFP-RBD was nearby in the cytosol (Fig. 4A`). This is the appropriate cytoplasmic location for GFP-RBD in an unstimulated cell. After five minutes of exposure of the cells to EGF, GFP-RBD (Fig. 4B`) could now be seen both at the plasma membrane and on many H-Ras-WT-coated, enlarged endosomes (Fig. 4B). Notably, within this short time frame, no significant binding of GFP-RBD to perinuclear membranes that might be Golgi was observed [44]. This result indicated that H-Ras-WT could become GTP-bound at the plasma membrane and on Arf6-associated vesicles that were already internal, or on endosomes that had entered the cell during the five minutes of EGF exposure.

Previous studies had shown that total H-Ras GTP binding remains elevated for several minutes after EGF stimulation, and returns to baseline within about one-half hour [12]. Similarly, in this experiment, after 10 minutes of exposure to EGF, GFP-RBD remained visible on both the plasma membrane and on many Arf6-Q67L vesicle membranes (Fig. 4C). Thus, during EGF stimulation, H-Ras continued to associate with, and retained GTP while bound to, Arf6 vesicles. There was no indication that endosomal H-Ras lost GTP more rapidly than H-Ras at the plasma membrane. After 30 minutes of EGF exposure, GFP-RBD binding to the plasma membrane and to Arf6 vesicles, did decline, indicating that H-Ras GTP binding had dropped (data not shown). These results indicated that vesicles of the Arf6-associated endocytic pathway did not initiate rapid down regulation of H-Ras activity.

**Very little Raf-1 binds to H-Ras61L on Arf6 endosomes**

The initial studies described above had indicated that early endosomes that contained EEA1 did not bind H-Ras, but did bind the H-Ras effector protein, Raf-1. The finding that
Arf6-associated vesicles harbored GTP-bound H-Ras raised the question of whether some Raf-1 might also be present on Arf6-coated vesicles. Because Arf6 is present both on the plasma membrane (where Raf-1 is known to associate with active H-Ras) and on endosomal membranes, immunofluorescence was used so that Arf6 endosomes could be examined specifically, rather than immuno-isolation from a mixture of membranes. Cells were co-transfected with DNAs for Arf6-Q67L (to generate visibly identifiable endosomes) and GFP-H-Ras61L, then were stained with antibodies for endogenous Raf-1. As before, the characteristic Arf6-Q67L vesicles and clustered endosomes were brightly rimmed with GFP-H-Ras61L (Fig. 5A and B). However, the Raf-1 antibody did not illuminate those clustered endosomes (Fig. 5A’ and B), and in only a few spots did endogenous Raf-1 and H-Ras61L co-localize on endosomes. As expected, however, some endogenous Raf-1 did co-localize with H-Ras61L at the plasma membrane (Fig. 5C). A second experiment was performed to examine more directly whether the smaller endosomes that displayed transfected Arf6-Q67L-GFP could bind Raf-1. Cells were transfected with cDNAs for H-Ras61L and Arf6-Q67L-GFP, and cells that expressed both proteins were identified (data not shown [see Immunofluorescence Methods]). In these cells, presumptive vesicular forms of endogenous Raf-1 were visible, but very few of the dots of endogenous Raf-1 aligned with dots of the Arf6, or with the clustered, larger, Arf-Q67L-stimulated endosomal structures (Fig. 5D). These results further suggested that Arf6 endosomes bound very little Raf-1 despite harboring an activated H-Ras61L.

**H-Ras is present in the perinuclear endosomal recycling center**

Although some H-Ras is present on Golgi membranes, recent studies have found that H-Ras persists in the perinuclear area despite clearing the H-Ras biosynthetic pathway by
exposing cells to cycloheximide [8, 9]. Our own studies (see below) confirmed that even after prolonged interruption of biosynthesis, H-Ras was present (i.e., either retained or continuously replaced) on perinuclear membranes. One source of this internal H-Ras is from previously synthesized protein that was de-palmitoylated at the plasma membrane and that recycles through the cytoplasm until it is re-palmitoylated and recaptured on endomembranes. The potential for an additional contribution of endosomal pathways, and in particular the perinuclear endosomal recycling center (ERC), to the internal pool of H-Ras was investigated.

In order to specifically distinguish the ERC from Golgi membranes, transferrin, a protein known to enter the ERC area (via the clathrin-mediated route), was used. In cells pretreated with cycloheximide for six hours, live-cell uptake of AlexaFluor-transferrin for 45 minutes at 37°C produced a strong perinuclear accumulation of transferrin in the ERC, along with numerous peripheral transferrin-laden vesicles that were traversing the cytoplasm (Fig. 6A’). In these cycloheximide-treated cells, the previously synthesized H-Ras61L (Fig. 6A) that was located on internal membranes co-localized entirely with the perinuclear concentration of AlexaFluor-transferrin, and not with a marker of the cis-Golgi, giantin (Fig. 6B andC). Transfected H-RasWT also co-aligned well with the transferring-marked ERC (data not shown). This indicated that the internal membranes populated by previously synthesized H-Ras were predominantly those of the ERC, and that, with this protocol, very little H-Ras was located on Golgi membranes. This data supported the recent report that H-Ras is associated with Rab11-positive vesicles in the recycling center [13]. Notably, at the periphery of the cell, the previously synthesized GFP-H-Ras61L was present in small vesicles that were separate from those that contained AlexaFluor-transferrin (Fig. 6D and E).
This corroborated the results shown above indicating that H-Ras61L could bind vesicles that were separate from those that carried transferrin. The ERC region was too densely packed with vesicles to determine if the H-Ras-containing vesicles simply intermingled or actually merged with transferrin-containing vesicles. These results indicated that, in addition to Arf6-associated endosomal membranes and membranes of the Golgi, H-Ras could distribute to a third type of internal membrane—the membranes of the ERC.

**K-Ras4B and Ext-Ras binding to Arf6 endosomes differs from H-Ras**

The route of the outward, biosynthetic trafficking of the non-palmitoylated K-Ras4B protein is unknown, but appears to differ from the vesicular mechanism used occasionally by H-Ras [6]. We therefore examined if these two Ras proteins also differed in their presence on Arf6-associated vesicles or the ERC. An activated GFP-K-Ras4B-12V protein could be clearly seen on the plasma membrane and on Arf6-Q67L-enlarged endosomes (Fig. 7A and A’). This result indicated that Arf6-associated vesicles could also bind activated K-Ras4B, and that this form of Ras was not excluded from this pathway. However, when the ERC was marked, by loading cells with AlexaFluor-transferrin (without Arf6-Q67L), activated K-Ras4B could not be seen in the ERC (Fig. 7B). Thus, K-Ras4B differed from H-Ras and did not bind either ERC or Golgi membranes.

To learn more about the properties that were responsible for the binding of Ras proteins to Arf6 endosomes, an H-Ras protein with C-terminal characteristics of both H-Ras (palmitates) and K-Ras4B (lysine residues) was examined [35]. This protein (Ext-Ras) is a version of H-Ras with a C-terminal extension of six lysines that act to prevent attachment of farnesyl. Because of its lack of farnesylation, Ext-Ras also fails to undergo proteolysis or methylation and does not interact with ER or Golgi membranes where these modifications
take place [35, 45]. Ext-Ras shows no evidence of binding to exocytic vesicles but does associate with the plasma membrane and become palmitoylated [35]. Interestingly, Ext-Ras differed from H-Ras and K-Ras4B, and was not detectable on Arf6-associated endosomes (Fig. 7C and C’). Furthermore, Ext-Ras61L did not co-localize with AlexaFluor-transferrin in the ERC (Fig. 7D). This was an important control because, although endocytosis via Arf6-associated vesicles can occur continuously, binding to these endosomes was neither universal nor inevitable for a palmitate-modified H-Ras protein.

**The C-terminal domain of H-Ras or K-Ras4B proteins can target GFP to the Arf6 endocytic pathway**

Because both GDP-bound and GTP-bound forms of H-Ras were found on Arf6-associated vesicles, it appeared that the Switch I and Switch II regions of the protein (which differ between the GDP vs GTP state) were not likely to be determinants of binding. To study the structural requirements for binding to Arf6 endosomes more directly, a GFP chimera (GFP-H-tail), having only the last ten C-terminal residues of H-Ras, was tested. Identical GFP-H-tail proteins have previously been shown to be correctly modified with farnesyl and palmitoyl lipids, and to be targeted to raft-like areas of the plasma membrane [21]. As they did with the full-length H-Ras protein, Arf6-Q67L endosomes bound this GFP-H-tail protein (Fig. 8A). Arf6-Q67L endosomes also bound a second chimeric version of GFP, GFP-K-tail, with the last twenty residues of K-Ras4B (Fig. 8B). This mirrored the ability of the full-length K-Ras4B protein to bind Arf6-Q67L endosomes. These results indicated that the (farnesylated, fully modified) C-termini of Ras proteins, with either palmitates or multiple lysine residues, were sufficient structurally to support binding to
Arf6-coated endosomes. Thus, ten Ras C-terminal residues (including lipid modifications) were sufficient to direct a protein to Arf6-associated vesicles.

**Discussion**

The studies here provide two notable findings—the absence of H-Ras from clathrin-dependent endosomes, and the identity of a second class of vesicles that do bind H-Ras. Normal, growth factor stimulated, and oncogenic forms of H-Ras bind clathrin-independent endosomes that contain the Arf6 protein. These endosomes are separate from, and in addition to, exocytic vesicles of the biosynthetic pathway. Arf6 endosomes provide a second, membrane-based mechanism for binding of H-Ras to internal membranes that is distinct from the deacylation-controlled cycle between the cell surface, cytosol, and internal membranes. Several other clathrin-independent endocytic routes are rapidly being defined [16, 25, 46, 47]. It will now be important to learn if H-Ras also utilizes these pathways, or only the Arf6-associated endosomes.

Arf6 endosomes bound both inactive and active forms of H-Ras, and thus provide a possible new platform for signaling within the cell. The nucleotide-independence of H-Ras endosome binding is consistent with previous reports [11] and with the constitutive nature of Arf6-mediated endocytosis [26, 48, 49]. Internal relocation of H-Ras via deacylation/re-acylation also operates for both GDP- and GTP-bound forms [8, 9]. Interestingly, palmitate turn-over is accelerated when H-Ras is in the GTP-bound form [37], suggesting that this form of relocation may be subject to control. Although Arf6 endocytosis occurs continuously, it can also be regulated [26, 41, 43]. It remains to be seen how stimulation of Arf6-associated endocytosis affects H-Ras-GTP that may bind those endosomes. Although the plasma membrane is widely viewed as a static, final destination for
Ras proteins, this report adds support to the idea that the amount of Ras at the plasma membrane (not just its activation state) may be dynamic and continuously adjustable, by both deacylation and endocytosis.

These studies also confirm the presence of H-Ras in the perinuclear recycling center [13] and thus reveal an additional challenge for studies that wish to measure H-Ras signaling from internal sites. Because the endocytic recycling center is often embedded within or near the Golgi, it may be necessary to distinguish H-Ras on ERC membranes from newly synthesized or re-acylated H-Ras on Golgi membranes [9].

**Properties of Ras proteins that bind to Arf6-associated vesicles**

Our results show that the seven, C-terminal residues of H-Ras, with the accompanying farnesyl, methyl, and palmitoyl groups, are sufficient to induce endosome binding, just as they also do for association with Golgi membranes [7, 9]. The H-Ras protein that is found on Arf6-associated vesicles is likely to be palmitoylated, as it continues to bind strongly to membranes. However, palmitoylation cannot be the sole determinant for Arf6-based endocytosis, because the Ext-Ras protein is palmitoylated on C-terminal cysteines, and it failed to enter endosomes. Neither is palmitate a requirement, as non-palmitoylated K-Ras4B could also bind.

Because the GFP-H-tail protein resides in low-density (“raft”) membrane microdomains [50], its internalization probably takes place from these domains. The GFP-K-tail protein and the full-length K-Ras4B-G12V protein, as well as GTP-bound forms of H-Ras, which reside outside of the raft-like domains [21] were also observed on Arf6-Q67L endosomes. These results are consistent with reports that Arf6-associated endosomes can internalize proteins from both types of domains [27].
**The role of endosomes in H-Ras signaling**

H-Ras was detected in the vesicle complex that contains insulin receptor, EEA-1 early endosomes, sorting endosomes, and lysosomes in insulin treated cells [57]. Previous study indicated that the cargos that endocytosed through clathrin-independent pathway fused vesicles internalized through clathrin-dependent pathway on sorting endosomes where further sorting the cargos for recycling back to the plasma membrane or to the late endosomes [31].

The separation of H-Ras from the clathrin-dependent pathway has special importance for interactions of H-Ras with its effector, Raf-1. Both in this work and in studies with insulin stimulated- [11] or angiotensin-stimulated [51] cells, activated Raf-1 has been found primarily on EEA1-containing endosomes. The presence of Raf-1 on endosomes derived from the clathrin-dependent pathway is also consistent with the report that H-Ras-stimulated Raf-1 signaling is decreased by a dominant negative dynamin mutant [17]. Combined with the H-Ras results of these studies, these reports suggest that at least a portion of H-Ras-triggered Raf-1 signals may occur on classical early endosomes, without H-Ras being present. This possibility is also consistent with the findings that H-Ras:Raf interactions at the plasma membrane are actually quite brief [52].

In contrast, very little Raf-1 was found on Arf6-associated vesicles that contained the activated form, H-Ras61L. Such a limitation in a major Ras effector pathway suggests that the spectrum of signals arising from endosomal H-Ras may differ from that of H-Ras on the plasma membrane. There is already precedent for selective association of nucleotide exchange factors (GEFs), effectors, and GTPase activating proteins (GAPs) with H-Ras that is present on Golgi membranes [1, 44, 53]. Furthermore, Arf6-associated endosomes also
bind other signaling proteins, such as Src and Rac [27, 41], which could influence or contribute to H-Ras signals that might arise from endosomes.

Ras signaling pathways have previously been linked functionally with Arf6. H-Ras stimulates phosphatidylinositol 3-kinase activity, which leads to recruitment and activation of the Arf6 nucleotide exchange factor, ARNO [31]. Reciprocally, inhibition of the extracellular signal-regulated kinases (ERKs) that are downstream of Ras proteins, specifically impedes Arf6-mediated, but not clathrin-mediated, endocytosis [54]. Arf6 and H-Ras collaborate in vesicular transport, through co-stimulation of phospholipase D activity needed for vesicle budding [55]. Both Arf6 and H-Ras proteins are also central to the pinocytosis and membrane ruffling that are caused by growth factors.

The identification of a specific class of endosomes to which H-Ras can bind connects nicely with the previous studies that H-Ras is found on recycling endosomes [13] and completes a framework for a new cycle of membrane-bound H-Ras from plasma membrane, through internal sites, and back. The studies reported here provide the physical foundation that will allow the reciprocal interactions of Arf6 and H-Ras, and the impact of those interactions on signaling, to be unraveled.

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References


**Note Added in Proof**

While this paper was in preparation, Julie G. Donaldson and colleagues reported that H-Ras enters cells via Arf6 associated endosomes (Porat-Shliom, N., Y. Kloog, and J.G. Donaldson, A Unique Platform for H-Ras Signaling Involving Clathrin-independent Endocytosis. *Mol Biol Cell*, 2007.).

**Figure Legends**

**Fig. 1. H-Ras is absent from early endocytic vesicles of the clathrin-dependent pathway.**

All scale bars in this figure = 5 µm.

(A) Merged images of adherent membrane fragment from sonicated cell expressing H-Ras61L. Cell membrane fragments were fixed, incubated with antibodies to H-Ras (green, arrow) and clathrin heavy chain (red, arrow head) and fluorescent secondary antibodies, and were then examined by indirect immunofluorescence microscopy.

(B) NIH 3T3 cells stably expressing H-RasWT cDNA were fixed and incubated
with antibodies to H-Ras (green, arrows) and EEA1 (red, arrow heads), and detected with AlexaFluor-labeled secondary antibodies. Vertical z-stack images of each color were deconvolved and merged.

(C) Merged images of adherent membrane fragment from sonicated cell expressing H-Ras61L. Cells were fixed and probed directly with antibodies to H-Ras (green, arrow) and EEA1 (red, arrow head), and detected with AlexaFluor-labeled secondary antibodies.

(D) NIH 3T3 cells stably expressing H-RasWT were treated with 10 ng/ml of EGF for 10 minutes before fixation and incubated with antibodies to H-Ras (green, arrows) and EEA1 (red, arrow heads), and detected with AlexaFluor labeled secondary antibodies. Vertical z-stack images of each color were deconvolved then merged.

(E) Merged images of AlexaFluor-transferrin (red, arrowheads) and GFP-H-Ras61L (green, arrows) in a cell transfected and grown for 18 hours, then treated with cycloheximide for 6 hours, chilled and exposed to transferrin, then warmed for 1 minute before fixation. Vertical z-stack images of each color were deconvolved before merging.

(F) Membranes from the post-nuclear supernatant (PNS) in NIH 3T3 cells stably expressing H-Ras-WT or H-Ras 61L were captured with EEA1 antibody (IP). Proteins were separated by SDS-PAGE and identified by immunoblotting with indicated antibodies. 95% of the sample was loaded in the IP lane, and 5% in the PNS lane.
**Fig. 2. H-Ras is present on endosomes that contain MHC-I and Arf6.** All scale bars represent 5 µm.

(A), (A’) and (A’’) HeLa cells were transfected with cDNA for GFP-H-Ras61L, incubated for 18 hours, and then treated with cycloheximide for 6 hours. During the last 30 minutes, anti-MHC-I monoclonal antibody was added to the culture medium. Cells were washed for 30 seconds with mild acid to remove surface-bound antibody, then were fixed, and stained with mouse secondary antibody (red). Vertical z-stack images of each color were deconvolved. The position of several vesicles containing both the internalized MHC-I (arrowheads) and GFP-H-Ras61L (arrows) are indicated. (A) Fluorescence image showing GFP-HRas61L. (A’) Fluorescence image showing MHC-I. (A’’) Merged image of (A) and (A’).

(B), (B’) and (B’’) HeLa cells were transfected with cDNA for Arf6-Q67L-GFP (green), incubated for 18 hours then treated with cycloheximide for 6 hours. During the last 30 minutes, anti-MHC-I was added to the culture medium. Cells were washed briefly with mild acid, fixed, incubated with mouse secondary antibody (red) to detect the anti-MHC-I, and imaged. Vertical z-stack images of each color were deconvolved and merged. (B) Fluorescence image showing Arf6-Q67L-GFP. (B’) Fluorescence image showing MHC-I. (B’’) Merged image of (B) and (B’).

(C) and (C’) Confocal image of transfected H-RasWT (C, red) on endosomes in cell expressing Arf6-WT-GFP (C’, green). Cells were incubated with anti-Ras antibodies and AlexaFluor-labeled secondary antibodies, and then examined by indirect immunofluorescence microscopy.
(D) Enlargement of merged H-Ras (red) and Arf6-WT-GFP (green) images, from another cell, to show the beaded appearance (arrow) of Arf6 and H-Ras on the endosome membrane.

(E), (E’) and (E’”) Confocal images of a cell transfected with cDNAs encoding Arf6-Q67L-GFP (E, green) and activated H-Ras61L (E’, red). (E’’) Merged image of (E) and (E’).

**Fig. 3. H-Ras is present on endosomal membranes that contain Arf6.**

(A) Cells were co-transfected with cDNAs encoding GFP-H-Ras61L and Arf6-Q67L-HA. Membrane fragments from the post-nuclear supernatant (PNS) were captured with anti-HA antibody (IP). Proteins present in the membranes were separated by SDS-PAGE and detected on immunoblots with anti-HA (for Arf6-HA) and anti-GFP (for GFP-H-Ras). Asterisk (*) shows position of antibody heavy chain from the anti-HA antibody used for immunoprecipitation.

(B) Cells stably expressing H-Ras61L were transfected with cDNA encoding Arf6-Q67L-GFP. Membranes from the post-nuclear supernatant (PNS) were captured with anti-Ras antibody (IP). Proteins present in the membranes were separated by SDS-PAGE and detected on immunoblots with anti-Ras and anti-GFP (for Arf-GFP) antibodies.

(C) Anti-HA antibody isolation performed on membrane fragments from the post-nuclear supernatant (PNS) of cells stably expressing GFP-H-Ras61L but no transfected Arf6 (IP). Proteins present in the sample were separated by SDS-PAGE and detected on immunoblots with anti-GFP (for GFP-H-Ras). Asterisk (*) shows
position of antibody heavy chain from the anti-HA antibody used for immunoprecipitation.

Fig. 4. EGF exposure results in GFP-RBD recruitment to enlarged endosomes in cells expressing H-RasWT and Arf6-Q67L. Confocal images of cells transfected with cDNAs encoding Arf6-Q67L-HA (unstained), cellular H-Ras (red), and the Ras-binding domain from Raf kinase (GFP-RBD, green) to detect GTP-bound H-Ras. At 24 hours after transfection, cells were exposed to EGF for the indicated times, fixed, and stained with anti-H-Ras. Arrows indicate H-Ras on plasma membrane or on enlarged endosomes that result from expression of Arf6-Q67L; arrowheads indicate GFP-RBD on plasma membrane or endosomes; chevrons indicate absence of GFP-RBD on membranes. Scale bars = 10 µm.

(A) and (A’) Control cell that was transfected with all three cDNAs, but not untreated with EGF. (A) Fluorescence image showing GFP-RBD. (A’) Fluorescence image showing H-RasWT.

(B) and (B’) Transfected cell treated with 5 ng/ml EGF for 5 minutes. (B) Fluorescence image showing GFP-RBD. (B’) Fluorescence image showing H-RasWT.

(C) Merged image of H-Ras-WT and GFP-RBD in transfected cell treated with 5 ng/ml EGF for 10 minutes. The chevron indicates lack of GFP-RBD on some endosomes after 10 minutes exposure to EGF.

Fig. 5. Very little Raf-1 is present on Arf6-containing endosomes. All scale bars in this figure = 10 µm.

(A) and (A’) NIH 3T3 cells were transfected with cDNAs for GFP-HRas61L (A,
green) and Arf6-Q67L-HA (unstained). Cells were fixed and incubated with anti-Raf-1 antibody, and AlexaFluor-labeled secondary antibody (A’, red). Vertical z-stack images of each color were deconvolved. Arrow indicates an endosome that contains GFP-HRas61L and chevron indicates that absence of endogenous Raf-1 on that endosome.

(B) Enlargement of merged box area of (A) and (A’). Arrows indicates endosomes that contain GFP-HRas61L but lack of Raf-1.

(C) Merged images of H-Ras61L (green) and endogenous Raf-1 (red) in NIH 3T3 cells that were transfected with cDNA of H-Ras61L. Vertical z-stack images of each color were deconvolved. Arrow shows both H-Ras61L and Raf-1 are located on the plasma membrane.

(D) Merged images of Arf6-Q67L-GFP (green) and endogenous Raf-1 (red) in cells that co-express transfected H-Ras61L and Arf6-Q67L-GFP. 24 hours after transfection, cells were fixed, and detected with antibody to Raf-1 and AlexaFluor-labeled secondary antibody. Vertical z-stack images of each color were deconvolved then merged. Arrowhead indicates an endosome with Arf6-Q67L-GFP that lacks Raf-1; chevron indicates Raf-1 on a separate structure that lacks Arf6.

**Fig. 6. H-Ras is present in the perinuclear endosomal recycling center.** All scale bars in this figure = 10 µm.

(A) and (A’) Confocal images of cell transfected with cDNA for H-Ras61L, treated with cycloheximide for 6 hr, exposed to AlexaFluor-transferrin (A, red) for 45 min, then fixed and stained with antibody against H-Ras (A’, green). Arrow indicates perinuclear accumulation of H-Ras61L; arrowhead indicates transferrin.
(B) Merged image of a cell transfected with cDNA for H-Ras61L, treated with cycloheximide for 6 hr, fixed, and stained with antibodies for H-Ras (green) and giantin (red).

(C) Boxed area of (B) is enlarged. Arrow indicates H-Ras61L is accumulated on the perinuclear region that does not contain giantin (arrow head).

(D) Cell treated as in (A) and (A’). AlexaFluor-transferrin is shown in red and H-Ras is shown in red.

(E) Boxed area of (D) is enlarged to show separate vesicles of red (transferrin, arrow head) and green (H-Ras, arrow).

**Fig. 7. Binding of K-Ras4B and Ext-Ras to endosomes and the endosomal recycling center differs from H-Ras.** All scale bars in this figure = 10 µm.

(A) and (A’) Confocal images of cell expressing GFP-K-Ras4B-12V (A, arrow; green) and Arf6-Q67L-HA (A’, arrowhead; red) on enlarged endosomes. Cell was transfected with cDNAs for GFP-K-Ras4B-12V and Arf6-Q67L-HA, fixed, and detected with anti-HA antibody and AlexaFluor-labeled secondary antibodies.

(B) Merged confocal images of a cell expressing GFP-K-Ras4B-12V (green) and exposed to AlexaFluor-transferrin (red) for 45 minutes, then fixed. Chevron indicates perinuclear accumulation of transferrin, but not K-Ras4B in the endosomal recycling center.

(C) and (C’) Confocal images showing Ext-RasWT (C, red) is not present on Arf6-WT-GFP (C’, green) enlarged endosomes (arrowheads; green). Chevrons in left panel indicate absence of Ext-Ras on Arf6-WT-coated endosomes that can be seen in right panel.
(D) Merged confocal images of a cell expressing Ext-Ras61L (green) after exposure to AlexaFluor-transferrin (red) for 45 minutes. Cell then was fixed and detected with antibody to H-Ras and AlexaFluor-labeled secondary antibodies. Chevron indicates perinuclear accumulation of transferrin, but not Ext-Ras61L, in the endosomal recycling center.

Fig. 8. C-terminal residues of H-Ras or K-Ras4B proteins can target GFP to Arf6 endosomes. All scale bars in this figure = 10 µm.

(A) Merged images of a cell expressing GFP-H-tail (arrow; green) and Arf6-Q67L-HA (red).

(B) Merged images of GFP-K-tail (arrow; green) on Arf6-Q67L-HA (red) endosomes. Vertical z-stack images of each color were deconvolved, then merged.
Figures

Fig. 1
Fig. 3

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CHAPTER 3. BIOLOGICAL ACTIVITY AND SIGNALING OF TWO H-RAS VARIANTS DESIGNED TO BE PREFERENTIALLY “IN” OR “OUT” OF RAFTS

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ABSTRACT

H-Ras protein has been shown to be associated with different microdomains of the plasma membrane. However, whether H-Ras activates and signals exclusively from raft or non-raft microdomains on the plasma membrane has not been thoroughly elucidated. Here two H-Ras variants were used: EHR-Ras, a N-terminal IBV E1 (QI) transmembrane domain targeted H-Ras lacking C-terminal cysteine lipidation sites; and Ext-Ras, a palmitoylated H-Ras that lacks a farnesyl group but instead has a C-terminal polybasic region. We found that EHR-Ras accumulated in non-raft microdomains, whereas Ext-Ras was found distributed predominantly in lipid raft containing membranes. Both EHR-Ras61L and Ext-Ras61L can activate most of the major Ras downstream signaling cascades including MAPK, PI3 kinase-AKT, and JNK pathways, though the extent of activation differs from

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that of H-Ras61L. Moreover, both EHR-Ras61L and Ext-Ras61L cause differentiation in PC12 cells and transformation of NIH 3T3 cells. Thus, our results indicate that H-Ras located either in lipid raft and non-raft microdomains can signal and has biological functions, and translocation between non-raft and raft microdomains is not a prerequisite for H-Ras mediated signaling.

**INTRODUCTION**

The biological composition of the plasma membrane is intricate because it is made of lipids that are arranged in distinct microdomains. Broadly, membrane domains can be divided into raft and non-raft microdomains based on their biophysical characteristics and molecular compositions. Raft microdomains are enriched with cholesterol and sphingolipids. They are also highly dynamic and exist in the liquid ordered phase (1–5). In contrast, non-rafts are enriched with unsaturated phospholipids and are less ordered than lipid rafts (6–8). To date, studies have shown that many proteins distribute themselves either in or out of rafts (9, 10). Among these proteins, perhaps the most well known is Ras. Ras is a small GTPase that can only signal when it is lipid modified at its C-terminus. This modification enables Ras to associate with the inner leaflet of the plasma membrane where the majority of Ras mediated signal transduction takes place. Like other GTPases, Ras cycles between its activated, GTP bound, and inactivated, GDP conformation. The microdomain localization of Ras in the plasma membrane is particularly important because the plasma membrane is the major platform from which the Ras signal emanates. While several different isoforms of Ras exist, studies using various methods (e.g., biochemical fractionation assays, electron microscopy studies, and biophysical methods) have shown that H-Ras is associated with raft and non-raft membrane localizations (11–15). However, whether different localizations of
H-Ras on the plasma membrane affect signaling initiation and biological functions has not been thoroughly elucidated. One popular model suggests that GTP activated H-Ras is sequestered on non-raft plasma membranes, while GDP bound H-Ras accumulates in raft microdomains (12, 13). This model indicates that although H-Ras associates with both plasma membrane microdomains, H-Ras only signals from non-raft domains and must shift from lipid rafts to non-rafts in order to activate its downstream signaling cascades. However, other studies have suggested that lipid rafts are also associated with Ras signaling (11, 16–18).

It has been well established that different lipid modifications of Ras proteins and the membrane targeting sequences of Ras can alter its subcellular localization and signaling ability. One H-Ras variant, Ext-Ras, is palmitoylated at its C-terminus, but is not farnesylated. Furthermore, Ext-Ras contains a polylysine sequence, which helps it to associate with the plasma membrane (19). Several studies have shown that the farnesylated peptides partition poorly into rafts, suggesting that farnesylated proteins are sequestered in non-raft membrane microdomains (20–23). Moreover, many acylated proteins were found to interact with membrane rafts (24). Because Ext-Ras lacks farnesyl but is palmitoylated, it may be expected that Ext-Ras will be included in raft microdomains. Previous results (19, 25) showed that the constitutively activated Ext-Ras61L variant has biological functions. Its expression resulted in neurite outgrowth in PC12 cells and transformed NIH 3T3 cells (19, 25). Also, a high level of signal activation was detected in the PI3 kinase pathway in PC12 cells transfected with Ext-Ras61L. These findings suggest that Ext-Ras61L, unlike H-Ras61L, may process signaling activity while associated with lipid rafts. Early reports indicated that Ext-Ras is localized both on the plasma membrane and in the cytosol (25), but
the specific microdomain of the plasma membrane on which it is localized has not been determined.

EHR-Ras is another H-Ras variant. This variant has a transmembrane domain from the E1 (Q1) protein of the infectious bronchitis virus (IBV) in the N-terminus, followed by a H-Ras sequence with the three cysteines mutated to serines in the C-terminus (26). These cysteine mutations inhibit lipid modifications of H-Ras, so that this Ras mutant cannot tether to the plasma membrane through the C-terminus. The transmembrane domain of EHR-Ras has been shown to target H-Ras to the plasma membrane (26, 27). Previous data suggested that single-spanning transmembrane peptides with short transmembrane domains tend to be excluded from the raft domains, while transmembrane peptides with long transmembrane domains are localized to the raft membranes (28, 29). The transmembrane spanning sequence of EHR-Ras (27, 30) is relatively short and its length is close to that of other known non-raft transmembrane peptides (31). Thus it is possible that EHR-Ras might also be targeted to non-raft membranes. Previous results show that the activated form of EHR-Ras, EHR-Ras61L, induced neurite outgrowth in PC12 cells and also caused membrane ruffling in REF-52 cells. This indicates that EHR-Ras61L can produce biological activity within these cells (26). However, the microdomain of the plasma membrane on which EHR-Ras is localized has not yet been identified.

In this study, we investigated whether or not the microdomain localizations impact H-Ras signaling initiation and function. We addressed this by using two H-Ras variants, Ext-Ras and EHR-Ras. We investigated their microdomain localizations and found that Ext-Ras is localized preferentially in the raft microdomains, and EHR-Ras is localized in the non-raft microdomains. Compared to other H-Ras chimeras that were designed to tether to
lipid rafts on the plasma membrane, Ext-Ras shows typical plasma membrane localizations and has a size and membrane anchor position similar to that of H-Ras. Other lipid raft located Ras chimeras, such as H-RasC184S and Raichu-tH, showed abundant distributions on the endomembranes (17, 32) and lipid raft located Ras chimeras LCK-Ras and Mys-Ha-Ras are tethered to the membrane through both their N-terminal and C-terminal membrane anchors (18, 33). We found that the two H-Ras variants that we used can signal and induce biological activity in NIH 3T3 and PC12 cells. These findings emphasize that both raft and non-raft microdomains allow H-Ras signaling and function, and the translocation of H-Ras between the lipid raft and non-raft microdomains is not required for generating H-Ras mediated signal transduction. In addition, these results demonstrated that both Ext-Ras and EHR-Ras are good models for studying raft and non-raft localized Ras.

MATERIALS AND METHODS

DNA constructs

The original DNA constructs of E1 (QI)-22-H-RasWT3S (EHR-RasWT) and E1 (QI)-22-H-Ras 61L3S (EHR-Ras61L) (34) in pcDNA1 vectors (Invitrogen) were obtained from Berthe Willumsen (University of Copenhagen, Denmark). EHR-RasWT was then cut out of pcDNA1 and inserted into pcDNA3 using Hind III and EcoR I. EHR-Ras61L was made using site directed mutagenesis to mutate the endogenous Gln at position 61 into a Leu. The sense oligo used was 5’ CTg gAT ACC gCC ggC CTg gAg gAg TAC AgC gCC 3’ and the antisense oligo used was 5’ggC gCT gTA CTC CTC CAg gCC ggC ggT ATC CAg 3’.

The construction of H-Ras and Ext-Ras DNAs in the pcDNA3 vector has been described previously (19). The DNA LYFP-GPI was obtained from the laboratory of A. Kenworthy (Vanderbilt University).
Cell culture and transfection

NIH 3T3 cells and 293T cells were cultured in a 37°C incubator in 10% CO₂ and placed in Dulbecco’s Modified Eagle’s medium supplemented with 10% calf serum plus penicillin, streptomycin, glutamine, and sodium pyruvate. The PC12 cells were grown in RMPI medium (Gibco) supplemented with 10% heat inactivated horse serum plus 5% fetal bovine serum and incubated in 5% CO₂. Stable NIH 3T3 cell lines were generated by transfection with the appropriate DNA (EHR-Ras61L or EHR-RasWT). G418 disulphate salt solution was added after 24 hours at a concentration of 750 µg/ml. The H-Ras61L stable cell line expressed the H-Ras61L in the pZIPneo vector.

NIH 3T3 cells and 293T cells were transfected with Effectene (Qiagen) or SuperFect (Qiagen) respectively according to the manufacturer’s recommendations and used after 24 hours. The transfection of PC12 cells was performed using Lipofectamine (Invitrogen) with 0.8 µg of DNA per well of a 12-well plate coated with 10 µg/ml laminin in growth medium.

Antibodies

The following antibodies were used for immunoblotting: mouse monoclonal anti-Ras (BD Bioscience 610010), mouse monoclonal anti-Ras 146-3E4 (Quality Biotech), rabbit polyclonal anti-caveolin (Transduction Laboratories C13630), mouse monoclonal anti-phospho-ERK (Thr 202/Tyr 204, Cell Signaling 9106), rabbit polyclonal anti-ERK1 (Santa Cruz Ssc-94), mouse monoclonal anti-phospho-Akt (Ser 473, Cell Signaling 4051), rabbit polyclonal anti-AKT (Cell Signaling 9272), mouse monoclonal anti-phospho-SAPK/JNK (Thr 183/Tyr 185, Cell Signaling 9255), and rabbit monoclonal anti-SAPK/JNK (Cell Signaling 9258). For immunofluorescence, rat monoclonal anti-Ras (sc-34) and rabbit polyclonal anti-c-Raf-1 (sc-227) were obtained from Santa Cruz.
Biotechnology; rabbit polyclonal anti-PI3 kinase p85 (06-195) was obtained from Upstate Biotechnology. Goat anti-rat IgG coupled to Alexa Fluor 488, goat anti-rabbit IgG coupled with Alexa Fluor 594, and donkey anti-rat IgG coupled with Alexa Fluor 594 were obtained from Molecular Probes and were used as secondary antibodies.

**Immunofluorescence**

All cells were plated on coverslips coated with poly-L-lysine (100 µg/ml) and fibronectin (500 µg/ml) in PBS. Cells were washed with PBS and then fixed with 4% paraformaldehyde for 10 minutes at room temperature. Cells were then permeabilized with -20°C methanol and quenched with 50 mM ammonium chloride in phosphate-buffered saline. The fixed cells were incubated with a blocking buffer for 30 minutes (2% horse serum and 0.4% bovine serum albumin in phosphate-buffered saline, pH 7.4) before undergoing a one-hour incubation with a blocking buffer containing the primary antibody. Secondary antibodies were also diluted (1:700) in blocking buffer for one hour. The experiments to produce adherent plasma membrane fragments were carried out exactly as described previously (14). After rinsing, the cover slips were mounted in Vectashield mounting medium (Vector Laboratories).

For photographing adherent membrane fragments, cells were examined using a 60x oil immersion Plan Apo lens or a 100x oil immersion Plan Fluor lens with an aperture adjustable from 1.3 to 0.6 on a Nikon Eclipse E800 microscope. The images were captured by a Spot RT digital camera (Roche Diagnostics) in color or in black and white, imported and overlaid in Adobe Photoshop 7.0. Other images were obtained with a Leica DMIRE2 microscope using a 100x oil immersion PL Fluotar lens with an aperture adjustable from 1.3 to 0.6 and a Q Imaging Retiga 1300 camera. All the images using the Leica DMIRE2
microscope were obtained in black and white, processed in Openlab3.5.1, converted to color, and then exported to Adobe Photoshop 7.0 to equalize image intensity prior to merging. For some experiments, as indicated in the figure legends, a series of z-stack images were acquired using a motorized stage on the Leica DMIRE2 microscope. These images were processed using Openlab’s Nearest Neighbor Deconvolution module and exported as above.

**Cell lysis**

NIH 3T3 cells were cultured in 100 mm tissue culture plates. Cells were lysed with 1.0 ml of lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% NP-40, and 1 mM Na$_3$VO$_4$, 1% protease inhibitor cocktail [Sigma]) on ice for 30 minutes. The lysates were then spun at 10,000 x g for 10 minutes, and the supernatant was collected. The 6x SDS-PAGE loading buffer (375 mM Tris-HCl, pH 6.8, 6% glycerol, 12% SDS, 15% β-mercaptoethanol, 0.15% bromophenol blue) was then added to the collected supernatant. Samples were boiled for five minutes, and proteins were separated by SDS-PAGE.

**Subcellular fractionation and density gradient membrane flotation**

Cells were lysed in hypotonic buffer (1 mM Tris, pH 7.4, 1 mM MgCl$_2$ in water) containing protease inhibitors and broken up with a 26-gauge needle. The lysates were then spun at 1300 x g for 10 minutes at 4°C to remove the nuclei. 5 M NaCl was added to the supernatant to a final concentration of 150mM. The supernatant was then spun at 100,000 x g for 30 minutes to separate the cytosolic (S100) and membrane fractions. The pellets (membrane bound fractions, P100) were resuspended in 0.2% Triton X-100 or 1% Triton X-100 on ice for 10 minutes. They were then spun again at 100,000 x g, and the detergent soluble (DS) and detergent resistant membrane (DRM) fractions were collected.
For a density gradient analysis, the membrane pellet (P100) was resuspended in Optiprep™ (60% [w/v] solution of iodixanol in water, density = 1.32g/ml, Sigma D-1556) to produce a final concentration of 40% Optiprep™ and then sonicated. Triton X-100 was added to sonicated samples at a final concentration of 0.2% or 1% for 30 minutes on ice. The samples containing Triton were loaded at the bottom of the centrifuge tube. Decreasing concentrations (from 37.5% to 20%) of Optiprep™ buffer, with no Triton, were then added carefully to the top of the Triton containing samples. The gradient was then centrifuged at 4°C for at least 16 hours at 100,000 x g. Samples were collected from each fraction from the top of the centrifuge tube, precipitated with trichloroacetic acid, and separated with SDS-PAGE.

**Western blots**

Cell lysates, subcellular fractions and Optiprep™ gradient samples were separated by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Pall Life Science) and then incubated in blocking buffer (Vector Labs 10x casein diluted to 1x) in TTBS (0.15 M NaCl, 0.05 M Tris-HCl, 0.05% Tween-20, pH 7.4) for 20 minutes at room temperature. PVDF membranes were washed in TTBS and incubated with primary antibodies overnight at 4°C. The proteins of interest were detected using horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies and the enhanced chemiluminescence substrates according to the manufacturer’s protocol (Pierce). X-ray films of Western blots were scanned with an HP 2300 series scanner/printer and the densitometry was quantified by ImageJ software (NIH).
**Detection of GM1**

Optiprep™ gradient samples were transferred to nitrocellulose membranes and then incubated in blocking buffer (Vector Labs 10× casein diluted to 1×) in TTBS (0.15 M NaCl, 0.05 M Tris-HCl, 0.05% Tween-20, pH 7.4) for 10 minutes at 4°C. Nitrocellulose membranes were then washed with TTBS. The gangliosides GM1 were detected with horseradish peroxidase-conjugated cholera toxin β subunit (Sigma C-4672, 1:10,000 dilution) and the enhanced chemiluminescence as described above for Western blot.

**Soft agar assay**

A bottom layer of agar media (1 portion 1.8% agar, 2 portions 2× NIH 3T3 media, see “Cell culture”) was plated in a 60 mm tissue culture dish and left to set overnight in the 37°C CO₂ incubator to keep the pH from becoming basic. Then NIH 3T3 cells were suspended as single cells in normal media (50,000 cells/ml), and 300µl of cells (15,000 cells) were added to 1 ml of agar media and carefully added to the base layer. All cell lines used stably expressed the desired protein. Colony formation was counted on the 14th day after being plated, and any clusters having two or more cells were counted as colony growth. As a control, the stable cell lines were also examined by immunofluorescence and stained with DAPI, and the percentage of cells expressing Ras proteins was calculated.

**Focus assay**

Cells were transfected with DNA encoding EHR-Ras61L, H-Ras61L, or EHR-RasWT. The amounts of DNA were tested at 3 ng and 30 ng/plate for H-Ras61L; 10 ng, 50 ng, and 100 ng/plate for EHR-Ras61L; 500 ng/plate for EHR-Ras61L. 12–17 days after transfection, plates were stained with crystal violet for visualization, and the number of foci was quantified.
RESULTS

Description of the different H-Ras variants

H-Ras accumulates in different membrane microdomains. It was our purpose to examine how the locations of H-Ras at different membrane sites of the plasma membrane affect signaling and biological functions. To do this, two DNA constructs were used, EHR-Ras and Ext-Ras; these were expected to tether to different microdomains of the membrane, non-rafts and rafts. Both the EHR-Ras and Ext-Ras maintain the effector binding regions of the H-Ras and preserve the GTP-binding properties of Ras (19, 26).

The logic in designing the EHR-Ras protein is as follows: For the EHR-Ras protein, a 3S (SMSSKSVLS in the C-terminus) mutated H-Ras (FIG. 1) was fused with the N-terminal E1 (QI, Gln37 → Ile) mutated transmembrane target signal of the infectious bronchitis virus (IBV) (30). The C-terminus of the 3S H-Ras does not associate with the plasma membrane (PM) because the 3S mutation eliminates H-Ras lipidation. However, the N-terminal E1 (QI) domain has been shown to effectively target proteins to the plasma membrane (26, 27, 30). In addition, the transmembrane portion of EHR-Ras will cause the protein to transit the Golgi and undergo exocytic transport to the plasma membrane (26). The 3S-mutated Ras portion of EHR-Ras is always located in the cytosol (FIG. 1). Between the N-terminal transmembrane domain and the 3S-mutated H-Ras is a 22 amino acid linker region (FIG. 1). This linker region functions as a spacer between the membrane targeting domain and the conserved domain (26, 27). The single transmembrane spanning sequence of the E1 (QI) domain is short, and we hypothesized that this sequence will cause this chimeric protein to localize to the non-raft microdomain of the plasma membrane (28, 29, 35).
We used the H-Ras variant Ext-Ras as a version of H-Ras that might be targeted to rafts. Compared to the normal H-Ras, the ‘X’ residue of CAAX motif in Ext-Ras was replaced with six lysine residues. To ensure association with membranes, six lysine residues were engineered in Ext-Ras to produce an ionic interaction with the plasma membrane (FIG. 1). Thus, Ext-Ras is not farnesylated, but can be palmitoylated due to the maintenance of the cysteine residues in the C-terminus (19). Ext-Ras was found to be distributed widely on the plasma membrane and, importantly, was absent from internal membranes (19, 25). Since palmitoylated proteins prefer to partition into raft domains (23), we expected that Ext-Ras would tether to the lipid raft regions of the plasma membrane. To ensure association with membranes, six lysine residues were engineered in Ext-Ras to produce an ionic interaction with the plasma membrane (FIG. 1).

**Expression of EHR-Ras and Ext-Ras in NIH 3T3 cells**

In order to check whether EHR-Ras and Ext-Ras were targeted as anticipated to either raft or non-raft microdomains, we first determined whether these proteins were expressed in the cells and localized to the plasma membrane. The DNAs of the EHR-Ras and Ext-Ras constructs were transfected into the NIH 3T3 cells, and the distributions of these Ras variants within the cells were detected by immunofluorescence with an anti-H-Ras antibody. EHR-RasWT was shown to be present on the plasma membrane (FIG. 2A). We also found some Ras staining on the ER and Golgi in addition to the plasma membrane staining in some other cells, as expected from the presence of a transmembrane domain that can direct transport through the conventional exocytic pathway (data not shown). EHR-Ras61L, the constitutively active form of EHR-Ras, is localized in the NIH 3T3 cells in a similar fashion as EHR-RasWT (data not shown). In order to monitor the locations of EHR-Ras on the native
plasma membranes, we adopted a technique in which the top layer of the cells was removed by sonication without any detergent or solvent treatment. Cells were grown on coverslips and then removed by sonication, leaving behind membrane fragments adhered to the coverslips. The localizations of Ras on the remaining single layer PM were detected by immunofluorescence. Using this technique, EHR-Ras61L localization on the native PM could be clearly seen (FIG. 2B).

Ext-Ras protein was localized to the PM, producing an outline of the cells as shown by the immunofluorescent image in FIG. 2C. A noticeable portion of Ext-Ras remained cytosolic as anticipated (19). We also found that the cellular localization of the GTP bound Ext-Ras, Ext-Ras61L, was similar to that of Ext-RasWT (data not shown). Localization of Ext-Ras on the PM was clearly observed in adherent membrane fragments (FIG. 2D). Plasma membrane association was also confirmed in 293T cells transfected with the EHR-Ras and Ext-Ras DNAs, with staining patterns identical to those observed for NIH 3T3 cells (data not shown). Overall, these results demonstrated that we have successfully expressed EHR-Ras and Ext-Ras in NIH 3T3 cells, and a substantial amount of each protein is localized to the plasma membrane.

**EHR-Ras associates with detergent sensitive membrane fractions whereas Ext-Ras preferentially associates with detergent resistant membrane fractions**

One characteristic of lipid rafts is that they are not fully solubilized by non-ionic detergents such as Triton X-100 at low temperatures; therefore, they are also called detergent resistant domains or DRMs. As one way to determine whether EHR-Ras or Ext-Ras was localized to lipid raft microdomains, we tested whether they were present in DRM or non-DRM membrane fractions. For this assay, cellular membranes (P100) from NIH 3T3
cells that stably expressed EHR-Ras61L or GFP-H-Ras 61L (positive control) were separated from the cytosol (S) by centrifugation and then exposed to different concentrations of cold Triton X-100 detergent to assess detergent resistance (lipid raft association). The membrane bound fraction (P100) was treated with 0.2% Triton X-100 on ice for 10 minutes to solubilize non-raft membranes, and then another high-speed spin was performed to separate insoluble components (raft domains) from the solubilized material. The detergent soluble membrane fractions (DS) and detergent resistant membrane fractions (DRM) were collected, and the Ras protein content in each fraction was examined by immunoblotting. Western blots of these fractions (FIG. 3A) clearly indicated that almost all EHR-Ras61L was associated with the detergent soluble membrane fractions (DS), and so was the non-raft localized GFP-H-Ras 61L, as anticipated. As expected, this fraction also contained a non-raft associated protein marker, the transferrin receptor. In contrast, the detergent resistant membrane fractions (DRM) contained most of the caveolin-1, a known protein marker for DRMs (FIG. 3A). The results showing the presence of GFP-H-Ras61L on the detergent soluble membrane fractions are in agreement with the current understanding of H-Ras61L localization on the non-raft membranes (12). The EHR-Ras61L protein was observed as multiple bands in the Western blot due to its multiple glycosylation states in the E1 (QI) extracellular region. A similar experiment with NIH 3T3 cells expressing EHR-RasWT showed that this protein was also entirely extracted into the detergent soluble fraction by treating the membranes with 0.2% Triton X-100 (data not shown).

To determine the membrane fractions that contain Ext-Ras, similar experiments were conducted in NIH 3T3 cells that were transfected with Ext-Ras61L. Cell lysates were prepared under the treatment of 0.2% ice cold Triton X-100 for 10 minutes, and then
centrifuged to separate the cytosolic/detergent soluble fraction from the pellets (0.2% Triton X-100 resistant fraction). The Western blot of these fractions showed that a considerable amount of Ext-Ras existed in the 0.2% Triton resistant fraction, although a portion of Ext-Ras was also present on the cytosolic/detergent soluble fraction as expected (data not shown) (19). In order to test whether the membrane domains that Ext-Ras associated with are resistant to a higher concentration of detergent and whether this is characteristic of other cell types, we conducted similar experiments in Cos-1 cells that were transfected with Ext-Ras61L or H-Ras61L (positive control) DNA. Cell lysates were centrifuged to separate the cytosolic fraction (S) from the membrane bound fraction. This membrane bound fraction (P100) was then treated with 1% Triton X-100 on ice for 10 minutes to solubilize non-DRM membranes (DS) and separate them from those that were detergent resistant (DRM). Another high-speed centrifugation was performed, and the DS and DRM fractions were collected. A Western blot of the samples clearly indicated that H-Ras61L had been completely dissolved in the 1% Triton X-100 (DS), whereas a portion of the Ext-Ras61L remained associated with the detergent resistant fractions (DRM, FIG. 4B). A considerable amount of Ext-Ras and H-Ras proteins was found in cytosolic fractions (S). This probably represents immature Ext-Ras and H-Ras proteins that lack lipid modifications and thus are not associated with the plasma membrane (19).

**EHR-Ras associates with non-raft membrane fractions whereas Ext-Ras associates with raft membrane fractions in density gradients**

As an additional test to determine how EHR-Ras and Ext-Ras proteins partition in membranes, density gradient with ultracentrifugation was used to isolate membrane fractions based on the buoyancy and detergent insolubility. Previously prepared membrane samples
(P100) were exposed to an ice-cold Triton X-100 extraction protocol before the gradient separation. The gradient ranged from 20–37.5% Optiprep™ with five intermediate progressions to allow for a well-defined separation between raft and non-raft associated proteins due to their buoyancy (raft) or lack thereof (non-raft). The markers caveolin-1 and ganglioside GM1 were used to monitor the localization of DRMs in the gradients.

In the gradients where membranes were pretreated with 0.2% Triton X-100, both caveolin and GM1, which remain in lipid raft containing fractions, were found in the least dense regions of the gradient (FIG. 4A). More than 70% of H-Ras61L was identified in the bottom fractions. About 80% EHR-RasWT was also identified in the more dense, non-raft fractions (FIG. 4A). When a similar experiment was performed in NIH 3T3 cells, EHR-Ras61L again was associated with non-raft fractions (data not shown). However, about 60% of Ext-Ras61L was detected in the more buoyant, lipid raft containing fractions (FIG. 4A).

For the gradients where membranes were pretreated with a higher concentration of detergent, 1% Triton X-100, about 90% of H-Ras61L was observed in the densest fractions, whereas 30% of Ext-Ras61L still migrated to the lighter, buoyant membrane fractions (FIG. 4B). The 1% Triton X-100 also extracted some of the caveolin to more dense fractions.

Taken together, these subcellular fractionation assays and density gradient experiments showed that the distribution of EHR-Ras and Ext-Ras at the cell membranes differ noticeably. EHR-Ras is distributed in the non-DRM, whereas Ext-Ras is distributed on the membrane fractions that are less sensitive to the detergent treatment (DRM), which likely represents the lipid rafts.
Ext-Ras binds tightly to detergent resistant microdomains, while EHR-Ras is easily extracted upon detergent treatment in native plasma membranes

From the previous subcellular fractionation assays of cell homogenates, we found that EHR-Ras was distributed on the detergent soluble membrane fractions (non-DRM), while the majority of the Ext-Ras was found in detergent resistant fractions (DRM). To determine how strongly these Ras variants bind to these microdomains in native cellular membranes, we examined their sensitivity to detergent extraction in the plasma membranes of intact cells.

NIH 3T3 cells co-expressing a glycosylphosphatidylinositol anchored YFP protein (LYFP-GPI), a lipid raft maker, along with EHR-Ras61L or EHR-RasWT, were treated with Triton X-100 before fixation and immunofluorescence. Treatment of membranes with ice cold 0.2% Triton X-100 does not dissociate the lipid rafts but solubilizes the rest of the membranes (non-rafts). Both EHR-Ras61L and EHR-RasWT were almost entirely dislodged from the membranes treated with 0.2% Triton X-100, while LYFP-GPI, a marker indicating the lipid raft regions of the cell, was retained (FIG. 5A). Similar results were obtained when 293T cells expressing EHR-Ras and LYFP-GPI were treated with either 0.2% or 1% Triton X-100 (data not shown). EHR-Ras proteins were completely extracted while LYFP-GPI was retained.

To test how strongly Ext-Ras binds to lipid rafts, NIH 3T3 cells were transfected with the Ext-Ras61L construct and cultured on coverslips. When the cells were treated with ice cold 0.2% Triton X-100 as described above for EHR-Ras61L, most of the cell body remained intact (data not shown). In order to see more clearly how strongly Ext-Ras binds to lipid rafts, in the following experiment on native plasma membranes, the cell body was removed by sonication, leaving adherent membrane fragments that were directly treated with ice cold
0.2% Triton X-100 or control buffer for five minutes and then fixed. The membrane fragments were labeled with antibodies to H-Ras and examined by immunofluorescence. This enabled us to detect the Ras proteins on the single layer of the plasma membrane in situ. After treatment of the sonicated membranes with 0.2% Triton X-100, much of the Ext-Ras61L remained undissolved (FIG. 5B). Even after treatment with a higher concentration (1%) of TX-100, Ext-Ras61L remained associated with the membrane fragments (FIG. 5B). Similar results were also found for NIH 3T3 cells expressing Ext-RasWT that were treated with 1% TX-100 (data not shown). In the control experiments for NIH 3T3 cells transfected with H-Ras61L (FIG. 5B), the H-Ras61L protein was partially extracted when membrane fragments were treated with 0.2% TX-100 and was completely dislodged under the treatment of 1% TX-100.

These experiments demonstrated that both the activated and inactivated forms of Ext-Ras associate tightly to the detergent resistant microdomain of the plasma membrane, whereas EHR-Ras localizes to a plasma membrane microdomain that is sensitive to the detergent. These results also support the idea that EHR-Ras is localized to non-raft microdomains, whereas Ext-Ras preferentially accumulates in lipid rafts of the native cellular membrane.

**Ext-Ras is not co-localized with caveolin-1**

Caveolae, a specialized type of lipid rafts, are small invaginations of the PM that are characterized by the presence of the protein caveolin-1 (36). Reports showed that H-Ras61L, which was localized to the non-DRM fractions of density gradients, was poorly co-aligned with caveolin-1 on adherent plasma membrane fragments (14). The previous experiments suggested that Ext-Ras is distributed on the DRMs. It would be of additional interest to know
on which subset of detergent resistant microdomains, caveolae or non-caveolae, Ext-Ras resides.

In order to document the native microdomain localizations of Ext-Ras clearly, we also used the sonication technique described above. Antibody staining of endogenous caveolin-1 and Ext-Ras61L showed numerous small dots on the membrane fragments, but there was no overlap between the Ext-Ras61L and caveolin-1 (FIG. 6 column 4), except on the edges of the membrane patch (FIG. 6 column 3). This overlap on the edges is attributed to a fold-back of the membrane fragment. In similar experiments with NIH 3T3 cells transfected with Ext-RasWT, Ext-Ras also did not co-align with the caveolin-1 on the plasma membrane fragments (data not shown). These results show that Ext-Ras is located on the non-caveolae microdomains of lipid rafts on the plasma membrane, which is the same as that for H-RasWT (14).

**EHR-Ras61L recruits Raf-1 to the plasma membrane while Ext-Ras61L is less efficient**

It has been previously demonstrated that oncogenic H-Ras has the ability to engage Ras effectors on the plasma membrane. We were interested in determining whether the EHR-Ras or Ext-Ras variants recruit Ras effectors to the plasma membrane, and if so, whether they recruit the same effectors that H-Ras recruits.

As a first approach to determine whether EHR-Ras61L and Ext-Ras61L recruit the downstream effector, Raf-1, to the plasma membrane from their different submembrane localizations, we utilized whole cell immunofluorescence to study the cells that were transfected with these Ras constructs.

In the cells expressing EHR-Ras61L proteins, both EHR-Ras61L and Raf-1 were evident on the plasma membrane (FIG. 7A, column 1). Raf-1 was also located on the
ER/Golgi membranes as well, where EHR-Ras was present. This result indicates that activated EHR-Ras has the ability to recruit Raf-1 on cellular membranes. In the untransfected cells, however, Raf-1 was not recruited to the plasma membrane or endomembrane, as expected (data not shown).

However, in a similar double-labeled immunofluorescence experiment with cells expressing Ext-Ras61L, little association of Raf-1 with the PM was seen (data not shown). There was particular difficulty in detecting Raf-1 at the plasma membrane in cells expressing Ext-Ras61L due to the significant amount of Ext-Ras61L that was present in the cytosol that could also bind Raf-1.

To visualize Ext-Ras61L and effectors that it might recruit to the plasma membrane more clearly, we attempted to utilize the adherent membrane fragment technique to monitor effector recruitment and determine whether the effectors Raf-1 and PI3-kinase co-localize with Ras proteins on native (without detergent treatment) plasma membranes. We first conducted a control experiment with H-Ras to test the validity of this technique for the study of effector recruitments. NIH 3T3 cells were transfected with H-RasWT and GFP-RBD (GFP tagged Ras-binding domain of Raf-1) and serum starved overnight before some of the cells were treated with EGF. Both the treated and non-treated cells were sonicated, washed, and fixed. The adherent cell membranes were stained with anti-Ras antibody. FIG. 7B (0 min) shows that before EGF treatment, though H-Ras was easily observed on the plasma membrane, little GFP-RBD was present. However, after the cells were treated with 10ng/ml EGF, GFP-RBD was clearly seen on the PM. These experiments recapitulate the current opinion (37, 38) that activated Ras (GTP bound) can recruit Raf via RBD to the plasma membrane. The ability of the sonicated membranes to retain Ras in a GTP-bound form that
was capable of associating with a Ras-binding domain of an effector led us to further utilize this technique in testing the recruitment of the full-length, endogenous effectors by Ras variants on the plasma membrane.

We therefore applied the adherent membrane fragment technique to the cells expressing Ext-Ras61L, H-Ras61L or H-RasWT respectively. As observed from the immunofluorescence of the cells, although the staining of the Ext-Ras61L was very intense, on adherent membrane fragments from cells expressing Ext-Ras61L, little Raf-1 was observed (FIG. 7A, column 2). In parallel, cells expressing H-Ras61L (FIG. 7A, columns 3) and H-RasWT (FIG. 7A, columns 4) were probed for recruitment of Raf-1 to the plasma membrane. On adherent membrane fragments, numerous green fluorescent patches were visible, marking the presence of H-RasWT or H-Ras61L. Punctuated staining of endogenous Raf-1 (red) on the PM was clearly seen in the cells that expressed the constitutively GTP bound H-Ras61L, and many of these dots co-localized with that of H-Ras61L. In contrast, few areas of Raf-1 staining were observed for membrane fragments from cells that expressed H-RasWT. This indicated that the translocation of Raf-1 to the PM is increased when H-Ras is activated. The staining for the endogenous Raf-1 in the cells expressing Ext-Ras61L gave a very different result from that of H-Ras61L; Raf-1 was poorly translocated to the plasma membrane in cells that expressed GTP bound Ext-Ras. Overall, these observations show that Raf-1 engages with activated EHR-Ras, which resides in non-raft microdomains of the plasma membrane, but does not engage as well with activated Ext-Ras that is accumulated on the lipid rafts.
Both EHR-Ras61L and Ext-Ras61L showed co-localization with p85 on the PM

We then examined the recruitment of another H-Ras effector, PI3 kinase. GTP-bound H-Ras can bind to the p110 α-catalytic subunit of PI3 kinase. We were interested to know whether the differentially targeted EHR-Ras61L and Ext-Ras61L proteins recruit PI3 kinase in similar or different manners. To determine how much PI3 kinase is present on the native PM and whether it co-localizes with these Ras variants, we again used the adherent membrane fragment technique. Antibody to the p85 regulatory subunit of PI3-kinase was used to detect the location of the PI3-kinase. In the NIH 3T3 cells expressing EHR-Ras61L, the non-raft localized Ras variant, a little p85 was present on the PM (FIG. 8A, column 1). For plasma membranes of cells expressing Ext-Ras61L, some p85 was present on the PM (FIG. 8A column 2). As a control experiment, membrane fragments from cells expressing H-Ras61L were also probed for the presence of p85 (FIG. 8A, column 3). Both H-Ras61L and p85 were present on the PM with many sites of co-localization observed.

In order to quantify the degree of co-localization between p85 and Ras variants such as EHR-Ras61L, Ext-Ras61L, and H-Ras61L, single layers of the plasma membranes that adhered to the coverslips were examined. The center of each membrane fragment was studied in order to exclude rolled or doubled over membranes. Digital thresholding was applied manually to the fluorescent images that revealed the p85 and H-Ras proteins of the cells. The images were further analyzed by integrated morphometry analysis (IMA) using MetaMorph™ 7.1.3 software (Molecular Devices, Downington, PA). Briefly, these methods enable the regions of interest to be isolated into closed regions based on the gray levels of each pixel, and false positives are then filtered out by the chosen threshold parameters (39–41). After that, the co-alignment of the pixels between each pair (p85 and H-Ras) of the
images was quantified by the "Measure Colocalization" subroutine in Metamorph™. A control experiment was also set up to test the threshold adjustment efficiency. Caveolin-1 on membrane fragments was labeled using both rabbit and mouse primary antibodies and detected with rabbit-specific and mouse-specific secondary antibody fluorescence probes (green and red). We adjusted the threshold in these control images so that the fluorescent signals that showed good intensity and relatively clear borders were selected and used while the background noise was filtered out. The co-alignment of the paired thresholded images that represented the green and red fluorescent signals from caveolin-1 was quantified. A 95% co-alignment was observed (data not shown). This number is very close to the 100% theoretical co-alignment and thus ensures that the threshold was adjusted effectively. The threshold was adjusted in a similar manner when measuring the co-alignment of the images produced from different Ras variants with staining that represented p85 and H-Ras proteins.

Co-alignment for p85 and EHR-Ras61L was about 7%; for p85 and Ext-Ras61L co-alignment was about 15%; for p85 and H-Ras61L co-alignment was about 25%. These data suggest that, although Ext-Ras61L and EHR-Ras61L recruit the p85-PI3 kinase effector to the plasma membrane, the amount of co-localization was lower for the H-Ras61L.

**Biochemical signaling of EHR-Ras61L and Ext-Ras61L**

Because EHR-Ras61L and Ext-Ras61L co-localize with and recruit Raf-1 and p85-PI3 kinase to the plasma membrane, we wanted to know whether these two Ras variants also trigger events downstream of these effectors in their associated signaling pathways. Raf-1 signals via the MAPK pathway, and its activity results in the activation of ERK1/2 observed by phosphorylation. Activation of PI3 kinase leads to phosphatidylinositol (3,4,5)-trisphosphate (PtdIns [3,4,5] P₃) production, which results in phosphorylation and
activation of AKT(42). In the JNK pathway, the activation of Ras can lead to the phosphorylation of c-Jun NH2 terminal kinases (JNK1 and JNK2) through the Ras-MEKK-JNK pathway (43).

To address the question of pathway activation, we utilized NIH 3T3 cells that stably expressed either EHR-Ras61L or Ext-Ras61L DNA and detected the activation of Ras downstream pathways by immunoblotting of cell lysates. Experimental results were compared to those of the control cells stably transfected with H-Ras61L. The relative expression level of each target protein was determined by Western blots with the anti-H-Ras antibody. We found that Ext-Ras61L had an expression level similar to that of H-Ras61L, while the EHR-Ras expression level was three to four times higher than that of H-Ras61L (FIG. 9A). EHR-Ras showed multiple bands due to its multiple glycosylation states.

We then examined whether EHR-Ras61L or Ext-Ras61L activates the ERK pathway using Western blots to detect ERK1/2 phosphorylation. As shown in FIG. 9B, cells expressing the constitutively activated EHR-Ras61L showed a high level of phosphorylated ERK1/2, but less than that of the control, H-Ras61L expressing cells. A low ERK1/2 activation was found in the stably transfected Ext-Ras61L cell line. This finding is in agreement with our previous work that showed low activation of the Raf/MEK/ERK pathway by Ext-Ras61L in PC12 cells (19).

In examining AKT pathway activation, results in FIG. 9C demonstrated that cells expressing Ext-Ras61L effectively activated AKT, and cells expressing EHR-Ras61L showed a weak AKT activation. For the control, a two-fold higher level of AKT activation was achieved in cells stably expressing H-Ras61L in comparison to cells expressing Ext-Ras61L.
The JNK pathway can also be activated by H-Ras in NIH 3T3 cells, although JNK phosphorylation can occur independently of Ras (44, 45). For Ext-Ras61L and EHR-Ras61L expressing cell lines, a considerable level of JNK activation was observed, with higher phosphorylation in Ext-Ras61L compared with EHR-Ras61L cell lines (FIG. 9D). Furthermore, activation of JNK in Ext-Ras61L cells was similar to that found for H-Ras61L cells. Though the signal intensities generated by these H-Ras variants, pERK, pAKT, and pJNK, were noticeably different, these findings do in fact demonstrate that both EHR-Ras61L and Ext-Ras61L variants, in their corresponding non-raft or raft microdomains, produce signals.

**Biological activity of EHR-Ras61L and Ext-Ras61L as seen in PC12 cells**

We examined whether the EHR-Ras61L and Ext-Ras61L variants that have the ability to activate downstream signaling pathways initiate a biological response. Activated H-Ras is known to cause several changes in PC12 cells including cessation of cell growth, rearrangement of the actin cytoskeleton, and cell differentiation as evidenced by neurite extension (46), whereas the non-activated H-Ras does not initiate these changes. We therefore tested the biological activity of EHR-Ras61L and Ext-Ras61L protein variants in PC12 cells by examining the morphology of the cells. For comparison, PC12 cells were stimulated with nerve growth factor (NGF) or transfected with H-Ras61L. Neurite-like extensions of the cell body were observed upon transfection with H-Ras61L. These were similar in shape and size to those caused by treatment with NGF (FIG. 10).

After transfecting cells with EHR-Ras61L, membrane extensions could be clearly seen within 72 hours (FIG. 10). These neurite extensions were different from those resulting from NGF treatment or transfection with H-Ras61L. EHR-Ras61L produced multiple neurite
outgrowths, and the cell bodies were very flattened. For cells transfected with Ext-Ras61L, a very different phenotype from H-Ras61L was observed. The cell bodies were broader, and the edges were ruffled with large lamellipodia. Cells that expressed Ext-Ras61L often generated 4–5 neurite extensions per cell (19). In addition, the neurites showed dramatically more kinks. Moreover, the time for generating extensions was shorter than that of cells expressing H-Ras61L (19). Despite the distinct difference in appearance of the neurite extensions, these findings show that EHR-Ras61L and Ext-Ras61L, which localized preferentially to non-raft or raft membrane microdomains, can induce extensions of neurites in PC12 cells.

**Loss of anchorage-dependant growth in EHR-Ras61L transfected cells**

Anchorage-independent growth is a hallmark of transformed cells, as compared to normal cultured cells, which must adhere to the bottom of the culture dish in order to grow. To determine if EHR-Ras61L expression triggers anchorage-independent growth, NIH 3T3 cells stably expressing EHR-Ras61L were plated in soft agar and cell colony growth was compared to cell colony growth on plates of cells transfected with GFP-H-Ras61L or EHR-RasWT. Results for similar experiments using Ext-Ras61L and Ext-RasWT have been published (19).

Cells transfected with EHR-Ras61L or GFP-H-Ras61L. Colonies that were clusters with two or more cells were counted as growth. The cells that expressed EHR-Ras61L and GFP-H-Ras61L showed 22% and 47.3% colony growth in the soft agar, respectively (FIG. 11D). The colonies formed by EHR-Ras61L were smaller and slower growing than those formed by H-Ras61L. In comparison, Ext-Ras61L-transformed cells readily formed colonies
in soft agar, similar to H-Ras61L expressing cells (19). For the control, EHR-RasWT expressing cells showed almost no growth in soft agar (see FIG. 11A and D).

Another assay for anchorage-independent growth is the focus assay. Transformed cells continue to grow, even under confluent conditions, by growing on top of each other in a pile called a focus. Untransformed cells are growth inhibited upon contact with neighboring cells. Thus, it is possible to quantify the level of transformation by determining the number of foci. After transfection of RAT1 cells with H-Ras61L, EHR-Ras61L, or EHR-RasWT, the cells were allowed to grow and foci were quantified twelve to seventeen days later. The plates of cells that expressed EHR-Ras61L formed a significant number of foci but less than that observed on plates of cells expressing H-Ras61L (Table 1). In contrast, no foci were produced after transfection with EHR-RasWT. The procedure was also performed in NIH 3T3 cells, and transformation was similarly confirmed (data not shown). Ext-Ras61L DNA also effectively transformed cells and caused robust focus formation. The transformation activity of the cells expressing Ext-Ras61L was comparable to that of the cells expressing H-Ras61L. Ext-RasWT produced almost no focus formation in NIH 3T3 cells (19).

Overall, these experiments clearly showed that both EHR-Ras61L and Ext-Ras61L, which reside in different membrane microdomains (raft or non-raft), prompted transformation. Thus, the non-raft localized EHR-Ras61L and raft localized Ext-Ras61L maintain aberrant growth characteristics of an oncogenic Ras protein in NIH 3T3 cells.

**DISCUSSION**

To date, many reports have indicated that H-Ras is associated with both raft and non-raft microdomains of the plasma membrane (47). As we gain more knowledge concerning the role membrane microdomain locations play in H-Ras signaling initiation and
biological functions, we will also better understand both the mechanism and the function of Ras signal transduction within the cell and the nature of the plasma membrane.

To study whether or not the microdomain localizations impact H-Ras signaling initiation and function, we used two H-Ras variants, EHR-Ras and Ext-Ras, designed to target different microdomains of the plasma membrane, non-raft and raft. We used several methods to study the microdomain targeting specificity of EHR-Ras and Ext-Ras: membrane fractionation, density gradient separation, and adherent native membrane immunofluorescence. Our results demonstrated that EHR-Ras is localized to the non-raft microdomains of the plasma membrane, and Ext-Ras is localized preferentially to the raft regions of the plasma membrane. Other reported H-Ras chimeras have been designed to tether to lipid rafts on the plasma membrane (17, 18, 32, 33). H-RasC184S, Raichu-tH, Mys-Ha-Ras, and LCK-Ras were all found to be associated with lipid rafts. In H-RasC184S, one palmitoylatable residue in the C-terminus of H-Ras, 184 cysteine, was mutated to serine (32). Raichu-tH contains an YFP, an H-Ras conserved region, a Ras-binding domain of Raf (RBD), and a CFP in its N-terminus. In the C-terminus, Raich-tH keeps the membrane anchor of H-Ras, which consists of the last nine amino acids. In Mys-Ha-Ras, the residues that enable myristoylation were linked to the N-terminal of H-Ras to generate this chimera. The residues that enable palmitoylation, myristoylation, and membrane targeting in LCK were fused to the N-terminus of a palmitoylation deficient H-Ras variant, H-Ras181, 184S, to generate the LCK-Ras. We showed that Ext-Ras is localized exclusively on the plasma membrane, whereas H-RasC184S and Raichu-tH chimera showed abundant distributions on the endomembranes (17, 32). Our results also demonstrated that, similarly to H-Ras, Ext-Ras also tethers to the plasma membrane via its C-terminus. In contrast, the Mys-Ha-Ras chimera
is tethered to the membrane through its N-terminal myristoylated residues in addition to the C-terminal lipid anchor sequence from H-Ras (33); the LCK-Ras chimera is tethered to the membrane both through its N-terminal lipid modification and C-terminal farnesylation motif (18). Moreover, since the Raichu-tH chimera also contains several additional tagged protein sequences, the size of this chimera protein is much larger than that of H-Ras (17). On the other hand, Ext-Ras has a molecular weight similar to that of H-Ras.

**E1 (QI) transmembrane sequence targets Ras to the non-raft domains whereas the C-terminal membrane anchor in Ext-Ras targets Ras to the lipid rafts**

Previously, the Donoghue group constructed EHR-Ras proteins to test whether these fusion Ras proteins had cell transformation ability in the absence of C-terminal lipid modifications and whether EHR-Ras could be targeted to the plasma membrane (27). However, which microdomain EHR-Ras resides in was not determined. Our results confirm that EHR-Ras is located on the plasma membrane. Moreover, we found that EHR-RasWT and EHR-Ras61L are present on the non-raft domains of the plasma membrane, which not only indicates the microdomain locations of EHR-Ras, but also shows the activation state of Ras does not affect EHR-Ras microdomain localization. The fact that EHR-Ras is localized to the non-raft membranes is due to its N-terminal transmembrane domain E1 (QI). The C-terminus (H-Ras 3S) of EHR-Ras cannot be post-translationally modified by addition of lipids and therefore cannot tether to the plasma membrane. Our results demonstrated that the E1 (QI) transmembrane sequence can be used to target proteins to the non-raft domains of the plasma membrane.

We also demonstrated that Ext-Ras resides primarily on the lipid rafts. The only sequence differences between Ext-Ras and H-Ras are as that Ext-Ras has an extension of six
lysine residues in the C-terminus, and Ext-Ras lacks the last three amino acids of the ‘CAAX’ farnesylation motif. Ext-Ras also keeps the cysteine previously used for farnesyl attachment as well as at the two natural cysteines in the H-Ras C-terminus, and therefore Ext-Ras is able to be palmitoylated (19). Thus, the membrane tethering of Ext-Ras is enabled due to its C-terminal palmitates and the added basic region. Compared with H-Ras61L, our data shows the microdomain localization of Ext-Ras61L is in the lipid rafts. Acylated (palmitoylated or myristoylated) protein motifs have been shown to favor lipid raft microdomains on the plasma membrane (10). However, how basic residues affect the lateral sequestration of plasma membrane microdomains has not been totally clarified. Peptides that have polylysine residues together with the farnesylated residues have been shown to associate with high affinity with specific anionic lipids (48). Other work has suggested that basic clusters on the myristoylated alanine-rich C kinase substrate (MARCKS) may attract lipid shells enriched in cholesterol (lipid rafts) on the cytoplasmic leaflet of the plasma membrane (49). We proposed that the basic residues in the Ext-Ras might contribute to raft localization. However, a non-palmitoylated Ext-Ras C181S/C184S/C186S mutant, which has the exact basic region that occurs in the normal Ext-Ras61L C-terminus, was not detected on the membrane fractions, but was found in the cytosolic fraction (19). Therefore, the basic region alone cannot support sufficient membrane interaction with lipid rafts, but can together with the palmitoylation sites. This is the first study to show that the C-terminal membrane anchor (polylysine and palmityolation residues) of Ext-Ras, preferentially targets H-Ras to lipid rafts.
The non-raft membranes allow EHR-Ras signaling, and the raft membranes allow Ext-Ras signaling

Our results demonstrated that both EHR-Ras and Ext-Ras have the ability to recruit effectors and activate downstream signaling cascades when they are in a GTP bound state. EHR-Ras61L recruited Raf-1 on the plasma membrane and exhibited about one-third of the activation of ERK1/2 as that of H-Ras61L. EHR-Ras61L also showed limited recruitment of PI3kinase-p85 on the PM and demonstrated low activation in the PI3-kinase-AKT pathway. Ext-Ras61L recruited some PI3kinase-p85 and showed considerable activation of the PI3-kinase-AKT pathway, but barely recruited Raf-1 on the PM and showed low activation of ERK1/2.

EHR-Ras61L, the non-raft localized H-Ras, recruits Raf-1 and activates ERK1/2 moderately in contrast to the raft-localized Ext-Ras. Our immunofluorescence and Western blot data on EHR-Ras indicated that translocation of Raf-1 to the plasma membrane or the activation of Raf-1 (in the MAPK pathway) could be independent of lipid rafts. Lipid rafts are rich in cholesterol, and cyclodextrin’s ability to absorb cholesterol can therefore disrupt lipid rafts. Other investigators have also shown that the activation of MAPK can take place in the membranes that were treated with cyclodextrin, implying that the non-raft microdomains allow the activation of this signaling cascade (50, 51). Together with the observations of other non-raft located H-Ras chimera (18), our results further demonstrate that H-Ras activated Raf-MEK-ERK cascades exist on the bulk of the plasma membrane without lipid rafts.

When Ras recruits Raf and activates its downstream MAPK signaling cascade, the Raf, MEK, and ERK kinases are organized in modules (52). We observed that Raf-1
recruitment and phosphorylation of ERK is much decreased for Ext-Ras61L, a raft localized Ras. The weak Raf-1 and ERK activation was also seen in other Ras constructs that were artificially targeted to the lipid raft microdomain using N-terminal membrane targeting signals (18, 33). Recently Eisenberg et al. research proposed that clustering of raft located proteins might limit the mobility of H-Ras and inhibit the downstream MEK/ERK signaling pathway (53). Further studies need to be done to explore how the signaling partners in the MAPK pathway are recruited in lipid rafts.

Our results demonstrate that the raft localized H-Ras, Ext-Ras61L, showed some colocalization with the PI3 kinase p85 subunit and can also activate the PI3 kinase-Akt pathway. One class of PI3 kinases, PI3 kinase α, is composed of a catalytic subunit of 110 kd (p110α) and a regulatory subunit of 85 kd (p85α). The p110α subunit interacts with activated Ras. Because p85α and p110α form a complex in vivo and the association between them is very tight (54, 55), we determined the degree of interaction between Ras and PI3 kinase-p110α by detecting the colocalization of Ras and PI3K-p85. The colocalization of p85-PI3K on the PM with Ext-Ras61L indicated that PI3 kinase can interact with H-Ras in a lipid raft environment. One product of the PI3 kinase reaction, PtdIns(3,4,5)P3, can bind to the pleckstrin homology domains (PH domains) of signaling proteins including AKT kinases. Therefore, the activation of AKT can be used to monitor PI3 kinase activity. Our finding that Ext-Ras61L activated the PI3 kinase-AKT pathway here in NIH 3T3 cells is supported by our lab’s previous results that showed Ext-Ras61L strongly activated the PI3 kinase pathway in the PC12 cells (25). The level of Ext-Ras activation of the PI3 kinase pathway is not the same in NIH 3T3 cells and PC12 cells. Why this is so is not currently known. Activation of AKT in rafts has also been demonstrated with a raft targeted Ras that used a N-terminal
myristoylation signal of LCK (18). These observations all suggested H-Ras activation of the PI3 kinase pathway does in fact occur in lipid rafts.

Our findings showed that the non-raft localized H-Ras, EHR-Ras61L, co-aligned less with PI3 kinase on the PM than H-Ras61L and Ext-Ras61L. In the NIH 3T3 cells that expressed EHR-Ras61L, we also observed low AKT activation, which is an indicator of low PI3 kinase activity. Earlier research also showed that the PI3 kinase activity initiated by EHR-Ras61L was weaker than that of H-Ras61L in 293T cells (26). The compromised AKT activation was also found in another non-raft targeted H-Ras that used a N-terminal transmembrane domain of the CD8α receptor (18). These results suggested that H-Ras-induced PI3 kinase activation can occur within non-raft microdomains, although the degree of activation may be decreased.

Thus, H-Ras signaling will still occur if H-Ras is preferentially located to the different plasma membrane microdomains. Furthermore, the translocation of H-Ras between raft and non-raft microdomains is not prerequisite for H-Ras signaling.

Though the effector domain structures and the general orientation of Ext-Ras and EHR-Ras should be similar to that of natural H-Ras, their membrane anchors and the part of the structures that are close to the membrane targeting motif may not be exactly the same as those of natural H-Ras. These features could play a role in how effectively Ras accesses effectors and signaling activators. For example, a recent study pointed out a new switch region in the H-Ras C-terminus that includes the hypervariable region (HVR). This switch region influences H-Ras membrane orientation and regulates MAPK signaling (56). In addition, the polybasic region in Ext-Ras may enhance the interaction with the phosphatidylinositol (4,5)-bisphosphate PtdIns (4,5) P₂ (57), which is a substrate for PI3
kinase, and this enhanced interaction may then increase the activation of PI3 kinase downstream cascades. Additional information is needed to determine whether this happens.

**Raft and non-raft located Ras variants both exhibit biological functions**

Although both the EHR and Ext proteins exhibit differences in their localization in either non-raft or raft microdomains, both EHR-Ras61L and Ext-Ras61L are biologically active. This is shown by the morphology and anchorage-independent growth (soft agar and focus assays) in NIH 3T3 cells, as well as by the differentiation of PC12 cells. There are observable biological differences between these Ras variants and H-Ras. This can be seen in both NIH 3T3 cells and PC12 cells. On soft agar, cells expressing EHR-Ras61L grow significantly slower than cells expressing H-Ras61L, and the cell clumps were significantly smaller than those of H-Ras61L or Ext-Ras61L. In PC12 cells, H-Ras 61L expression results in a few, relatively straight neurite protrusions per cell. EHR-Ras61L expression results in many neurite outgrowths, but the projections often took 24 hours longer to appear than those of cells expressing H-Ras61L. Ext-Ras61L caused a differentiated phenotype with exaggerated neuritic structures, with most of them exhibiting a kinked morphology, and Ext-Ras61L neurite growth was very fast. These differences in phenotypes may result from differences in the pathways related to H-Ras activation. Our findings suggest that activated H-Ras, whether residing in raft or non-raft microdomains, is capable of stimulating cell transformation. However, the different cellular phenotypes observed may result from the activation of different combinations of downstream signaling cascades.

Taken together, the results show that we have successfully targeted two H-Ras protein variants, Ext-Ras and EHR-Ras, to localize preferentially in two different membrane microdomains. Our results from the investigation of Ext-Ras and EHR-Ras suggest that both
the non-raft and raft microdomains allow H-Ras signaling and biological function, although the extent of downstream signaling is not equivalent for these Ras variants: Ext-Ras61L activates the PI3 kinase-AKT pathway but decreases the recruitment of Raf-1, whereas EHR-Ras activates the MAPK pathway but limits AKT activation in NIH 3T3 cells. Thus, the previous models that assumed a shifting of raft to non-raft for H-Ras signaling may need to be modified, since such shifting may not be a prerequisite for Ras activation, signaling, and biological function. Additionally, we have demonstrated that the E1 (QI) transmembrane sequence in EHR-Ras targets Ras proteins to the non-raft domains of the plasma membrane, and the C-terminal membrane anchor (polybasic residues together with the palmitoylation sites) in Ext-Ras can target Ras to the lipid rafts. Because EHR-Ras and Ext-Ras variants can be successfully targeted to non-raft or raft domains, these constructs can serve as models in the study of Ras functioning in different microdomain localizations.

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**FIGURE LEGENDS**

**FIG. 1.** Illustration of the interactions of H-Ras, Ext-Ras, and EHR-Ras with the plasma membrane. In this study, several Ras variants were utilized: H-RasWT, H-Ras61L, EHR-RasWT, EHR-Ras61L, Ext-RasWT and Ext-Ras61L. The
C-terminus of EHR-Ras was made from H-Ras61L-3S, which is cytosolic due to three cysteines to serine substitutions, 3S (SMSSKSVLS), at the C-terminus, which alters or abolishes the post-translational processing and lipid modification of the protein. EHR-Ras also has an N-terminal IBV E1 (QI) transmembrane anchor in addition to its 3S mutations in the C-terminus. The linker region between the transmembrane domain and the Ras protein in EHR-Ras is 22-amino acids in length. Ext-Ras has three Cys in C-terminus, but lacks the ‘CAAX’ motif for farnesylation. Instead, it has a polybasic region in the C-terminus. This Ext-Ras variant can attach to the plasma membrane. All Ras variants were made in both a wild type (WT) and the oncogenic variant, which contains the point mutation Gln61 to Leu (designated as 61L).

**FIG. 2. EHR-Ras and Ext-Ras are located on plasma membrane.** All scale bars in this figure = 10 µm. NIH 3T3 cells were transfected with EHR-RasWT. (A) or Ext-RasWT (C) DNA for 24 hours, then fixed, permeabilized, and stained with an H-Ras antibody then with appropriate secondary antibodies labeled with fluorescent probes (red). Adherent basal plasma membrane fragments from NIH 3T3 cells expressing EHR-Ras61L (B) or Ext-Ras61L (D) were prepared by sonication, then fixed and stained directly with an antibody to H-Ras and AlexaFluor-labeled secondary antibody (green).

**FIG. 3. Fractionation assays show EHR-Ras associates with detergent sensitive membrane fractions whereas Ext-Ras preferentially associates with detergent resistant membrane fractions.** (A) Cytosolic (S) and membrane fractions from NIH 3T3 cells expressing EHR-RasWT or GFP-H-Ras61L protein were prepared
Membrane fractions were then pretreated with 0.2% Triton X-100 for 30 min on ice and subjected to centrifugation to separate detergent soluble (DS) and detergent resistant membrane (DRM) fractions. Each fraction was acetone precipitated, separated by SDS-PAGE, and then subjected to immunoblotting with anti-H-Ras antibody to detect Ras. EHR-Ras exhibits multiple bands due to multiple glycosylation states for E1 (QI). An anti-caveolin-1 immunoblot identified the lipid raft fractions, and an anti-transferrin receptor antibody identified the non-raft fractions. The molecular weight of the EHR-Ras upper band is ~40 kd, and the molecular weight of the GFP-Ras band is 48 kd. (B) Cytosolic (S) and membrane fractions from Cos-1 cells expressing the Ext-Ras61L or H-Ras61L protein were prepared as described in “Material and Methods”. Membrane fractions were then pretreated with 1 % Triton X-100 for one hour on ice and then were subjected to centrifugation and separated as detergent soluble (DS) and detergent resistant membranes (DRM). Each fraction was acetone precipitated, separated by SDS-PAGE, then immunoblotted with anti-H-Ras antibody to detect Ras.

**FIG. 4.** EHR-Ras and Ext-Ras show different distributions on density gradients. (A) P-100 crude membrane fractions prepared from NIH 3T3 cells expressing either H-Ras61L or ExtRas-61L, and 293T cells expressing EHR-RasWT proteins were pretreated with 0.2% Triton X-100 for 30 min on ice and all extracts were subjected to centrifugation on detergent-free Optiprep gradients as described under "Material and Methods." EHR-Ras was detected in the different fractions by immunoblotting with an anti-H-Ras antibody. EHR-Ras displays multiple bands due to multiple
glycosylation states for E1 (QI). Anti-caveolin-1 immunoblotting and anti-GM1 dot blot identified the lipid raft fractions. The immunoblots were scanned and the band densities were quantified by Image J software. (B) Membrane fractions from NIH 3T3 cells expressing either H-Ras61L or Ext-Ras61L protein were solubilized in 1 % Triton X-100 for 30 min on ice and then were resuspended in detergent-free Optiprep gradients as described in "Material and Methods." The immunoblots were probed with anti-H-Ras antibody to identify H-Ras in the fractions. Anti-caveolin-1 immunoblotting identified the fractions containing lipid rafts. The immunoblots were scanned and the band densities were quantified by Image J software. The distribution of Ras proteins in gradient fractions was calculated.

**FIG. 5.** EHR-Ras is extracted from membranes treated with 0.2% Triton, while Ext-Ras61L is resistant to 1% Triton. All scale bars in this figure = 10 µm. (A) Merged images of NIH 3T3 cells co-expressing EHR-Ras61L or EHR-RasWT (red) with LYFP-GPI (green). Intact cells were treated with detergent-free buffer (no TX), permeabilized with methanol before fixation for the control groups. For the experimental groups, cells were treated with ice-cold 0.2% Triton X-100 (TX) for 5 minutes to extract non-raft regions of the membrane before fixation and staining with antibody to H-Ras and appropriate secondary antibodies labeled with fluorescent probes (red). Most of the red is washed off after Triton treatment. The glycosylphosphatidylinositol anchored proteins (GPI) remain on the raft regions of the membrane. LYFP-GPI was used for co-transfection to ensure that protein localized to a raft domain was not removed by this treatment. (B) Adherent membrane fragments from sonicated NIH 3T3 cells expressing Ext-Ras61L or
H-Ras61L were exposed to cold 0.2% or 1% Triton X-100 for 5 min, washed, then fixed in paraformaldehyde and stained with H-Ras antibody then with appropriate secondary antibodies labeled with fluorescent probes. Ext-Ras61L is mostly retained on cellular membranes after Triton treatment. H-Ras61L is completely extracted after 1% Triton treatment.

**FIG. 6. Ext-Ras does not co-localize with caveolin on native membranes.** All scale bars in this figure = 10 µm. Adherent membrane fragments from sonicated NIH 3T3 cells expressing Ext-Ras61L were fixed with formaldehyde and stained directly with anti-H-Ras (green) and anti-caveolin-1 (red) antibodies and then with the appropriate fluorescent secondary antibodies. Merged image and zoomed inset show little overlap between Ext-Ras61L and caveolin-1.

**FIG. 7. Raf-1 co-localizes with EHR-Ras61L on the plasma membrane but not with Ext-Ras61L.** All scale bars in this figure = 10 µm. (A) Column 1: Intact NIH 3T3 cells stably expressing EHR-Ras61L were fixed, then EHR-Ras61L and endogenous Raf-1 (red) detected by appropriate primary antibodies and fluorescent antibodies, with vertical z-stack images of each color deconvolved. Arrows indicate the presence of Ras and Raf-1 on the plasma membrane. Columns 2, 3, 4: NIH 3T3 cells were transfected with Ext-Ras61L, H-Ras61L or H-RasWT respectively. In column 2, after transfection with H-RasWT for 12 hours, cells were serum starved for 12 hours. Then the adherent membrane fragments from sonicated cells were labeled with anti-H-Ras antibody (green), anti-Raf-1 (red) antibody and detected with AlexaFluor-labeled secondary antibodies. In column 3 and 4, after 24 hours of transfection with H-Ras61L or Ext-Ras61L, adherent membranes fragments from
sonicated cells were incubated with anti-H-Ras (green), anti-Raf-1 (red) antibodies and detected with appropriate fluorescent secondary antibodies. (B) NIH 3T3 cells were co-transfected with H-RasWT and GFP-RBD (green) for 12 hours in complete media and then cultured in a serum-starved media for 12 hours. A 0 min sample was then collected. Remaining cells were stimulated with 10 ng/ml EGF for 10 min, and then a sample was collected. Cell samples were sonicated and the adherent membrane fragments from these cells were washed, fixed and labeled with H-Ras antibody then with appropriate fluorescent antibodies (red).

**FIG. 8.** The co-localizations of EHR-Ras61L, Ext-Ras61L and H-Ras61L with p85 on the plasma membrane. All scale bars in this figure = 10 µm. Columns show images of NIH 3T3 transfected with DNA of EHR-Ras61L, Ext-Ras61L or H-Ras61L. After 24 hours, cells were sonicated, fixed, and then labeled. On these adherent membrane cell fragments, H-Ras61L, Ext-Ras61L or EHR-Ras61L was detected with anti-H-Ras antibody (green), while p85 was detected using anti-PI3 kinase p85 antibody (red). Merged images and zoomed insets show co-localizations of Ras and their corresponding effector proteins. The extent of co-localization was measured using Metamorph™ software with EHR-Ras61L and p85 demonstrating approximately 7% co-localization; Ext-Ras61L and p85, 15%; and H-Ras61L and p85, 25% on the displayed and zoomed images.

**FIG. 9.** Activation of effector proteins by EHR-Ras61L and Ext-Ras61L. (A) NIH 3T3 cells stably expressing the H-Ras61L, Ext-Ras61L or EHR-Ras61L proteins were grown to 10^7 per 100 mm tissue culture plate. Cells were lysed as described on “Material and Methods”. Lysates from each cell line were loaded in each lane of the
SDS-polyacrylamide gel from the same experiment and the blot is probed with anti-Ras antibody and examined by immunoblotting. The bottom panel shows the endogenous tubulin level of each sample as a loading control. (B) Activation of ERK1, 2 by H-Ras61L, Ext-Ras61L or EHR-Ras61L proteins. NIH 3T3 cells stably expressing the indicated Ras proteins were lysed and processed as described in “Material and Methods”. The lysates from each cell line were loaded in each lane of the SDS-polyacrylamide gel. Total ERK, phospho-ERK and Ras were detected by immunoblotting from the same lysate for each cell line, and scanned images were quantified by using the Image J software. The ratio between phospho-ERK to total ERK was normalized for Ras levels. ERK activation level for cells stably expressing H-Ras61L was set to 1.00. The same calculation method was used for Ext-Ras61L and EHR-Ras61L experiments. The graph shows the average ± S.D. of 4 independent experiments. (C) Activation of AKT by H-Ras61L, Ext-Ras61L or EHR-Ras61L proteins. NIH 3T3 cells stably expressing the indicated proteins were lysed as described in “Material and Methods”. The Ras expression level for each cell line was as shown in (A). Lysates from H-Ras61L, EHR-Ras61L and Ext-Ras61L were loaded in each lane of the SDS-polyacrylamide gel from the same experiment. Total AKT, phospho-AKT, and Ras were detected by immunoblotting from the same lysate for each cell line, and scanned images were quantified by using the Image J software. The values show the relative activation level of AKT in cells expressing each Ras variant, normalized for Ras levels. The AKT activation level for cells stably expressing H-Ras61L was set to 1.00. The graph represents the mean ± S.D. of 4 independent experiments. (D) Activation of JNK by H-Ras61L,
Ext-Ras61L or EHR-Ras61L proteins. NIH 3T3 cells stably expressing the indicated proteins were lysed and processed as described in “Material and Methods”. The Ras expression level in each cell line was shown in FIG. 10A. Lysates from each cell line were loaded in each lane of the SDS-polyacrylamide gel from the same experiment. Total JNK, phospho-JNK, and Ras were detected by immunoblotting from the same lysate for each cell line, and scanned images were quantified by using the Image J software. The ratio between phospho-JNK and total JNK was normalized for Ras levels. The JNK activation level for cells stably expressing H-Ras61L was set to 1.00. The same calculation method was used for Ext-Ras61L and EHR-Ras61L experiments. These values showed the ranges (mean ± S.D.) of two independent experiments.

**FIG. 10. Biological activity of EHR-Ras61L and Ext-Ras61L in PC12 cells.** Cells after activation with NGF. Four days after transfection with Ext-Ras61L, GFP H-Ras 61L, or EHR-Ras61L. PC12 cells showed a growth of neurite extension.

**FIG. 11. Growth in soft agar and loss of anchorage-dependent growth in EHR-Ras61L.**

Two examples of typical colonies expressing (A) EHR-RasWT, (B) EHR-Ras61L, (C) GFP-H-Ras61L, after growth in soft agar for 14 days. (D) Percentages of cells that showed colony growth in soft agar. Cells transfected with EHR-RasWT showed no colony growth (0%), whereas cells transfected with EHR-Ras61L or GFP-H-Ras61L showed robust colony growth, 22% and 47.3%, respectively. The percentages of cells expressing each Ras protein are as follows: 52.9% for EHR-RasWT, 75.4% for EHR-Ras61L and 88.9% for GFP-H-Ras61L. NIH 3T3 cells stably expressing EHR-WTRas, EHR-Ras61L, or GFP-H-Ras61L were plated in
soft agar in order to assess their ability to grow in an anchorage-independent manner. On the 14th day after plating, cells were counted and clusters with two or more cells were counted as colony growth. The graphs for EHR-RasWT and GFP-H-Ras61L represent the mean ± S.D. for three plates from the same experiment.

**TABLE LEGENDS**

**Table 1. Transformation of RAT1 cells by EHR-Ras proteins.** RAT1 cells were transfected with H-Ras61L and EHR-Ras61L DNA. Amount of DNA used varied from 3–100 ng/plate for H-Ras61L and 10–100 ng/plate for EHR-Ras61L. Two to four plates of each DNA concentration were counted. Foci of transformed cells were counted after 12–17 days of transfection. Data presented here show the average number of foci counted, normalized to 1 µg of DNA.
FIGURES

EXTRACELLULAR

PLASMA MEMBRANE

EHR-RasWT

EHR-Ras61L

N

N

N

N

W T

H-RasWT

H-Ras61L

3S

3S

3S

3S

C

C

C

C

TM

TM

W T

WT

61L

61L

61L

61L

H-Ras61L-3S

Ext-RasWT

Ext-Ras61L

Modified from Hart et al., 2000 Ext Cell Res.

FIG. 1
FIG. 2
<table>
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<th>EHR-Ras61L</th>
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<td>NTX 0.2% TX</td>
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<td>S   DS  DRM</td>
</tr>
<tr>
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<td>GFP-H-Ras61L</td>
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**FIG. 3A**

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<td>S   DS  DRM</td>
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</table>

**FIG. 3B**
FIG. 5A
FIG. 7A

FIG. 7B
FIG. 9
FIG. 10
FIG. 11A–C

FIG. 11D
TABLES

Table 1

Transformation of RAT1 cells by EHR-Ras proteins

<table>
<thead>
<tr>
<th>DNA transfected</th>
<th>Average No. foci/µg</th>
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<tbody>
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CHAPTER 4. GENERAL CONCLUSIONS

Overview

This dissertation describes one mechanism behind H-Ras endocytosis. This dissertation also describes the distribution of H-Ras on the plasma membrane microdomains and its subsequent activation of the signal transduction cascades. In the area of H-Ras endocytosis, this work provides evidence for a novel, clathrin-independent mechanism. In the area of H-Ras membrane microlocalization, this work provides evidence that both raft and non-raft microdomains serve as H-Ras signaling platforms. These discoveries expand our current understanding of H-Ras trafficking and localization at the sub-cellular level and thus provide new sites and directions for future research.

Summary of Our Studies

Clathrin-independent endosomes associated with Arf6 bind both active and inactive forms of H-Ras

Until recently, H-Ras has been shown to be present on the vesicles that are characterized as endosomes in addition to some of the more traditional sub-cellular locations such as the plasma membrane [1, 2]. However, how H-Ras reaches the endosomes and what type of endosomes H-Ras binds is not fully illustrated.

H-Ras is endocytosed through a clathrin-independent pathway

We discovered that H-Ras traffics within the cell through a clathrin-independent pathway. Neither the GDP bound form nor the GTP bound form of H-Ras is co-localized with clathrin or EEA-1, either on the plasma membrane or in the cytosol. This indicates that H-Ras utilizes the clathrin-independent pathway at early and later stages of its internalization, and the activation of H-Ras does not change the usage of this pathway. Thus,
this study demonstrates a new route by which H-Ras is distributed on the endosomal membrane after H-Ras reaches the plasma membrane. This clathrin-independent endocytosis pathway may be another microenvironment that allows the interaction between H-Ras, lipids, and other proteins.

**H-Ras binds to the Arf6 endosomes**

The Arf6 endosomes provide a good platform for studying the H-Ras endocytosis pathway. Using this platform, we found that although GTP bound H-Ras is located on the Arf6 endosomes, the recruitment of one of the Ras effectors, Raf-1, on these endosomes is limited. This observation indicated that H-Ras can initiate signaling on the Arf6 endosomes and selectively recruits the signaling partner. This finding adds to the already complex nature of Ras signaling and highlights the uniqueness of the Arf6 endosomes as potential signaling platforms. Recent studies from Porat-Shliom *et al.* [3] showed that H-Ras is sequestered in Arf6Q67L vacuoles; the Arf6Q67L vacuoles recruit the active form of the Ras effectors ERK and AKT but not Rab5. Our studies, together with the work from Porat-Shliom *et al.* [3], further demonstrate that H-Ras can cooperate with Arf6 in signaling, vesicular trafficking, and biological functions.

**Properties of H-Ras and K-Ras proteins in endocytosis**

We observed that both H-Ras and K-Ras bind to Arf6 endosomes. K-Ras has been found to be released from the plasma membrane and localized to the mitochondria through phosphorylation of Serine 181 in its C-terminal basic region by protein kinase C [4]. In rat hippocampal neurons, K-Ras was found to escape from the plasma membrane and relocate to the Golgi apparatus though binding with the Ca²⁺/calmodulin at the K-Ras C-terminus upon glutamate stimulation [5]. This dissertation describes a new trafficking pathway in which
K-Ras is distributed on endosomal membranes in a different manner from those previously described pathways.

My work demonstrated that both the palmitoylated Ras, H-Ras (or H-Ras tail), and the non-palmitoylated Ras, K-Ras (or K-Ras tail), are distributed on the Arf6 endosomes. This finding illustrates that the mechanism by which H-Ras and K-Ras localize on the Arf6 endosomal membranes is different from the other recently studied de-palmitoylation/re-palmitoylation mechanism [6]. In that mechanism, de-palmitoylation causes H-Ras and N-Ras to dissociate from the plasma membrane. Ras proteins can then be randomly captured at the Golgi apparatus, get re-palmitoylated, and then travel through the secretory pathway back to the PM. The studies described here also show that Ext-Ras, an H-Ras variant in which the C-terminal farnesylation motif has been replaced with multiple lysine residues, is not found on the Arf6 endosomes. These results indicate that the N-terminal conserved region of Ras does not appear to control endocytosis, but the C-terminal farnesylation motif might play a role in relocating H-Ras to the Arf6 endosomes.

In this study, K-Ras was not found on the endosomal recycling center (ERC), which suggests that K-Ras does not reach the ERC during endocytosis. Though the mechanism behind K-Ras recycling has not yet been fully understood, it is possible that K-Ras may disassociate from the endosomes prior to reaching the ERC and perhaps traffic back to the PM via non-vesicular means. This study also verifies that H-Ras utilizes the perinuclear recycling center during endocytosis. H-Ras and K-Ras are highly homologous over the N-terminal 165 residues. However, the recycling of H-Ras and K-Ras exhibits an obvious difference. The difference between H-Ras and K-Ras during the recycling could be due to their last 24 C-terminal residues by several possibilities: (a) C-terminal palmitoylation may
be needed for Ras to distribute on the ERC, (b) the polybasic region of the C-terminus might prevent K-Ras from associating with the ERC, and (c) other residues in the C-terminal hypervariable region (HVR) might influence the association of Ras with the ERC.

Activation of different isoforms of Ras—H-Ras, K-Ras, and N-Ras—result in various responses at the molecular, cellular, and even the organismal levels [7, 8]. This study helps us further understand that these variations may be due to the different ways these isoforms of Ras act in the endocytic pathways. Moreover, this study provides clues as to why their endocytosis and recycling mechanisms are different and could be used as a basis to study the trafficking of other proteins in the Ras GTPase super family.

**Concluding remarks**

This research work reveals novel trafficking pathways for H-Ras and K-Ras endocytosis. Together with other new findings on H-Ras localization on the ERC and on recycling endosomes [9], this study completes a loop describing how membrane bound H-Ras endocytoses and recycles back to the plasma membrane. Thus, the entire landscape of H-Ras trafficking outward-bound to the plasma membrane and inward-bound to the cytosol becomes better outlined.

Ras proteins have essential roles in activation of their downstream signaling cascades, which in turn control cell proliferation, migration, survival, and differentiation [10]. Mutations in Ras genes can cause their related signaling network to become out of control and lead to the malignant growth of cells—tumors [11]. In humans, 20% of all cancers have been found to contain the mutations of Ras proteins [12]. Originally, the plasma membrane was thought to be the final destination for Ras proteins where they could produce signals. Studies from Porat-Shliom *et al.* [3] showed that activation of Ras stimulates plasma
membrane ruffling and macropinocytosis. They also found the active form of H-Ras can recruit some of its downstream effectors when trapped on Arf6Q67L vacuoles during a certain stage of macropinosome maturation. These findings and our work on Ras endocytosis and recycling provide evidence for a new platform for controlling Ras signaling level and signaling specificity in a spatial and temporal manner. Thus future cancer therapies will need to take into account the fact that Ras signaling also occurs during endocytosis.

Arf6 protein has been shown to function in membrane trafficking, actin remodeling, and cellular endocytosis through the interactions with its effector proteins and membrane lipids [13, 14]. Arf6 protein has also been found to be involved in the invasion and metastasis of breast cancer cells [15]. This observation of the intracellular association of Ras proteins with Arf6 may lead to more insights in the study of Arf6 GTPase induced tumor invasions.

Mutations of different Ras isoforms—H-Ras, N-Ras and K-Ras—have been found in different types of cancers [16]. These isoforms of Ras differ primarily in their C-terminal sequences. Our results illustrate that the specific amino acid sequence and lipid modifications in the C-terminal tails of Ras proteins control the endocytosis and trafficking of Ras proteins. Thus, this finding provides a basis for further studying Ras signal transduction and cancers caused by the mutations of these Ras proteins.

**Biological activity and signaling of two H-Ras variants designed to be preferentially “in” or “out” of rafts**

“Raft” and “non-raft” are two microdomains of the plasma membrane [17, 18]. H-Ras has been found localized on the microdomains on the plasma membrane [19–22]. However, whether H-Ras signals and produces biological function exclusively from a raft or non-raft microdomain has not been thoroughly elucidated [20, 23–26]. In order to study this, we
utilized two H-Ras variants (EHR-Ras and Ext-Ras) that were designed to reside in non-raft or raft microdomains respectively, and we found the following to be true.

*Both non-raft and raft microdomains allow H-Ras signaling*

Using subcellular fractionation assays, density gradients, immunofluorescence, and detergent treatment methods, we were able to show that EHR-Ras that is located on the plasma membrane accumulates exclusively in non-raft microdomains. Ext-Ras, which is also located on the plasma membrane, is found distributed predominantly in lipid raft containing membranes. Both EHR-Ras61L and Ext-Ras61L co-localize with their effectors, such as Raf-1 or PI3 kinase, on the plasma membrane and induce the signal transduction in the MAPK kinase, PI3 kinase-AKT and JNK pathways. These results demonstrate that both non-raft and raft microdomains allow H-Ras signaling. They also clarify that the translocations of H-Ras between these microdomains are not a prerequisite for the signaling initiation by H-Ras. Additionally, they indicate that membrane rafts, which serve as signaling platforms for many signaling proteins [27–29], also provide the necessary microenvironment for H-Ras signaling initiation and activation of its downstream cascades.

We also found that the extent of downstream signaling activation in different signaling pathways varies between these H-Ras variants. Ext-Ras61L activates the PI3 kinase-AKT pathway but decreases the recruitment of Raf-1, whereas EHR-Ras activates the MAPK pathway but limits AKT activation in NIH 3T3 cells. These results indicate that the membrane environment and the positions where these H-Ras variants tether may influence the signaling level. A recent study pointed out a new switch region in the H-Ras C-terminus that includes the hypervariable region (HVR). This switch region influences H-Ras
membrane orientation and regulates MAPK signaling [30]. Therefore, these findings further demonstrate the signaling complexity of H-Ras on the plasma membrane.

*Both lipid raft and non-raft microdomains allow the biological activity of H-Ras*

EHR-Ras61L and Ext-Ras61L, which are localized to non-raft or raft membrane microdomains respectively, cause potent transformation of NIH 3T3 cells and differentiation of PC12 cells. In addition, EHR-Ras61L and Ext-Ras61L cause aberrant growth characteristics of an oncogenic Ras protein in PC12 cells. In PC12 cells, EHR-Ras61L or Ext-Ras61L expression results in differentiated phenotypes with exaggerated neuritic structures and broad cell bodies. Therefore, they reveal that both raft and non-raft microdomains not only allow H-Ras signaling but also enable its biological functions.

*The N-terminus of EHR-Ras targets H-Ras to the non-raft microdomain and the C-terminus of Ext-Ras targets H-Ras to the membrane rafts*

Our experimental data showed that EHR-Ras and Ext-Ras localized to the non-raft and raft microdomains, respectively. Several other groups also made Ras chimeras that were designed to localize to lipid rafts: Raichu-tH [26] and LCK-Ras [31]. Raich-tH contains an YFP, an H-Ras conserved region, a Ras-binding domain of Raf (RBD), and a CFP in its N-terminus. In the C-terminus, Raich-tH keeps the membrane anchor of H-Ras, which consists of the last nine amino acids. The residues that enable palmitoylation, myristoylation, and membrane targeting in LCK were fused to the N-terminus of a palmitoylation deficient H-Ras variant, H-Ras181, 184S, to generate the LCK-Ras. Compared to Raichu-tH, Ext-Ras displays membrane localizations exclusively on the plasma membrane, while the Raichu-tH shows abundant distribution on the endomembranes as well as on the plasma membrane [26]. Compared to LCK-Ras, Ext-Ras is tethered to the plasma membrane via its C-terminus,
which is closer to the tethering sequence position of H-Ras than LCK-Ras; LCK-Ras is tethered to the membrane by the N-terminal lipid modified residues of LCK, and the C-terminal farnesylated residues form H-Ras181, 184S [31].

These results demonstrate for the first time that the E1 (QI) transmembrane sequence in EHR-Ras may be used to target proteins to the non-raft domain of the plasma membrane, and the C-terminal membrane anchor (polybasic residues together with the palmitoylation sites) in Ext-Ras can target H-Ras to lipid rafts. These discoveries help us to understand what kind of amino acid sequence and lipid modification can be targeted to the different microdomains of the plasma membrane and therefore further reveal the preferences of the interactions between membrane lipids and specific amino acid sequences. Additionally, these studies also provide a basis for research on other membrane bound proteins.

Concluding remarks

H-Ras regulates cell growth, proliferation, and survival [10]. Mutations of H-Ras proteins are often found in thyroid, bladder, and kidney tumors [16]. The plasma membrane is a major platform where H-Ras signals and produces biological functions [32]. In this work, we demonstrate that both non-raft and raft microdomains support H-Ras signaling and induction of cell biological activity by utilizing two new H-Ras variants, EHR-Ras and Ext-Ras. Previous models, which assumed that shifting from raft to non-raft domains was required for H-Ras signaling initiation [33], may need to be modified, since such shifting is not a prerequisite for signaling. In summary, this work further clarifies our understanding of how H-Ras initiates signals and activates its downstream cascades on the plasma membrane microdomains and increases our knowledge of the cell membrane organization. Additionally, this work suggests directions and models for future research in studying how the membrane
lipids, proteins, and Ras membrane anchors affect Ras signaling. Finally, this research also provides clues for further studying of carcinogenesis caused by Ras proteins.

**Extension of Research**

In order to further study the subcellular localizations of H-Ras and the Ras signaling, several future research directions could be considered:

1. How does H-Ras interact with the Arf6 endosomes?
   a. Does any region of H-Ras other than the C-terminus affect the interaction with Arf6 endosomes?
   b. Do any intracellular molecules such as lipids or proteins affect H-Ras binding to Arf6 endosomes?

2. How does the localization of H-Ras on the Arf6 endosomes affect the signaling specificity of H-Ras?
   a. Can H-Ras recruit other effector proteins at this location?
   b. Can H-Ras interact with the scaffold proteins (i.e., KSR), GEFs, or any other proteins that may help or inhibit H-Ras signaling at this location?

3. During endocytosis, how do Ras and Arf6 cooperate in signaling and biological activity?
   a. Will Ras regulate the Arf6 signaling activation in this pathway?

4. What is the detailed mechanism of K-Ras endocytosis?

5. Does N-Ras endocytose through a clathrin-independent pathway?

6. How are Ras downstream signaling modules (i.e., MAPK cascades) assembled in the non-raft and raft domains of the plasma membrane?
References


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