Localization of two transmembrane H-Ras proteins

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Localization of two transmembrane H-Ras proteins

by

Jessica Lynne Price

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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Major: Biochemistry

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Andy Norris
Linda Ambrosio

Iowa State University

Ames, Iowa

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Graduate College
Iowa State University

This is to certify that the master's thesis of

Jessica Lynne Price

has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy
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## CHAPTER 2: A TRANSMEMBRANE H-RAS WITH MINIMAL CELLULAR EXPRESSION

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CHAPTER 1: GENERAL INTRODUCTION

G proteins

There are two classes of GTP binding proteins (G proteins) -- heterotrimeric and small G proteins. Heterotrimeric G proteins consist of three subunits: α, β, and γ. The α subunits bind GTP and catalyze the enzymatic reaction to return it to GDP. Upon activation by GTP, the α subunit dissociates from the β/γ subunit and both are able to activate their downstream effectors. (36) The small G proteins are monomeric and able to both bind GTP and downstream effectors. The first small G proteins discovered, and some of the best studied, were the mammalian Ras proteins (K-Ras, H-Ras and N-Ras). Other members of the Ras super-family include Rho, Ran, Rab and Sar/Arf.

Properties of G proteins

All G proteins cycle between an activated state, with GTP bound, and an inactive state, with GDP bound. While G proteins are able to undergo this cycle without assistance, the rate is so slow that help is often required to allow the proteins to be functional in living cells. An inactivated G protein must interact with a guanine nucleotide exchange factor (GEF) in order to exchange its GDP for a GTP. The binding of the GTP causes a conformational change in the protein, allowing it to interact with its downstream effectors. In the course of time, the intrinsic GTPase activity will inactivate the protein. Often, especially in small G proteins, the intrinsic rate is too slow, so GTPase activating proteins (GAPs) catalyze the cleavage of the phosphate bond to form GDP by insertion of an arginine side-chain into
the active site of Ras (1). By this rapid switching between GTP and GDP states, G proteins are able to pass signals from their upstream activators to their downstream effectors.

Most small G proteins, including K-, H-, and N-Ras, contain signals at their C-terminus that lead to posttranslational modifications. These modifications include the addition of lipid moieties (the isoprenoid farnesyl or geranylgeranyl, and the fatty acid palmitate) that help to attach the small G proteins to the membrane. Even though these specific moieties are not required (10), membrane localization is necessary for activity of Ras (37).

**Ras subfamily**

In humans and other mammals, there are three different genes that code for the small G protein Ras (H-Ras, K-Ras, and N-Ras), that after translation and alternative splicing form four different proteins (H-Ras, K-RasA, K-RasB, and N-Ras). Viral forms of H-Ras and K-Ras were discovered in the 1960’s (12) and afterwards, as altered forms of cellular Ras proteins in human tumors (33). Further research has shown that Ras is expressed in all eukaryotes, including yeast and insects, and in most mammalian cell types (11). The genes for Ras proteins are mutated in about 90% of human pancreatic cancers, 35% of lung cancers, about 60% of thyroid cancers, and to a significant level in many other kinds of cancer (11). Ras GTPase activity is almost always the mutational point in human tumors (11). One such mutation is a glutamine to leucine mutation at the 61st amino acid (AA).

**Ras homology**

Ras shares 40-50% homology with its other family members, such as Rap and Ral (12). Between the four Ras proteins, there is a high level of homology—about 85% sequence identity (Figure 1.1). The N-terminus, especially the GTP binding domain, is extremely con-
served, while the C-terminus contains most of the variation in a domain called the hypervariable region (HVR). Within the HVR is the signal sequence for membrane localization called a CAAX (Cysteine, Aliphatic AA, Aliphatic AA, X any AA) box.

![Comparative structures of Ras proteins with various domains labeled.](image)

Figure 1.1 - Comparative structures of Ras proteins with various domains labeled. Yellow and cyan indicate a different amino acid than is found in H-Ras. Cyan also indicates the hypervariable region (HVR). The CAAX box is the signal sequence for membrane localization.

**Cellular processing of Ras**

In order for Ras to become active, it must be posttranscriptionally modified, although the modifications differ depending on the C-terminus. All Ras proteins contain a CAAX sequence, this is a sequence specific signal instructing farnesyl transferase to add a farnesyl moiety to the cysteine (26). The AAX portion of the sequence is then cleaved by a protease and replaced by a carboxymethyl group on the prenylated cysteine’s carboxyl group. (26) After this point, there are no further modifications to K-Ras4B. N-Ras and H-Ras receive one or two palmitates respectively, on cysteines near the CAAX motif, through a thioester linkage. Palmitoylation is a readily reversible reaction, a dynamic property which may be important for Ras signaling (20).

The above-mentioned posttranslational modifications are necessary for proper localization of Ras proteins. All native Ras proteins function at the cytoplasmic side of the
plasma membrane, although the path taken to arrive at the membrane is different. K-Ras4B, since it receives no further modifications, goes straight out to the plasma membrane through an unknown, golgi-independent pathway. However, both H-Ras and N-Ras appear to adhere to the cytoplasmic surface of vesicles that traffic from the endoplasmic reticulum (ER) through the golgi network and onwards to the plasma membrane. (26) Originally it was believed that signaling could occur only at the plasma membrane, but recent evidence indicates that Ras might be able to signal from endomembranes (5)

**Biological effects of activated Ras**

Once activated, Ras causes different biological effects in diverse cell types. In NIH3T3 mouse fibroblasts, activated H-Ras will cause transformation (31). This is evidenced by a spindle-shaped appearance and tumor-like properties, including a loss of contact inhibition of growth, permitting foci formation. Contact inhibition is a property of untransformed cells that tells the cells to cease cellular replication upon close contact with neighboring cells. Untransformed cells also need to adhere to a solid surface, unlike their transformed counterparts that gain the ability to grow suspended in agar.

In adrenal pheochromocytoma (PC-12) cells, the expression of activated Ras results in yet another phenotype. The cells will differentiate, which can be seen by the outgrowth of neurites. The neurites formed by Ras activation are comparable to those caused by nerve growth factor (NGF). An average of two neurites can be found on PC12 cells exposed to NGF, extending thin and straight, away from the cell. (4)

**Ras effectors**

To cause these diverse effects in cells, Ras must interact with several different downstream effectors. The two principle effectors of Ras are the serine-specific protein kinase Raf
and phosphatidylinositol 3- kinase (PI3K). Activation of Raf is the beginning of a signal cascade involving mitogen-activated protein kinase (MAPK) and leading to the regulation of gene transcription (35). The activation of PI3K also causes a different signaling cascade to occur, that results in the formation of 3' phosphorylated phosphatidylinositols and their recruitment to the plasma membrane and subsequently the activation of other proteins, such as Akt and Btk (12).

In addition, Ras interacts with many other proteins that are important for its regulation, such as GEFs and GAPs. There are a number of GEFs that will bind to and activate Ras proteins. The GEFs Sos1 and RasGRP1 interact with all types of Ras. Other GEFs are more specific and will only interact with particular Ras proteins. For example, RasGRF1 is able to activate H-Ras, but does not interact with N- or K-Ras. GAPs are also important regulators of Ras. The best-studied GTPase activating protein for Ras is p120 RasGAP. (12)

**Detergent resistant membranes**

The plasma membrane was once thought to have the properties of a fluid mosaic (28). Proteins and lipids were able to freely move throughout the plane of the membrane, proteins floating in a sea of lipid. This model has recently been shown to be incomplete, due to the discovery of different microdomains within the membrane (21). These domains contain lipids that resist extraction by cold detergent, and hence are known as detergent resistant membranes (DRMs). These domains are also known by many other names, including lipid rafts, glycolipid-enriched membranes, and detergent insoluble glycolipid-rich domains. Many new discoveries are showing the importance of these domains for various cellular functions, in-
eluding cell adhesion (15), synaptic transmission (34), viral budding (22), and signal trans-
duction (6).

Properties of DRMs

The existence of detergent resistant microdomains was first proposed during the
1990’s (7). This indicated that DRMs had more structure, a liquid ordered phase, than the
sea of lipid they were floating in, which has earned them one of their common names: lipid
rafts (14). A closer investigation of these regions indicated that DRMs seemed to be similar
in composition to caveolae-- invaginations in the plasma membrane which were thought to
mediate the transport of molecules across the membrane (29). In many cases DRMs are
similar to caveolae, but cells that do not express caveolin may contain DRMs (13), so the two
are not equivalent.

Composition of DRMs

The composition of DRMs differs dramatically from the remainder of the plasma
membrane. In general the plasma membrane is made up of glycoprophospholipids and low lev­
els of cholesterol (21). In contrast, the major components of both DRMs and caveolae are
sphingolipids, cholesterol, glycosylphosphatidylinositol (GPI) anchored proteins (8) and
caveolin (Figure 1.2). The partitioning of sphingolipids and cholesterol to DRMs decreases
their fluidity. Sphingolipids, whose tails are usually completely saturated, decrease the fluidi­
ity by being able to pack more tightly than their non-raft counterparts, which are often un­
saturated (7). In addition to this, cholesterol is able fill in small gaps in between phospholipid head groups, and provide for the increased stability of DRMs and caveolae (21).
In many cell types, the most common protein in DRMs is caveolin. Caveolin is an integral plasma membrane protein between 21 and 24 kDa, the major component of caveolae. By binding to cholesterol, caveolin is able to selectively partition to the liquid ordered phase and act as a scaffold for other proteins, including signaling proteins (7). Among the signaling proteins that interact and partition with caveolin, at least part of the time, are H-Ras, adenylyl cyclase, heterotrimeric G proteins, as well as insulin and epidermal growth factor (EGF) receptors (29).

**Isolation of DRMs**

Several techniques have been designed to isolate and study DRMs. One of the first, and most frequently used methods, is a density gradient. In this technique the membranes, which have been isolated from the whole cells, are placed in detergent (often Triton X-100)
at the bottom of a sucrose or a carbonate gradient and spun in an ultracentrifuge for at least 14 hours. After this treatment, the resistant membranes are still intact and thus display the low density of all intact membranes by rising to the top of the gradient (2, 27). The detergent will disrupt nonresistant membranes, leaving the non-DRM proteins on the bottom of the gradient. Detergent extraction can also be used in immunofluorescence to detect DRMs in whole cells.

An important question has been raised regarding DRMs: Are they just artifacts that arise from the detergent extraction itself (21)? It is possible that by adding detergent to the cells that proteins and lipids are brought together that normally have no association with each other. To address this issue several studies have used non-detergent methods to isolate microdomains with a similar composition to DRMs (3, 19).

Several labs have now used a powerful technique to study DRMs and caveolae, called fluorescence resonance energy transfer (FRET). Using derivatives of GFP as acceptors and donors it is possible to measure distances between labeled proteins to determine their colocalization. Since FRET can only occur between molecules 10 nm or less apart, it is much more precise than standard microscopy. (18)

**Ras and DRMs**

There is a lot of debate in the scientific community regarding Ras and its interactions with DRMs. Several different studies have shown that H-Ras does localize, to some extent, with DRMs (9). Ras has also been shown to directly interact with caveolin, thus providing a mechanism for the partitioning of Ras to DRMs (30). A model has been put forth suggesting
Ras is associated with DRMs only while it is inactive and is released from these domains upon activation (23).

**Background of chimeric proteins**

Since Ras may be found in DRMs, several questions can be asked. Is Ras able to signal when it is specifically localized outside of those domains? Is DRM localization necessary for H-Ras to become active? Can Ras activators and downstream effectors locate Ras if it is not in a DRM? Does the activity of Ras, without an activating mutation, increase upon removal from DRMs? To answer these questions several different chimeric Ras proteins were studied.

**E1 infectious bronchitis virus chimera**

A chimeric, transmembrane H-Ras protein was designed during the late 1990’s (16). This protein was created with the external and first transmembrane (TM) domains of the E1 protein from the avian infectious bronchitis virus (IBV) attached to a cytoplasmic domain consisting of either H-Ras wt (EHRwt) or H-Ras 61L (EHR61L). E1 is an N-terminally glycosylated protein that is incorporated into the envelope of the virus. The E1 protein localizes to the cis-golgi in infected cells, but a Q→I mutation in the first transmembrane domain will allow the protein to be transported through the golgi network and out to the plasma membrane (32). The specific localization of EHR within the plasma membrane, either in or out of DRMs, was unknown.
**CD45 chimera**

CD45 is a tyrosine phosphatase that is expressed in human B and T cells. It is a vital regulator of receptors on the cells that control the immune system response to antigens. There are several splice variants, depending on which exon(s), if any, (A, B, or C) are expressed at the N-terminus of the protein. B220, the isoform of CD45 that was used to construct the chimera with H-Ras 61L (CHR61L), contains all three exons. (25)

With the goal of generating a non-DRM construct, CD45 was selected for the construction of the chimeric Ras. The rationale for this was due to several previous studies that showed that CD45 does not localize to DRMs (17, 24). The making of this protein was similar to that of EHR proteins-- using the extracellular and transmembrane domains with a short linker between the TM and Ras.

**Purpose of chimeric Ras proteins**

By studying several different chimeric Ras proteins we desire to learn how Ras functions outside of DRMs. By showing that the chimeric Ras proteins are or are not able to function outside of DRMs we will be able to better understand cellular localization of Ras. We may then be able to better explain its oncogenic properties. We will also be able to examine how its signaling differs from native and constitutively activate Ras. Similarities and differences in signaling between the chimeric Ras proteins and the constitutively activated Ras will provide insight as to how Ras typically interacts with DRMs.
Organization of thesis

The general introduction found above gives background for all of the chapters of this thesis that follow. Both chapter 2 and chapter 3 are written in publication format. The second chapter has been written in the format used by Molecular Cellular Biology and the third chapter has been written with the intent of submitting it soon to the same or comparable journal. I did all of the research and writing for the third chapter, except the focus assay experiments in NIH 3T3 cells, which were done in Berthe Willumsen’s lab, University of Denmark, Copenhagen. The final chapter contains general conclusions that can be drawn from this research, as well as future directions.

List of abbreviations

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AA</td>
<td>amino acid</td>
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<tr>
<td>CHR</td>
<td>CD45/H-Ras chimera</td>
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<td>DRM</td>
<td>detergent resistant membrane</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>EHR</td>
<td>E1/H-Ras chimera</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
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<td>GAP</td>
<td>GTPase activating protein</td>
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<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
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<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>HVR</td>
<td>hypervariable region</td>
</tr>
<tr>
<td>IBV</td>
<td>infectious bronchitis virus</td>
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<tr>
<td>MAPK</td>
<td>mitogen activating protein kinase</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
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<td>TM</td>
<td>transmembrane</td>
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CHAPTER 2: A TRANSMEMBRANE H-RAS WITH MINIMAL CELLULAR EXPRESSION

A paper written in the format of Molecular Cellular Biology

Jessica Price and Janice Buss

Abstract

H-Ras is a 21kD protein encoded by the H-ras human oncogene. While it is understood that H-Ras must localize at a membrane, most commonly the plasma membrane, the precise localization is not well understood. Within the plasma membrane there are discrete regions that are known as detergent resistant membranes (DRMs). The current debate is if H-Ras interacts with or localizes in DRMs. This study examines a transmembrane version of H-Ras that is to be specifically directed to non-DRM regions by the transmembrane and extracellular domains from CD45. The DNA was properly constructed and transfected into various cell lines but gave only minimal cellular expression. After optimizing many different cellular and experimental conditions it was determined that this particular non-DRM H-Ras construct is not expressed at useful levels. Further study will need to be done to determine if its non-DRM status causes the lack of expression.

Introduction

H-Ras (21kD) is an important member of a class of proteins called small G proteins. H-Ras, along with the other Ras proteins of the mammalian cell (K-Ras4A, K-Ras4B and N-Ras), binds GTP in its active state. By the mechanism of its intrinsic GTPase activity and helper GTPase activating proteins (GAPs) it converts the GTP to GDP and thus becomes in-
active. To then reactivate the protein guanine nucleotide exchange factors (GEFs) are then recruited to exchange the GDP for a GTP. While active, Ras initiates its signal cascades, thereby regulating gene expression and the cell cycle. The standard GDP/GTP cycle can be broken by certain mutations to the Ras protein, causing it to remain in the active, GTP-bound form. One such mutation is the Gln61Leu mutation, in the GTP binding domain. (5)

In recent years it has been shown that the plasma membrane is not a random conglomeration of lipid and protein as was once predicted, but there are discrete domains within the membrane (8). These domains, which can be isolated using their detergent (Triton X-100 or Brij) resistant feature, contain elevated levels of cholesterol and sphingolipids both of which contribute to their less fluid state (4). There is still much controversy over the nature of these detergent resistant membranes (DRMs) and their role in the cell (12). Recent evidence indicates that they may play a role in signal transduction by acting as a platform where signaling proteins can congregate (3). H-Ras is hypothesized to interact with DRMs, although its exact mechanism is still being debated (1, 9).

Seeking to better understand the importance of the interaction between H-Ras and DRMs we decided to examine the biological implications of prohibiting H-Ras from interacting with DRMs. To achieve this goal we made three important modifications to H-Ras. The first was the addition of the extracellular and transmembrane domains of CD45 to the N-terminus of Ras. CD45 has been known to localize outside of DRMs (7, 10), thus making it an excellent choice for these experiments. The second modification was to the C-terminus of Ras. There are three cysteines that each receives either a farnesyl or a palmitate upon cellular posttranslational modification of H-Ras (13). These lipids are the factors that normally cause H-Ras to be localized at the plasma membrane (13). In order to ensure that these lipids
do not influence the localization of the new transmembrane protein, these cysteines were all mutated to serines, thus eliminating the lipid binding. The third change was the previously mentioned Q61L mutation.

**Materials and methods**

**DNA**

The construction of CHR61L began with CD45 cDNA of the mouse B220 isoform of the CD45 protein, obtained from Bart Sefton (Salk Institute). Human H-Ras has been modified by the following mutations: Q61L, causing constitutive activation, C181S, C184S and C186S, preventing posttranslational modification of protein by isoprenoids and thus eliminating endogenous membrane binding. Delta CD45 (ΔCD45) is defined as the external and transmembrane domains of full length CD45. B220 cDNA in pBluescript (Stratagene) was cut with Cla I. The 3’ overhang was filled in using a Klenow reaction kit (Promega), and then it was cut with Xho I. pcDNA3 (Invitrogen) was cut using EcoR V, a blunt cutter, and Xho I, and was then ligated to B220 cDNA that had been prepped in the above-mentioned manner. While the Klenow reaction should have destroyed the Cla I site, upon ligation with the EcoR V blunt end the Cla I site was inadvertently recreated. This is not an issue for further cloning due to its Dam methylation sensitive nature.

To generate ΔCD45, PCR was used to engineer a Cla I site, a stop codon, and a Xho I site after the transmembrane domains of B220. Based on the research of Hart and Donaghue (6) Ras was specifically placed 42 base pairs after the transmembrane in order to ensure it was not too proximal to the plasma membrane. The sense oligo used was 5’ CgA AAT TAA TAC gAC TCA CTA TAg 3’ and the antisense oligo was 5’ CgC gCg CTC gAg CgC gCg
CgC TCA ATC gAT ATT ggA TCT TTT CTT gCg 3’. The ΔCD45 PCR product was then inserted into pcDNA3 using Xho I and EcoR I.

PCR was also performed on H-Ras to engineer Cla I and Xho I sites in the proper locations, 5’ and 3’ respectively, to attach it to ΔCD45. The sense oligo used for the PCR was 5’ CgC gCg ATC gAT ACA gAA TAC AAg CTT gTg gTg g 3’ and the antisense oligo was 5’ CTA gAT gCA TgC TCg AgT CA 3’. Ras and ΔCD45 were both cut with Cla I and Xho I and were ligated together.

Other DNAs used include GFP H-Ras 61L, EHR61L (see Chapter 3, from B. Wilmusen, University of Denmark), H-Ras Ext61L (H-Ras 61L with a polybasic tail after the CAAX box and known to localize in DRMs (2)).

**Cell culture**

NIH3T3 and Cos1 cells were grown in DMEM (Gibco) + 10% calf serum. 293T cells were grown in DMEM + 10% fetal bovine serum. PC-12 cells were grown in RPMI (Gibco) + 10% heat inactivated horse serum + 5% fetal bovine serum. All cells were grown in 5% CO₂ except 293T cells, which were grown in 10% CO₂. Stable NIH3T3 cell lines were formed one of two ways. The first method was direct application of 1000µg/mL G418 to the cells that had been transfected with the desired construct in a neomycin resistant vector (pEGFP-C3 for GFP H-Ras 61L and pcDNA3 for other Ras). The second involved a co-transfection of the appropriate DNA with pIRESpuro, an empty vector containing the gene for puromycin resistance. Puromycin was then added at a concentration of 1µg/mL.
**Antibodies**

Antibodies used for western blotting and immunofluorescence are: Ras 146-3E4 (Quality Biotech); Ras Y13-238 (Santa Cruz); Ras Y13-172 (C. Der, University of North Carolina); anti-caveolin (Transduction Laboratories); CD45 M-20 (Santa Cruz); CD45 RA3-6B2 (Santa Cruz); CD45 35-Z6 (Santa Cruz), CD45 I3/2.3 (B. Sefton, Salk Institute). Appropriate secondary antibodies were used (Molecular Probes).

**Transfection**

Most transfections of CHR61L were performed using Superfect (Qiagen), with 1.6µg of DNA used in each well of a 12-well plate. All transfections in PC-12 cells were performed using Lipofectamine (Invitrogen), with 0.8µg of DNA used per well of a 12-well plate. Other transfection reagents used to improve protein expression include Effectene (Qiagen), Perfectin (Gene Therapy Systems) and Lipofectamine Plus (Invitrogen). These were all used according to their manufacturer’s directions.

**Immunofluorescence**

All cells were grown on coverslips coated with poly-L-lysine (100µg/mL) and/or fibronectin (50µg/mL) in PBS. Cells were washed with PBS and then fixed with 4% paraformaldehyde for 5 minutes on ice. Cells were then permeabilized with cold methanol for 30 seconds. Formaldehyde was quenched (1% milk, 150mM NaOAc in PBS, pH7) and cells were washed (1% milk in PBS) before incubating in primary antibody (diluted 1:400 in wash buffer). Secondary antibodies were also diluted (1:700) in wash buffer. After rinsing, cells were mounted on microscope slides using 4µl Vectashield (Vectorlabs). Variations on this
protocol include the length of time for each step of the procedure and using 5% goat serum with 0.4% BSA in PBS as a blocker instead of milk containing solutions.

**In vitro translation and western blot**

Translation was performed *in vitro* using the TNT T7 Quick Coupled Transcription/translation System (Promega). Added to the kit were 1µg DNA and 10 µCurie [S35] methionine and then the samples were incubated at 30°C for 90 minutes. These samples or cellular lysates were run on SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) gels. The gels were transferred to PVDF membrane and were either exposed overnight by autoradiography or probed with the appropriate antibody and detected by chemiluminescence (ECL).

**Results**

**Construction of CHR61L**

The CD45: H-Ras 61L (CHR61L) chimeric protein was designed from full-length cDNA of the B220 isoform of CD45 in the pcDNA3 vector. A truncated form of CD45, called ΔCD45, was created containing only the extracellular and transmembrane domains of CD45. After engineering appropriate restriction sites it is possible for H-Ras to be inserted just downstream from the transmembrane domain. Figure 2.1A shows the plasma map of CHR61L in pcDNA3, while Figure 2.1B gives an illustration of CHR61L attachment to the plasma membrane.
**In vitro translation**

To confirm the construction of CHR61L an *in vitro* system was used to express the protein. The rabbit reticulocyte both transcribed and translated the DNA to make the appropriate protein. As indicated in the Materials and Methods, $^{35}$S labeled methionine was added to the system incorporation in to the protein, thus providing a means to detect the protein. The samples were then run on a SDS-PAGE gel and detected via autoradiography. Expression of all CD45 constructs, full length CD45, ΔCD45, and CHR61L, as well as an H-Ras positive control were assayed (Figure 2.2A). Clean, well-defined bands were detected at the expected sizes for all constructs. These results indicate that CHR61L and its precursors were correctly made and able to be expressed. It should be noted that even in the *in vitro* system the expression of all CD45 constructs, including CHR61L, had much lower expression than the Ras positive control. The cause of this decreased expression remains unknown.

In order to ensure that the proper, full-length proteins were translated the membrane was probed with a Ras antibody (Figure 2.2B). Once again clean bands were
detected at the proper sizes in lanes 3 and 4. By using the Ras 146 antibody it was also possible to conclude that there was no premature termination of translation, since its epitope is located at the C terminus of Ras (Figure 2.1B). This evidence, as well as the autoradiographic data, conclusively demonstrates that proteins of the proper size and content can be made from the DNA constructs. Attempts were also made, unsuccessfully, to detect the CD45 containing proteins using CD45 antibody (data not shown).

Detection via western blot

Western blot was used to undertake the detection of CHR61L in vivo. Plasmids containing the CD45 or CHR61L gene were transfected into NIH3T3 cells. Cells were lysed, run on a SDS-PAGE gel, transferred to PVDF and blotted with the CD45 antibody 35-Z6 (see figure 2.1B for epitope information). While, CD45 could be detected in commercially available Jurkat cell lysate (Figure 2.3, lane 1), it was not found in the NIH3T3 cells that had been transfected with either full length CD45 (Figure 2.3, lanes 2-4) or with CHR61L (Figure 2.3, lanes 5-7).
In addition to attempting to detect CHR61L with the CD45 antibody 35-Z6, several other antibodies were, directed to CD45, as well as the Ras domain, used (see Materials and Methods and Figure 2.1B). However, none of these improved the detection of CHR61L. Additional attempts were made in order to improve protein expression and detection. These variables included using different cell types (NIH3T3, Cos1, 293T), transfection reagent (Superperfect, Lipofectamine, Effectene, Perfectin, Lipofectamine Plus), adjusting concentrations of antibodies, altering the blocker and antibody diluent (milk, casein, BSA and TTBS), and incubation time of membrane in antibody. Also, cotransfection of CHR61L with full length CD45 or with H-Ras was performed with no increased expression of CHR61L. While some of these variations gave slightly better results, the overriding observation was low, sporadic detection of any CD45 construct.

**Detection via immunofluorescence**

Another method used to detect CHR61L in vivo was immunofluorescence. This method allows indirect visualization of protein in intact cells using antibodies. Initial attempts at detecting CHR61L in NIH3T3 cells were not very successful, thus many different variables were examined in hopes of improving the transfection efficiency and expression. These variables included changing the cell type (Cos1, PC-12, and 293T cells), immunofluo-
rescence protocol (different time lengths, blockers, etc), transfection reagent (including Effectene, Superfect, Lipofectamine, Lipofectamine Plus, and Perfectin), amount of DNA per well and antibody (both to the CD45 and the Ras portions of CHR61L). A second means of verifying transfection efficiency is by transfecting PC-12 cells with activated Ras and looking at their differentiation. CHR61L did not cause the differentiation that one would expect. This could be because of its non-DRM characteristic, of its low expression, or toxicity.

Many of the changes to the above mentioned variables were not advantageous to the expression of CHR61L, although a few were. Antibodies to the Ras portion of the chimeric protein were much better at detecting CHR61L than antibodies to the external domain of CD45; the Ras 172 antibody gave the strongest signal. Superfect led to the strongest expression and made the cells the least sick. Cotransfections of CHR61L with H-Ras wt or with full length CD45 were also performed, although no better results were attained. Overall there were small successes in detecting CHR61L (Figure 2.4), but they were not large enough to draw any conclusions as to the biology or biochemistry of the protein.

A.  

B.  

Figure 2.4 – Images were taken by confocal microscopy of one of the few (A) NIH3T3 and (B) Cos1 cells brightly expressing CHR61L. Green staining was done using Ras 172 antibody and red staining was done using an anti-caveolin antibody.
**Stable cell lines**

In an effort to increase the percentage of NIH3T3 cells expressing CHR61L, they were put under selection using the antibiotic G418, resistance to which is provided by the neo' gene in the pcDNA3 plasmid. Although there were some cells expressing CHR61L just after transfection, no cells were expressing the protein after several weeks of selection. This seems to indicate that CHR61L is at least somewhat toxic to the cells, since they found some means to resist the antibiotic without expressing the desired protein.

A second method of selection was also used, which involved cotransfecting CHR61L with the empty vector pIRESpuro, thereby conferring puromycin resistance to transfected cells. Puromycin was added to the media and several weeks later the cells were fixed and visualized, either by autofluorescence (GFP61L) or immunofluorescence (CHR61L, EHR61L) to check percentage of cells expressing protein. This second selection method was more successful, but when compared to stable cells lines from other Ras constructs the signal was extremely dim and with only a minimal number of cells were expressing the protein (Figure 2.5).

**Discussion**

Several different techniques were used to study the *in vivo* expression of CHR61L and its CD45 precursors. After much work and optimization of multiple parameters the detectable expression of these proteins remained minimal. As shown by the rabbit reticulocyte system this low expression was not due to errors in the construction of the protein. Therefore either the CHR61L protein conformation was altered beyond recognition or the protein has mild to high level of toxicity in the cells.
It seems reasonable that there is some level of toxicity conferred by CHR61L to the cells. There are two possible sources of this toxicity. The first is that CD45, which is not natively found in any of the cell lines studied, was toxic and therefore the CD45 chimeric protein was also toxic. The second option is that the non-DRM status of the chimeric protein resulted in the reduced health of the cells. Based on the data obtained thus far it is impossible to decide which possible source is correct, although since full length CD45 was also undetectable in vivo the former options seems more plausible. Further testing must be done using a different non-DRM protein to determine the biological effects of Ras plasma membrane distribution.

References


CHAPTER 3: TRANSFORMATION AND DIFFERENTIATION
BY ACTIVATED, NON-DRM H-RAS

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Abstract

H-Ras, a small G protein, can activate several downstream effectors and cause diverse effects in the cell. These effects include cell cycle and gene transcription regulation. In order to produce these effects H-Ras must localize at the plasma membrane via three lipid modifications, although its precise location, in or outside of detergent resistant membranes (DRMs), is still being debated. A transmembrane Ras (EHRwt or EHR61L, a continuously activated version) was designed to localize to the plasma membrane in a lipid-independent manner. Both EHR proteins were found, by detergent extraction, to be located outside of DRMs. Based on this localization, the question was asked if Ras would still be able to find its proper downstream effectors. EHR61L causes both transformation of 3T3 cells and differentiation of PC-12 cells, in a similar manner to normal activated H-Ras. The rates of cell and neurite growth caused by EHR61L seemed to be slower than its lipid modified counterpart. EHRwt, while being localized in non-DRMs, does not become activated in either cell type.

Introduction

H-Ras, a 21kDa membrane bound protein, is a founding member of a subclass of small G proteins. Small G proteins are part of a larger class of proteins called GTP binding proteins (G proteins), a grouping that also contains heterotrimeric G proteins. All small G
proteins share common characteristics and H-Ras is no exception. These properties include the ability to bind GTP and thus become activated, a low level of GTPase activity to return the protein to an inactive state and the ability to activate several different signaling cascades (18).

An understanding of the downstream effectors of a protein is vital when examining its biology. H-Ras activates many different pathways in cells, including several that lead to cell growth (7), at least one that causes differentiation of nerve cells and at least one that leads to apoptosis (6). While H-Ras is able to activate all of these pathways, they are difficult to examine unless there is an activating mutation in the protein. Mutations of this sort are often found in the GTP binding domain and prohibit the conversion of GTP to GDP, leaving the protein in a continuously activated state (6). One such mutation is a glutamine to leucine switch at position 61.

Detergent resistant membranes (DRMs) are defined as domains within the plasma membrane that have a different composition and less fluidity than the rest of the plasma membrane (10). DRMs contain high levels of sphingolipids and cholesterol, both of which decrease the fluidity of these domains (4). The protein composition of DRMs is also different from the remaining PM. DRMs often contain a structural protein called caveolin (16) and many proteins that are anchored to the membrane via glycosylphosphatidylinositol (GPI) (15). There are also many different signaling proteins that have been shown to interact with DRMs, lending to the idea that DRMs may serve as signaling platforms (3).

There has been a lot of study recently regarding Ras and DRMs. There is a significant amount of evidence showing that Ras does interact with DRMs, although to what extent remains unknown (5, 9, 10). A recent model suggested by John Hancock et al states that
Ras associates with DRMs only while its inactive and upon activation it repartitions to non-DRMs (14). H-Ras has also been shown to interact with caveolin, a major DRM component of some cell types (17).

These studies bring up several questions. Is it necessary for H-Ras to localize to DRMs in order to be activated? Must H-Ras associate with DRMs at all? What are the consequences of Ras being exclusively localized in DRMs? Could H-Ras be active and function normally if it was entirely localized to non-DRM regions of the membrane? Answers to these questions will enhance our current understanding of how H-Ras interacts with these areas of the membrane.

One good way to specifically localize a membrane protein to a region is by creating a chimeric, transmembrane protein. In this study a variant of the E1 protein of the avian infectious bronchitis virus was used as the transmembrane anchor for Ras (12). It was left to determine the location of the chimeric protein in the membrane and its biological activity and answer at least some of the above stated questions.

**Materials and methods**

**DNA**

The DNA of E1(QI)-22-H Ras wt.3S (EHRwt) and E1(QI)-22-H Ras 61L.3S (EHR61L) (12) were obtained from Berthe Willumsen (University of Copenhagen, Denmark) in pcDNA1 (Invitrogen) and transferred to pcDNA3 (Invitrogen). H-Ras is human and either has an activating Gln61Leu mutation (EHR61L) or the native sequence in that location, as well as Cys181Ser, Cys184Ser and Cys186Ser mutations, preventing posttranslational modification of protein by isoprenoids. EHRwt was cut out of pcDNA1 using Hind III
and EcoR I and inserted into pcDNA3, which had been cut using the same restriction enzymes. EHR61L was made using site directed mutagenesis to mutate the endogenous Gln at position 61 to a Leu. The sense oligo used was 5’ CTg gAT ACC gCC ggC CTg gAg gAg TAC AgC gCC 3’. The antisense oligo used was 5’ggC gCT gTA CTC CTC CAg gCC ggC ggT ATC CAg 3’.

Other DNAs used include GFP H-Ras 61L, H-Ras 61L, H-Ras Ext61L (H-Ras 61L with a polybasic tail after the CAAX box and is known to localize in DRMs (2)), LYFP-GPI (A. Kenworthy, Vanderbilt), and GFP gt46 (A. Kenworthy, Vanderbilt).

**Cell culture**

NIH3T3 cells were grown in DMEM (Gibco) + 10% calf serum. 293T cells were grown in DMEM + 10% fetal bovine serum. PC-12 cells were grown in RPMI (Gibco) + 10% heat inactivated horse serum + 5% fetal bovine serum. All cells were grown in 5% CO₂, except 293T cells, which were grown in 10% CO₂. Stable NIH3T3 cell lines were formed by a cotransfection of the appropriate DNA (GFP H-Ras 61L, EHR61L, or EHRwt) with pIRE-Spuro (Clontech), an empty vector containing the gene for puromycin resistance. Puromycin was added after 48 hours at a concentration of 1µg/mL.

**Antibodies**

Antibodies used for western blotting and immunofluorescence are: Ras 146-3E4 (Quality Biotech); Ras Y13-238 (Santa Cruz); anti-caveolin (Transduction Laboratories); anti-transferrin receptor (Zymed Laboratories). Appropriate secondary antibodies were used (Molecular Probes).
**Transfection**

All transfections of PC-12 cells were performed using Lipofectamine (Invitrogen) with 0.8µg of DNA per well of a 12-well plate coated with 10µg/mL laminin in PBS. For NIH3T3 and 293T cells other transfection reagents (Effectene and Superfect from Qiagen) were used according to their manufacturer’s recommendations.

**Immunofluorescence**

All cells were grown on coverslips coated with poly-L-lysine (100µg/mL) and/or fibronectin (50µg/mL) in PBS. Cells were washed with PBS and then fixed with 4% paraformaldehyde for 5 minutes on ice. Cells were then permeabilized with cold methanol for 30 seconds. Formaldehyde was quenched (1% milk, 150mM NaOAc in PBS, pH7) and cells were washed (1% milk in PBS) before incubating in primary antibody (diluted 1:400 in wash buffer). Secondary antibodies were also diluted (1:700) in wash buffer. After rinsing cells were mounted on microscope slides using 4µl Vectashield (Vectorlabs).

**Subcellular fractionation and density gradient membrane flotation**

Cells were lysed in hypotonic buffer (1mM Tris, pH 7.4; 1mM MgCl2 in water) containing protease inhibitors and broken up with a 26 gauge needle. The lysates were then spun at 4,000 rpm for 10 minutes at 4°C to remove the nuclei. 5M NaCl was added to the supernatant to a final concentration of 150mM. The supernatant was then spun at 33,000 rpm for 30 minutes to separate the cytosolic and membrane fractions. The P100 (membrane bound) fraction was resuspended in water or 0.2% Triton X-100 on ice. If the pellet was resus-
pended in Triton, it was spun again at 33,000 rpm and the S100 and P100 fractions were collected.

For a density gradient, the first membrane pellet was resuspended in Optiprep (40%) and sonicated. Triton was added to the fraction to a final concentration of 0.2%. Decreasing concentrations of Optiprep (from 37.5% to 20%) were added carefully to the top of the sample. The gradient was then centrifuged at 4°C for at least 16 hours at 30,000 rpm. Samples were concentrated with tricholoracetic acid and separated by SDS-PAGE.

**Western blot**

Cell lysates, S/P fractions or Optiprep gradient samples were run on SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) gels. Unless otherwise noted, all samples were run on 15% gels. The gels were transferred to PVDF membrane for 40 minutes at 70 milliamps using a semi-dry transfer apparatus and were probed with the appropriate antibody and detected by chemiluminescence.

**Soft agar assay**

A bottom layer of agar media (1 portion 1.8% agar:2 portions 2X NIH3T3 media, see Cell Culture) was plated in a 60mm tissue culture dish and left to set overnight. Then NIH3T3 cells (50,000 cells/mL) were suspended as single cells in normal media. 300µL of cells (15,000 cells) were added to 1mL of agar media and carefully added to the base layer. All cell lines used were stably expressing the desired protein. Cells were counted on the 14th day after being plated and any clusters having two or more cells were counted as colonies. As a control, the stable cell lines were also examined by immunofluorescence and stained with DAPI and the percentage of cells expressing protein were then counted.
Focus assay

Focus assay experiments were performed by Berthe Willumsen and colleagues at the University of Copenhagen.

Results

Preparation of EHR61L and EHRwt

The EHRwt DNA construct was received from Berthe Willumsen (University of Copenhagen) who had obtained it from the Donoghue lab that had constructed it originally (11, 12). EHR61L was designed in pcDNA3 from EHRwt by site directed mutagenesis (see Materials and Methods for details). Both constructs were sequenced and found to contain no inadvertent point mutations. Figure 3.1, demonstrates the interactions of H-Ras wt, H-Ras 61L, H-Ras 61L-3S, EHRwt, and EHR61L.

Immunofluorescence to determine localization

Several different techniques were used to examine the EHR proteins and determine if they localized with DRMs. 293T cells expressing EHR61L or EHRwt, as well as LYFP-GPI (a known DRM marker), were treated with Triton X-100 before fixation and immunofluorescence. Cells were cotransfected with the GPI construct in order to ensure that DRMs were not disrupted while the rest of the membrane was solubilized. Results indicate that at 1% Triton X-100 both EHR proteins were entirely extracted while the LYFP-GPI was retained (data not shown). Even at extremely low amounts of Triton X-100 (0.2%) both EHR61L and EHRwt were almost entirely dislodged from the membrane, while LYFP-GPI remained intact (Figure 3.2).
Figure 3.1 – Illustration, modified from Hart and Donoghue (12), of H-Ras wt, H-Ras 61L, H-Ras 61L-3S, EHRwt, and EHR61L interacting with the plasma membrane. Due to its three serine mutations on its C-terminus, H-Ras 61L-3S is unable to localize to the membrane.

Figure 3.2 – Transiently transfected 293T cells expressing either EHR61L or EHRwt (red) and LYFP-GPI (green). Cells were treated with either completely no Triton (CNT), as a control, or 0.2% Triton to extract non-DRM regions of the membrane.
A similar experiment with NIH3T3 cells, using caveolin as the DRM marker, showed comparable results were obtained. Even at low percentages of Triton X-100 the transmembrane Ras was extracted while the caveolin remained intact (data not shown). Both of these experiments point to EHR61L and EHRwt being localized to non-DRM regions of the plasma membrane.

**Ultracentrifugation to determine localization**

Ultracentrifugation was used to confirm the non-DRM status of the EHR proteins. NIH3T3 cells that were stably expressing EHR61L or GFP H-Ras 61L (positive control) were spun to separate the cytosolic S100 fractions from the membrane bound P100 fractions. As expected the majority of each of the Ras proteins was found in the P100 fraction (Figure 3.3). This fraction was then treated with 0.2% Triton X-100 to solubilize non-DRM membranes and separate them from those that are detergent resistant. Another high-speed spin was performed and the S100 and P100 fractions were collected. A western blot of the samples clearly indicates that EHR61L and GFP H-Ras 61L had been solubilized by the Triton while the caveolin (a DRM marker) was still membrane bound (Figure 3.3). EHR61L gives multiple bands due to its multiple glycosylation states. A similar experiment was done with EHRwt, giving the same results (data not shown).

An additional ultracentrifugation technique was performed using sucrose gradients with Triton X-100. The gradients ranged from 40-20% sucrose with five intermediate progressions to allow for well defined separation of DRM and non-DRM proteins due to their buoyancy (DRMs) or lack thereof (non-DRM). As seen below in Figure 3.4, the DRM marker caveolin, which remains in DRMs, rose up to the least dense regions of the gradient,
while EHRwt remained in the more dense regions. These same results were also seen with EHR61L (data not shown). This is consistent with what is expected from a non-DRM protein. These results were seen in both 293T and NIH3T3 cells.

Figure 3.3 – S/P fractionation of stable NIH3T3 cells expressing either EHR61L or GFP61L. Lysates in the CNT (completely no Triton) lanes were separated in a standard S/P fractionation. The P100 fraction of an identical fraction was then resuspended in 0.2% Triton and fractionated again. EHR61L has multiple bands due to its various glycosylation states. Blots were probed with transferrin receptor and caveolin to ensure that known non-DRM and DRM proteins, respectively, were in the proper locations.

Figure 3.4 – 293T cells expressing EHRwt were centrifuged at high speeds in order to separate DRM proteins from those which had been extracted with 0.2% Triton. Caveolin was used as a DRM marker to ensure that DRMs remained intact.
**Treatment of cells with cyclodextran to assess EHR localization**

Stable NIH3T3 cells expressing either EHR61L or EHRwt were transfected with LYFP-GPI and then treated with methyl-β-cyclodextran (MBCD) to absorb the cholesterol out of the plasma membrane and result in DRM disruption. Examination using a fluorescent microscope showed that LYFP-GPI had coalesced (Figure 3.5B). The surprising observation was that distinct dark spots could be seen in the Ras staining (Figure 3.5A). It is interesting to note that these exactly corresponded with the GPI location (Figure 3.5C); EHR61L was specifically excluded from these clusters of GPI (Figure 3.5A and B). While these results can not be fully explained, it is clear that EHR61L is acting in a very different manner than the DRM marker or the control (Figure 3.5 D and E). Once again lending support to the idea that EHR61L is not in a DRM.

![Image](image-url)

Figure 3.5 – 2% cyclodextran was added to NIH3T3 cells expressing EHR61L to extract cholesterol from the plasma membrane and thus disrupt DRMs. EHR61L was detected using H-Ras 146 antibody and labeled red. LYFP-GPI is green and was used as a DRM marker.
**Biological activity of EHR61L as seen in NIH3T3 cells**

The comparison of the morphology between the two stable cell lines, one expressing EHR61L and the other expressing EHRwt, provided some dramatic evidence for the biological activity of EHR61L. The EHR61L cells were very long and spindle-shaped while the EHRwt cells were much more flat. The EHRwt cells would also cease cellular division upon contact with their neighbors in contrast to the EHR61L cells which seemed to migrate towards each other, preferring to grow atop one another. It is important to note that while there were distinct morphological differences between the EHR61L and the EHRwt, the transforming ability of the EHR61L cells was decreased in comparison to their GFP H-Ras61L and H-Ras Ext 61L counterparts (data not shown).

![EHR61L and EHRwt](image)

**Figure 3.6** – NIH3T3 cells stably expressing the labeled protein, colored green. The cells were also costained for caveolin, colored red. The morphologies of the EHR61L and the EHRwt are distinctly different in shape and behavior. The EHR61L also have a decrease in their expression of caveolin.

Another interesting morphological feature of the EHR61L stable cells is their down regulation of caveolin. According to several studies published in the late 1990’s (8, 13), caveolin may be down regulated in transformed cells (see Figure 3.6 above). While we have
not seen this in all transformed cell lines, there is a definite decrease in its expression in EHR61L cells.

**Biological activity of EHR61L as seen in PC-12 cells**

Activated H-Ras has been known to cause PC-12 cells to cease growth and differentiate by sending out neurites (1). Thus, another test for the biological activity of the EHR proteins was to see if it could cause differentiation in PC-12 cells. It is also important to compare the phenotype of the cells after transfection as one means of determining if EHR is behaving in a standard manner.

In order to compare the cells, several standards were needed, these included nerve growth factor (NGF), H-Ras 61L and H-Ras Ext 61L. The neurites caused by H-Ras 61L are similar in shape and size to those caused by NGF (Figure 3.7) -- rather straight with only a few neurites per cell. Another important comparison is to H-Ras Ext 61L, an activated H-Ras protein which is known to localize in DRMs (2). As seen in Figure 3.7, H-Ras Ext 61L causes a very different phenotype than either NGF or H-Ras 61L. The cell bodies are much broader with some ruffling of the edges and the neurites have dramatically more kinks.

After transfecting cells with EHR61L clear neurites could be seen within 72 hours (Figure 3.7). These neurites were very different from those caused by NGF or H-Ras 61L, but appeared to be much more similar to those caused by H-Ras Ext 61L. This indicates that possibly a non-standard pathway is being activated and should be investigated further. EHRwt showed no differentiation (data not shown).
Figure 3.7 – Typical cells after activation with NGF, or 4 days after transfection with H-Ras Ext61L, GFP H-Ras 61L or EHR61L.

**Loss of anchorage-dependant growth**

NIH 3T3 cells that were stably expressing Ras were plated in soft agar in order to assess their ability to grow in an anchorage-independent manner. Anchorage-independent growth is a hallmark of transformed cells, differing from normal culture-grown cells, which must be adherent to the bottom of the culture dish in order to grow.

Three different stably expressing cell lines were plated in the soft agar, GFP H-Ras 61L, EHR61L and EHRwt. The EHRwt formed a very low level of colonies (see Figures 8a and 9), 11.21% of the cells showed growth. The EHR61L (Figures 8b and 9) and the GFP H-Ras 61L (Figures 8c and 9) both showed comparable levels of growth in the soft agar, with 59.15% and 62.69%, respectively, of the cells growing after normalization based upon the
number of cells expressing the desired protein. Even though there was some growth seen in the EHRwt, the level is significantly lower than that of either activated Ras protein. It should also be noted that the colonies formed by EHR61L were smaller and slower growing than those from H-Ras 61L.

![Image of photographs](image-url)

Figure 3.8 – Photographs of (A) EHRwt, (B) EHR61L, (C) GFP H-Ras 61L after growing in soft agar for 14 days.

![Image of graph](image-url)

Figure 3.9 – Percentages of cells that grew in soft agar, after correction for the total number of cells expressing each protein.
Another assay for anchorage-independent growth is a focus assay. Transformed cells will continue to grow, even under confluent conditions, by growing on top of each other in a pile called a focus. Untransformed cells will be growth inhibited upon contact with neighboring cells. Thus it is possible to quantify the level of transformation by the amount of foci and compare it to a standard. After transiently transfecting NIH3T3 cells with either v-H-Ras (a viral, active form of H-Ras), EHR61L or EHRwt, the cells were allowed to grow. At a reasonable time point the foci were quantified and it was found that EHR61L formed a significant amount of foci, although the number of foci was less than those caused by H-Ras 61L. EHRwt gave no foci. (personal communication, B. Willumsen)

Discussion

Based upon the above data, several important conclusions can be drawn. First is that both EHRwt and EHR61L localize to the plasma membrane, in non-DRM regions. Strong evidence supporting this has been seen in a number of different cell types, and by using multiple techniques. Both proteins behave exactly as one would expect of non-DRM proteins.

Certainty of the localization of EHR proteins is vital for interpreting their biological activity. Despite its non-DRM status, EHR61L is biologically active. This was shown by morphology, anchorage-independent growth (soft agar and focus assays), and its down regulation of caveolin in NIH3T3 cells, as well as the differentiation of PC-12 cells. In these ways, EHR61L is very similar to H-Ras 61L.

While the activation seen by both EHR61L and H-Ras 61L is very similar, there are some significant differences. Cells expressing EHR61L are generally slower growing than their H-Ras 61L counterparts. This was seen in both NIH3T3 cells and PC-12 cells. In the
soft agar assay the EHR61L cell clumps were considerably smaller than those caused by H-Ras 61L. In PC-12 cells there was clear neurite formation, but it often took 24 hours longer to appear.

The other major difference is in the phenotype of PC-12 cells after differentiation. H-Ras 61L gives few, very straight neurites per cell. EHR61L on the other hand gives many outgrowths, most of which are kinked. This phenotype is much more similar to the one given by H-Ras Ext 61L. This in interesting since H-Ras Ext 61L is strongly associated with DRM (2). This may imply that it is the cycling between DRM and non-DRM regions that is important for normal Ras functions.

An interesting question was also answered by EHRwt. It is feasible that by permanently localizing H-Ras in non-DRM regions of the membrane that the EHRwt protein could gain activity by not cycling between GTP and GDP bound states. This would mean that it would stay in the GTP bound form and thus become constitutively active. It turns out that this is not the case. There is no elevated level of activity of EHRwt over H-Ras wt.

Even though Ras signaling may be aberrant when the protein is specifically excluded from DRM, it is still present. This is important for understanding how Ras interacts with the plasma membrane and its microdomains. Any information we can glean about Ras and its role in the cell will eventually lead to the development of methods to help control its signaling, and thus its sometimes disastrous effects on the human body.

References


CHAPTER 4: GENERAL CONCLUSIONS

The study of Ras is important for the future treatment of diseases which are caused by its oncogenic counterparts. It is necessary to learn everything that can be learned about it in order to be able to best treat these life threatening diseases. One of the characteristics that researchers are still trying to grasp is that of the address of Ras. Just as it is important to know the address of a person to whom you wish to send a package, it is just as important to know the address of Ras if one desires to treat it.

The research examined above in detail has been an attempt to look at a possible address of H-Ras, in this case localization outside of DRMs. The question at hand is if H-Ras can live and work outside of the shelter of a DRM. While the first attempt at finding a non-DRM was not successful, the EHR proteins definitively show that not only can H-Ras live outside of a DRM, but it thrives. EHR61L is able to do the same or similar work as its non-transmembrane counterpart: the work of differentiating PC-12 cells, transforming NIH3T3 cells, growing foci and continuing to grow even upon placement in soft agar.

The foundation has been laid for looking at Ras plasma membrane interactions on a much deeper level. From here it will be important to closely examine the Ras signaling pathways to determine what, if any, differences there between EHR61L, H-Ras 61L and Ext H-Ras 61L (a strong DRM Ras). One theory is that EHR61L is superactive. This can be looked by looking at the levels of phosphorylation of its downstream effectors. Ras activates many pathways, including at least one for apoptosis, so superactivity or preferential of an apoptotic pathway might explain why EHR61L lags behind H-Ras 61L in growth. One way
of looking at this would be to compare the number of G418 colonies formed by each protein. This would show if there is toxicity caused by the EHR protein.

A solid foundation has been laid by this research into investigating the role of DRMs in Ras cellular function. From here it will be important to build upon that foundation and learn as much as we can about these interactions. It is through this learning that we will hopefully gain the knowledge needed to effectively treat human tumors that are caused by oncogenic Ras.