The Role of ERp57 in Hras Intracellular Trafficking and Function.

Jaime Lyn Parman
East Tennessee State University

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The Role of ERp57 in Hras Intracellular Trafficking and Function

A thesis
presented to
the faculty of the Department of Biochemistry
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Master of Science in Biomedical Science

by
Jaime Parman
December 2003

Dr. Antonio Rusinol, Chair
Dr. Douglas Thewke
Dr. Robert Schoborg

Keywords: ras proteins, farnesylation, palmitoylation, polybasic domain,
intracellular traffic, ERp57, CAAX box
ABSTRACT

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by

Jaime Parman

Ras is a central player in signal transduction that mediates cellular proliferation and differentiation. Recent evidence has shown that lipid and non-lipid modified domains participate in Ras traffic and that plasma membrane association is mediated by vectorial vesicular transport from the endomembrane system. ERp57, an ER chaperone, has been shown to specifically bind farnesylated Hras but not non-farnesylated Hras. The objective of this study was to determine if ERp57 participates in Ras trafficking and function. First, the effect of ERp57 knock down by siRNA technology on Hras function was studied; there was a reduction in ERp57 cellular levels that led to a decrease of active ras. Second, specific anti-ERp57 antibodies were delivered into 3T3 cells expressing GFP-ras chimeras to observe the effect on intracellular trafficking. Anti-ERp57 antibodies blocked Hras plasma membrane localization but not Kras suggesting that ERp57 may be involved in Hras intracellular trafficking and function.
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Antonio Rusinol. Your guidance and devotion to your students and your career encouraged me to attend graduate school. When I thought graduate school could never be a possibility, you gave me the encouragement and support I needed to achieve my goals. With your continued support and friendship, I was able to succeed as a graduate student. I can honestly say had I not met you I would not be the scientist that I am today. I am truly thankful for everything you have done for me.

I would also like to extend my thanks to my committee members, Dr. Douglas Thewke and Dr. Robert Schoborg. Your advice and expertise has helped me tremendously. I appreciate the time and effort you have both spent in helping me complete my graduate degree.

I would also like to thank my family and friends. Your continuous support and encouragement have given me the strength to finish this long journey.
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CHAPTER 1
INTRODUCTION

Ras is a monomeric GTP-binding protein that is a member of the G-protein superfamily. Ras serves as a molecular switch that alternates between the active form bound with GTP and the inactive form bound with GDP. Mutations that constitutively activate ras proteins have been detected in 30% of all human cancers (Sinensky 2000a). The ras family of proteins is a central player in the transduction of signals that mediate cellular proliferation and differentiation. Ras mitogenic signal transduction has been implicated in many cancers, due to either a direct mutation of the ras protein, or the loss of functioning GTPase activating proteins (GAP), which terminate the signaling of active ras.

There are 3 major isoforms of ras, Hras, Nras, and Kras4B. All 3 isoforms have a 90% sequence homology. Hras, Nras, and Kras contain highly conserved domains that interact with effectors, exchange factors, and guanine-nucleotides. The area in which the isoforms differ greatly is the hypervariable region, which includes the last 23-25 carboxy-terminal amino acid residues of the protein. The hypervariable region contains the signal sequences that are required for ras plasma membrane localization. There are 2 domains of the hypervariable region, the linker domain and the membrane-targeting domain. The membrane-targeting domain contains the two signal sequences that cooperate in targeting ras to the plasma membrane (Prior and Hancock 2001). The first signal sequence is the same for all 3 isoforms of ras, which is prenylation, the sequential post-translational modification of the CAAX box. The second signal sequence in Hras and Nras is the palmitoylation of cysteines and a polybasic domain in Kras (Figure 1). There is evidence that the linker domain contains plasma membrane targeting information (Prior et al. 2001). The linker domain also regulates the interaction of Hras with lipid rafts in the plasma membrane and plays a role in regulating the interactions with effector proteins (Jaumot et al. 2002).
Plasma membrane localization is essential for the biological activity of ras (Willumsen et al. 1984). Therefore, the mechanism of ras plasma membrane localization has attracted considerable attention. The details of ras intracellular trafficking to the plasma membrane are not entirely understood. However, a model for ras trafficking has been proposed and is supported by compelling data (Figure 2)(Apolloni et al. 2000).
As mentioned previously, there are 2 signal sequences that are required and must cooperate for plasma membrane localization of ras. The first signal, the CAAX box (C=cysteine, A=aliphatic amino acid, X=serine or methionine) is common to all 3 forms of ras and is located at the C-terminus. The CAAX box is sequentially modified, as the cysteine is farnesylated by an enzyme known as farnesyltransferase (Reiss et al. 1990). Ras then travels to the cytosolic side of the endoplasmic reticulum (ER) where an endoprotease, RCE1, cleaves AAX, the last 3 residues from the ras protein, leaving the farnesylated cysteine at the C-terminus (Boyartchuk et al. 1997). The C-terminal cysteine is then carboxymethylated by a prenylcysteine carboxymethyltransferase (pcCMT) (Dai et al. 1998). The processed CAAX box must now act
in conjunction with a second signal to target ras to the plasma membrane (Choy et al. 1999; Apolloni et al. 2000; Hancock 1990). Kras is thought to leave this trafficking pathway at this point because it contains a polybasic domain, a stretch of 6 lysines (175-180), in the hypervariable region, as its second signal (Hancock 1990). Hras and Nras are further modified to produce the second signal, the palmitoylation of cysteine 181 in Nras or cysteines 181 and 184 in Hras, which are found in the hypervariable region of the protein (Hancock 1990). Hras and Nras then enter the secretory pathway by trafficking through the Golgi to the plasma membrane (Choy et al. 1999; Apolloni et al. 2000), whereas, Kras bypasses the Golgi and traffics to the plasma membrane through an unidentified pathway (Apolloni et al. 2000). Since the CAAX-processing enzymes are all ER residents (Dai et al. 1998) and recent data suggest that palmitoylation occurs at the ER (Choy et al. 1999), the final processing of the CAAX box must occur on the cytosolic face of the ER membranes. However, this raises the question of how trafficking of ras proteins from the ER to the plasma membrane occurs after the post-translational modifications. Recent studies, using green fluorescent protein (GFP) fused to ras protein creating Ras-GFP chimeras have shown that Nras and Hras are found throughout the secretory pathway (Choy et al. 1999; Apolloni et al. 2000). To test whether GFP-Nras and GFP-Hras use vesicular transport to access the plasma membrane experiments were conducted using Brefeldin A, a drug that disassembles the Golgi apparatus. The results of these experiments revealed a pronounced accumulation of these proteins in the ER (Choy et al. 1999; Apolloni et al. 2000), indicating that vesicular transport is required for plasma membrane localization of Hras and Nras. A 15°C temperature block, which impairs transport from the ER to the Golgi, was also used to compare the trafficking of Hras and Kras (Choy et al. 1999). These results demonstrated an accumulation of Hras in the intermediate compartment between the ER and the
Golgi. In contrast, however, there was no accumulation of Kras, presumably due to the polybasic domain excluding Kras from the Golgi (Apolloni et al. 2000). Because the temperature block or treatment with Brefeldin A did not affect the trafficking of Kras to the plasma membrane, it can be concluded that palmitoylated Hras and Nras proteins use vesicular trafficking through the secretory pathway to access the plasma membrane, whereas Kras does not (Choy et al. 1999; Apolloni et al. 2000). Although Hras and Nras use vesicular transport to access the plasma membrane, they are not traditionally packaged on the inside of the vesicles; instead, Hras and Nras must be transported on the cytoplasmic face of the transport vesicles (Choy et al. 1999). This is where the differential role of the second signal (palmitoylation or a polybasic domain) may become critical in signaling release from the ER compartment (Apolloni et al. 2000).

Recent evidence of the role of the carboxy-terminal amino acids of Hras in plasma membrane localization shows that both lipid-modified and non-lipid-modified domains play a role in Hras movement throughout the cell and plasma membrane association (Sinensky 2000a). In addition, Choy et al. discovered that the C-terminus CAAX box is sufficient to target proteins to the endomembrane system and that localization to the plasma membrane requires a second signal (Choy et al. 1999). Given that plasma membrane localization of Hras is mediated by vesicular transport suggests that prenylated cytosolic Hras interacts with specific proteins in the endomembrane system, which makes Hras accessible for further processing. Because the plasma membrane is the ultimate destination of Hras, it has been hypothesized that there is a protein that recognizes both the plasma membrane and the farnesylation of Hras (Rusinol et al. manuscript under revision). Evidence to support this hypothesis for the existence of such a protein has been presented by Siddiqui et al. (1998). Siddiqui et al. proposed the “two-site” hypothesis where
partner proteins specifically bind to prenylated proteins through the recognition of both the lipid substituents and the primary sequence of the prenylated protein partner. Previous work by Rusinol et al. (manuscript under revision) used a combination of ion exchange and affinity chromatography to identify a prenylation-dependent Hras binding protein, which was confirmed by ligand blotting (Rusinol et al. manuscript under revision). A 58kDa band was observed as having the highest binding activity to farnesylated Hras. This 58kDA band showed nearly a 100% sequence homology with a previously cloned protein, which has been identified by multiple laboratories (Mazzarella et al. 1994; Bourdi et al. 1995,). This protein has been given multiple names, P58, ER60, Grp58, and ERp57, Rusinol et al. refers to this protein as ERp57 as will I (Rusinol et al. manuscript under revision). Bourdi et al. isolated the human clone of ERp57 (1995). ERp57 is a member of the protein disulfide isomerase (PDI) family of enzymes. ERp57 is found mostly in the ER, but recent evidence has indicated that it is also present in the cytosol, plasma membrane and nucleus (Johnson et al. 1992; Guo et al. 2002; Coppari et al. 2002). PDI, the best-known member of the family, and ERp57 have been shown to be involved in the proper folding and formation of disulfide bonds of proteins that are synthesized in the rough ER. They also function as chaperones and redox-catalysts, which are essential for cell viability (Turano et al. 2002, Coppari et al. 2002). Rusinol et al. (2003) has shown that ERp57 specifically binds to farnesylated Hras but not non-farnesylated Hras. In this study ERp57 has been described as a functional partner for Hras, which is consistent with the “two-site” hypothesis. The objective of the present investigation was to test the hypothesis that ERp57 participates in Hras trafficking to the plasma membrane and therefore is necessary for Hras function. Primarily two approaches were used to test this hypothesis. The first approach was to study the effect of an ERp57 knock down by siRNA technology on Hras function. In this
system, the reduction of ERp57 cellular levels led to a decrease of GTP-bound ras as shown by interaction with raf1. The second approach was to deliver specific anti-ERp57 antibodies into live 3T3 cells expressing GFP chimeras with ras and control proteins and then observe the effect on intracellular trafficking. In this system, anti-ERp57 antibodies blocked Hras localization to the plasma membrane but not Kras, suggesting that ERp57 may be involved in Hras intracellular trafficking and function.
CHAPTER 2  
MATERIALS AND METHODS

Plasmids

pEGFP was from Clontech; Kras-GFP was a gift from Dr. Patrick Casey, Duke University. Hras-GFP fusion protein was created from Hras in pSP64 (Promega, Madison, WI) by cutting with HindIII, which gives 2 fragments. The larger of the 2 fragments (3.6Kb) was gel purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Digestion of the 3.6Kb fragment with restriction enzyme BamHI gave 2 fragments in agarose gel electrophoresis, a 686bp and a 2.3Kb fragment. The 686bp fragment (ras) was gel purified and directionally ligated into the vector pEGFP-C3 (Clontech, Palo Alto, CA) to yield the finished construct, which was confirmed by restriction enzyme analysis.

DNA and Protein Transfections

DNA transfections were done by using GeneJammer™ from Stratagene (La Jolla, CA). Protein transfections were done using ProJect™ from Pierce (Rockford, IL) or Bioproter™ from Sigma (St. Louis, MO). All transfections were performed according to manufacturer’s instructions.

Electrophoresis and Transfer to PVDF Membranes

One-dimensional SDS-PAGE was performed under reducing conditions on a 4-12%gradient Bis-Tris NuPAGE precast slab gel from Invitrogen (Carlsbad, CA). Electrophoresis was performed at 200 volts for 45 minutes using the Novex Shurelock System from Invitrogen. Broad range pre-stained protein markers from Invitrogen were used to estimate the relative molecular masses of the proteins. Electrophoretic transfer to PVDF membranes
(Millipore) was performed in 20mM Tris-HCL and 0.15M glycine 10% methanol buffer, pH 8.1, for 60 minutes at 60 volts.

**Western Blot**

After transfer to PVDF, membranes were blocked for 1 hour in 5% nonfat milk in tris-buffered saline containing 0.1%Tween 20 (TBS-T). PVDF membranes were then probed with antibodies. Membranes were incubated with primary antibody; diluted 1:1000 in 5% nonfat milk in TBS-T solution, either anti-ERp57 (Stressgen, Victoria, BC), anti-Hras (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Kras (Calbiochem, San Diego, CA) or anti-Pan-ras (Oncogene™, San Diego, CA), for 1 hour and washed 3 times for 5 minutes each. After washing, membranes were then incubated with secondary antibody; diluted 1:25000 in 5% nonfat milk in TBS-T solution, either goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA) or goat anti-mouse IgG-HRP (Pierce, Rockford, IL), for 1 hour and washed as before. Antibody-bound proteins were detected by chemiluminescence from Pierce. The membranes were then exposed to X-ray film to visualize the proteins.

**Immunofluorescence**

3T3 cells were grown on glass coverslips overnight. After washing with PBS, cells were fixed in freshly prepared 4% paraformaldehyde in PBS, pH 7.3. After fixation, cells were incubated for 1 hour with anti-human ERp57 specific rabbit polyclonal antibodies (Stressgen, Victoria, BC), diluted 1:100 in 5% BSA in PBS, rinsed 3 times with PBS, and then stained for 1 hour with FITC-conjugated anti-rabbit IgG or Texas Red-conjugated anti-rabbit IgG (Stressgen, Victoria, BC), diluted 1:500 in 5% BSA in PBS. For co-localization experiments, cells were also incubated with mouse monoclonal anti-PMCA ATPase antibodies (Affinity BioReagents, Golden, CO) and Texas Red- conjugated anti-mouse IgG (Stressgen, Victoria, BC). Cells were
then mounted in a special medium for fluorescence (Prolong Anti-fade, Molecular Probes) and observed. Confocal images were obtained by digital deconvolution of 10-slice stacks acquired on a Nikon Diaphot 200 equipped with a Photometrics Sensys cooled CCD digital camera or Nikon D100, Oncor Z-drive and Oncor image software.

Co-immunoprecipitations

Co-immunoprecipitation assays were performed using Seize™ Coated Plate immunoprecipitation Kits from Pierce (Rockford, IL) according to manufacturer’s instructions. 3T3 cells were grown briefly in DMEM 10% FBS medium and then scraped and solubilized in a buffer containing Triton X-100 to a protein concentration of 1mg/ml. Cell lysates were then incubated with primary antibodies, either anti-Hras or anti-Kras, immobilized onto 96-well plates coated with protein A/G. After binding, washing, and elution, the co-presence of ras and ERp57 in the precipitates was examined by immunoblotting.

VSVG Intracellular Trafficking

Dr. Jennifer Lippincott-Schwartz (National Institutes of Health, Bethesda, MD) kindly provided a vector expressing a temperature sensitive VSVG-GFP chimera (Presley et al. 1997). 3T3 cells were transiently transfected with VSVG-GFP and cultured at 40°C. After 16 hours at 40°C, cells were transfected with anti-ERp57 antiserum and incubated for 6 hours, then switched to 32°C for 3 hours. VSVG-GFP localization was then determined by fluorescence microscopy and digital deconvolution as described previously.

siRNA Construction and Transfections

pSilencer™ from Ambion (Austin, TX) was used in siRNA experiments. Target sites for siRNA in ERp57 were selected using Ambion’s siRNA target finder and design tool that follows the current guidelines for siRNA design. Based on the target sequences, 5 complementary
oligonucleotide pairs (4 with correct sequences plus 1 randomized sequence) were custom synthesized. The oligos were then allowed to anneal, creating ApaI and EcoRI overhangs. siRNA generating vectors were constructed by ligating the annealed oligos into linearized pSilencer vector. The finished siRNA plasmid was transfected into 3T3 cells as described above.

Detection of Ras Activation

Active ras in 3T3 cell lysates was detected using the EZ-Detect™ Ras Activation Kit from Pierce (Rockford, IL). Cell lysates were treated according to manufacturer’s instructions. Then the pulled-down active ras was detected by Western Blot using anti-ras antibody.
CHAPTER 3

RESULTS

Previous experiments by Rusinol et al. used HeLa cells in ERp57 localization experiments. The Ras-GFP chimeras that were used in this study are stably expressed in 3T3 cells, because 3T3 cells are larger and allow for easier observation of intracellular trafficking. Therefore, it was necessary to determine where ERp57 localizes in 3T3 cells. This was determined by standard immunofluorescence, which is explained in materials and methods. The results show that ERp57 is localized mostly in the ER but is also present in the plasma membrane of 3T3 cells as indicated by the arrows in figure 3.
Figure 3: Detection of ERp57 in 3T3 Cells. A representative cell of several experiments is shown. Localization of ERp57 was observed mostly in the ER but is also seen in the plasma membrane of 3T3 cells as indicated by the arrows.

To confirm that ERp57 was in fact localized in the plasma membrane, a co-localization experiment was performed as a control, using an anti-PMCA ATPase antibody as a plasma membrane marker. Figure 4 shows the results of the co-localization between ERp57 and the calcium pump of the plasma membrane (PMCA ATPase) in 3T3 cells. PMCA-ATPase showed a typical plasma membrane staining whereas ERp57 showed a cytoplasmic distribution (mostly ER) but also showed plasma membrane staining which is seen with the co-localization of PMCA-ATPase in many areas. These results are consistent with partial ERp57 localization to the plasma membrane as previously reported in other cell types (Wyse et al. 2001; Guo et al. 2002) as well as the isolation of ERp57 from our purified plasma membrane preparations.
Since trafficking of Hras begins in the ER and terminates in the plasma membrane, the co-localization of ERp57 at the plasma membrane is consistent with the hypothesis that ERp57 plays a role in Hras trafficking.

Figure 4: Subcellular Localization of ERp57 in 3T3 Cells. The subcellular distributions of ERp57 and plasma membrane calcium ATPase (PMCA-ATPase) were determined in 3T3 cells by indirect immunofluorescence as described in Materials and Methods. Cells were visualized by fluorescence microscopy and digital deconvolution. Images represent single digitally deconvolved sections. Shown is a representative cell of several experiments. The panel on the right is an overlay of the ERp57 and PMCA ATPase images and shows numerous areas of co-localization in the plasma membrane (yellow).
Data from Rusinol et al. have shown that ERp57 and Hras interact in vitro, but not in vivo (Rusinol et al. manuscript under revision). To determine if ERp57 is able to associate with Hras in vivo, co-immunoprecipitation experiments were performed. As described in materials and methods, 3T3 cells were solubilized in Triton-X100 buffer and centrifuged to remove the insoluble fraction. The cell lysate (soluble fraction) was immunoprecipitated with monoclonal anti-Hras and anti-Kras antibodies. The immunoprecipitates were then analyzed by immunoblotting for the presence of Hras, Kras and ERp57. Figure 5 shows that the anti-Hras antibodies immunoprecipitated Hras and ERp57 (Figure 5A) and that the anti-Kras antibodies immunoprecipitated Kras but not ERp57 (Figure 5B). The specificity of this reaction was confirmed by showing that the anti-ras antibodies did not precipitate other molecular chaperones, such as GRP94 and PDI (Figure 5C).
Figure 5: Co-immunoprecipitation of ERp57 and Ras. 3T3 lysates were immunoprecipitated with rabbit polyclonal anti-Hras (A) rabbit polyclonal anti-Kras (B) or mouse monoclonal anti-pan-ras antibodies (C). A) The immunoprecipitates from anti-Hras antibodies were separated by SDS-PAGE, blotted, and probed with rabbit anti-Hras and rabbit anti-ERp57 antibodies. B) The immunoprecipitates from anti-Kras antibodies were probed with rabbit anti-Kras and rabbit anti-ERp57 antibodies. A control lane in which anti-ras antibodies were omitted showed no detectable ERp57. TCL: positive control with equivalent amount of total cell lysate loaded on the gel. NR: TCL after immunoprecipitation with a pre-immune antibody. Anti-H: TCL after immunoprecipitation with anti-Hras antibodies. Anti-K: TCL after immunoprecipitation with anti-Kras antibodies. IP (A): ERp57 and Hras were immunoprecipititated with anti-Hras antibodies. IP (B): Kras was immunoprecipitated with anti-Kras antibodies, but no ERp57 was present. C) The immunoprecipitates formed by treatment with anti-pan-ras antibodies were tested for the presence of other chaperones. IP: immunoprecipitates were blotted and probed for GRp94 and protein disulfide isomerase. TCL: equivalent amount of total cell lysate.
Role of ERp57 in Hras Trafficking

In order to test the hypothesis of the involvement of ERp57 in Hras intracellular trafficking and function, we reduced the cellular levels of ERp57 using 2 methods, siRNA technology and peptide mediated protein delivery. siRNA is a technique that is used to reduce the expression of a specific gene in living cells by introducing a homologous dsRNA. Once inside the cell, the dsRNAs are cleaved into short 21-25 nucleotide siRNAs. The siRNAs assemble into an RNA-induced silencing complex (RISC) where it is unwound and activated. This allows the siRNA antisense strand and mRNA to bind to their complementary transcript by base pairing interactions. Gene silencing is the result of sequence specific cleavage of the mRNA-siRNA-RISC complex. This technology was used to study the functional consequences of reducing ERp57 expression. There are 5 ways to produce siRNAs; we chose to use a siRNA expression plasmid, the pSilencer™ vector from Ambion (Austin, TX). Target sequences were designed to reduce the cellular expression of ERp57. The knockdown experiments were performed by ligating the target sequences into the pSilencer™ vector then transfecting 3T3 cells and 3T3 cells expressing GFP-Hras chimeras with the pSilencer™ construct. The total cell expression of ERp57 in the transfected cells was then analyzed by Western blot (Figure 6). Figure 6 shows that the cellular levels of ERp57 in 3T3 cells are reduced by siRNA expression over time. The transfected cells were observed by fluorescence microscopy at the different time points to observe the effect of reduced ERp57 levels, by siRNA expression, on Hras plasma membrane localization. Figure 7 indicates that the reduction of ERp57 blocks the intracellular trafficking of Hras-GFP to the plasma membrane.
Figure 6: ERp57 Levels in 3T3 Cells Are Reduced by siRNA Expression. Cells were transfected with empty pSilencer vector as a control and with the pSilencer-ERp57 siRNA construct then lysed at different time points. Total cells lysates were separated by SDS-PAGE and then analyzed by Western Blot using anti-ERp57 antibodies. The level of ERp57 in 3T3 cells was drastically reduced, 48 hours after transfection, by siRNA expression, as compared to the control where the level of ERp57 is constant.

The transfected cells were observed by fluorescence microscopy at the different time points to observe the effect of reduced ERp57 levels, by siRNA expression, on Hras plasma membrane localization (Figure 7). Figure 7 indicates that the reduction of ERp57 blocks the intracellular trafficking of Hras-GFP to the plasma membrane.
Figure 7: Reduction of ERp57 Expression Blocks Hras-GFP Intracellular Trafficking. 3T3 cells stably expressing Hras-GFP were transfected with the pSilencer-ERp57 siRNA randomized sequence (control) and the pSilencer-ERp57 siRNA construct. Cells were observed by fluorescence microscopy. Shown is a representative cell of several experiments. This figure shows that 48 hours after transfection, Hras-GFP traffic to the plasma membrane is blocked as compared to the control where there is clear plasma membrane localization.

Because the reduced levels of ERp57 had such a dramatic effect on Hras-GFP trafficking to the plasma membrane, we examined whether the reduction ERp57 expression had any effect on the activation of ras in 3T3 cells. The EZ-Detect™ Ras Activation Kit from Pierce (Rockford, IL) was used for this experiment. The kit uses the Ras-binding domain of Raf1, and specifically “pulls down” active ras (GTP bound) out of cell lysates. The proteins that were “pulled down” were separated by SDS-PAGE and then analyzed by Western Blot using anti-ras antibodies, which is shown in figure 8.
Figure 8: Reduction of ERp57 Expression Hinders Ras Activation. Cells were transfected with empty pSilencer vector as the control and the pSilencer-ERp57 siRNA construct. Cells were lysed at different time points then analyzed by Western Blot using anti-ras antibodies. The amount of active ras in the cell lysate was then determined using the Raf-1 binding domain of ras. This figure shows that overtime the amount of active ras in 3T3 cells was decreased due to reduction of ERp57 expression.

In the second approach, the ability of ERp57 antibodies to inhibit the trafficking of Hras was confirmed in whole cells. This was done by examining the effect of transfection with ERp57 antibodies on the intracellular localization of Hras-GFP chimeras expressed in 3T3 cells. ERp57 antibodies were introduced into the cells by a peptide carrier technology (Morris et al. 2001), in which proteins are delivered into the cytosol of transfected cells. Figure 9 shows the effect of ERp57 antibodies on the intracellular localization of GFP chimeras with full-length Hras and an Hras 21a.a. C-terminal fragment (HrasCT). Control experiments were done by transfecting the Hras-GFP or HrasCT-GFP cells with non-immune serum, which shows plasma membrane localization as well as cytoplasmic staining (Figure 9A, C). Our results were consistent with previous results reported by Choy et al. for expression of these GFP constructs in
3T3 cells (Choy et al. 1999). However, transfection with the ERp57 antibody resulted in the accumulation of Hras-GFP in intracellular vesicles, possibly the ER, in both constructs (Figure 9B, D).

Figure 9: Anti-ERp57 Antibodies Block the Trafficking of Full-length Hras and HrasCT-GFP to the Plasma Membrane. 3T3 cells stably expressing full-length Hras-GFP or HrasCT-GFP were transfected with anti-ERp57 antibodies. The location of the GFP chimeras was then assessed by fluorescence microscopy followed by digital deconvolution of the acquired images. Shown is a representative cell of several experiments. When cells expressing full-length Hras-GFP (A) or HrasCT-GFP (C) were transfected with the protein delivery reagent only, clear plasma membrane localization was observed. When cells were transfected with 2µg/ml of anti-ERp57 antibodies, traffic of both chimeras to the plasma membrane was completely blocked (B and D).
To examine the specificity of the inhibition of Hras trafficking by ERp57 antibodies, relative to another protein that uses the secretory pathway to access the plasma membrane, the effect of transfection with ERp57 antibodies on the trafficking of VSVG protein was examined. For the studying of this protein, we used a previously reported system (Presley et al. 1997), in which a temperature sensitive mutant of VSVG (tsVSVG) was used. At a nonpermissive temperature of 40°C the tsVSVG-GFP is unable to exit the ER (Figure 10A). However, at the permissive temperature of 32°C the tsVSVG-GFP is allowed to traffic using the secretory pathway to show clear plasma membrane and Golgi localization (Presley et al. 1997)(Figure 10C). The tsVSVG-GFP was transiently expressed in 3T3 cells then transfected with anti-ERp57 antibody (Figure 10B, D). Co-transfection with Texas-Red conjugated anti-rabbit IgG was used to monitor transfection of the antibody into individual cells. Texas-Red positive cells were then examined for the localization of the green tsVSVG. The results observed in Figure 10 show that the cells transfected with anti-ERp57 antibodies and switched to 32 °C had no effect on the transport of VSVG-GFP to the plasma membrane (Figure 10D).
Figure 10: Anti-ERp57 Antibodies Do Not Affect tsVSVG Traffic to the Plasma Membrane. 3T3 cells transiently expressing tsVSVG and cultured at 40 °C were transfected with pre-immune antiserum (not shown) or antiERp57 antibodies. Texas Red-mouse IgG was also included as a marker for transfection (A, C). Cells were incubated for 6h at 40 °C in regular growth medium. Cells were then maintained at 40 °C (B), or switched to 32 °C (D). VSVG-GFP traffic to plasma membrane was determined in Texas-red positive cells after 3h by fluorescence microscopy. Images represent single digitally deconvolved sections. Shown is a representative cell of several experiments. This experiment indicates that ERp57 antibodies did not block plasma membrane localization of tsVSVG at the permissive temperature (D).
As previously mentioned, Kras is farnesylated but contains a different second signal sequence from Hras for targeting the protein to the plasma membrane and does not traffic to the plasma membrane through the secretory pathway. Because ERp57 specifically recognizes farnesylated Hras, it was of interest to determine whether ERp57 played a role in Kras traffic as well. This would be allowed to happen if, for example, ERp57 had a role in transporting ras proteins between various ER enzymes that were involved in their post-translational modification. This idea was tested using the same peptide carrier technology as described above. ERp57 antibodies were transfected into 3T3 cells stably expressing Kras-GFP. Co-transfection with Texas-Red conjugated anti-mouse IgG was used to monitor transfection of the antibody into individual cells. Texas-Red positive cells were then examined by fluorescent microscopy to determine the effect on the localization of the Kras-GFP. The results shown in Figure 11 indicate that the transfection of Kras-GFP cells with ERp57 antibodies had no affect on the plasma membrane localization of Kras.
Figure 11: Anti-ERp57 Antibodies Do Not Affect Kras Traffic to the Plasma Membrane. 3T3 cells stably expressing Kras-GFP (A) were transfected with Texas-Red mouse IgG as a marker for transfection (B) and with anti-ERp57 antibodies (C). The red cells were then examined for the localization of the green Kras-GFP by fluorescence microscopy followed by digital deconvolution of the acquired images. Shown is a representative cell of several experiments. This experiment shows that blocking ERp57 function has no impact in Kras plasma membrane localization.

This is consistent also with the hypothesis that Hras and Kras traffic to the plasma membrane via different routes. Together with the results shown in Figure 9, these data strongly suggest a role for ERp57 in the specific targeting of Hras to the plasma membrane. This also suggests that although ERp57 recognizes the farnesylated Hras, ERp57 must interact with Hras somewhere within the secretory pathway because Kras is farnesylated as well, but ERp57 antibodies do not affect its trafficking. This result is consistent with our previous result shown in figure 5B, that Kras and ERp57 do not interact as shown by co-immunoprecipitation and ligand blot.
Previous studies reported from Siddiqui et al. suggested the “two site” hypothesis, where partner proteins specifically bind to prenylated proteins through the recognition of both the lipid substituents and the primary sequence of the prenylated protein partner (Siddiqui et al. 1998; Sinensky 2000b). The present study was motivated by this hypothesis, and supported by previous data, that post-translational lipidation of proteins serves to mediate heterodimeric protein-protein interactions (Rusinol et al. manuscript under revision).

Consistent with the “two-site” hypothesis, the current study describes a functional partner for Hras. ERp57 has been identified as the heterodimer for Hras because of its ability to bind farnesylated Hras relative to non-farnesylated Hras by ligand blotting. Data from previous work have shown that Hras and ERp57 interact in vitro, but do not prove that the 2 proteins associate in vivo in 3T3 cells or HeLa cells (Rusinol et al. manuscript under revision). The results of the co-immunoprecipitation experiments in this study (Figure 5), where the anti-Hras antibody precipitated ERp57, indicate that ERp57 and ras do interact in vivo in 3T3 cells. The siRNA experiments also indicate that there is a connection between ERp57 and ras localization because the reduction of cellular levels of ERp57 hindered the localization of Hras at the plasma membrane in Hras-GFP cells. This suggests that ERp57 may also be required for the correct folding of another protein that targets Hras.

Hras has been proven to traffic from the ER to the plasma membrane using the secretory pathway (Choy et al. 1999; Apolloni et al. 2000). The blockage of Hras trafficking by anti-ERp57 antibodies can be observed in vitro and in vivo. The specificity of this interaction was confirmed in respect to VSVG protein. The results of this experiment argue against the blockage
of Hras trafficking being a consequence of a global inhibition of the secretory pathway. Many laboratories have demonstrated that Hras plasma membrane localization is dependent on farnesylation (Willumsen et al. 1984; Cox et al. 1995). Other studies have also shown that mutations present in the hypervariable region of Hras do not affect its lipidation but do prevent correct targeting to the plasma membrane (Willumsen et al. 1996; Jaumot et al. 2002). However, based on our current study we cannot eliminate the possibility that the acyl group of Hras is the second recognition site for ERp57.

Our observations suggest a binding protein that mediates Hras trafficking, ERp57, that recognizes both the lipid moiety and possibly another structural element of Hras. The specificity for inhibition of Hras trafficking relative to Kras trafficking by ERp57 antibodies supports these observations. The CAAX box of Hras and Kras are both sequentially modified in the same manner; however, farnesylation alone is not sufficient enough to target the proteins to the plasma membrane. Hras and Kras both must ultimately arrive at the plasma membrane for function, but how they reach this final destination is very different from each other. It is believed that the second signal sequence in the hypervariable region, palmitoylation for Hras and Nras or the polybasic domain for Kras, serves as a sorting signal that specifies which pathway for the protein to take (Hancock et al. 1990; Apolloni et al. 2000). Although Hras and Kras have many similarities, Kras is known to traffic by a different mechanism from Hras (Thissen et al. 1997; Apolloni et al. 2000; Chen et al. 2000). Thus, we have demonstrated that the specificity for Hras trafficking on ERp57 compared to VSVG and Kras are consistent with the functional role of ERp57 as a chaperone molecule for Hras trafficking operating through the “two-site” recognition mechanism (Rusinol et al. manuscript under revision).
ERp57 has been shown to associate with other molecular chaperones such as, calnexin and calreticulin, this occurs in the lumen of the ER (Zapun et al. 1998). It has also been reported that ERp57 is associated with the plasma membrane lipid rafts (Guo et al. 2002). The lipid rafts present in the plasma membrane of cells have also been shown as an anchoring point for Hras in the plasma membrane (Prior et al. 2001). The interaction of ERp57 and Hras would not be expected to occur in the lumen of the ER because the CAAX processing and plasma membrane targeting of Hras takes place on the cytoplasmic side of the ER. This was demonstrated in the present study by the trafficking of full-length Hras and the C-terminal 21 a.a. of Hras was blocked by the delivery of anti-ERp57 antibodies into the cytosol of 3T3 cells. This suggests that the site of Hras-ERp57 interaction is located on the cytoplasmic side of the ER/Golgi or the cytosol (Rusinol et al. manuscript under revision).

The mechanism by which ERp57 participates in Hras traffic is not obvious from the present study. However, because the ERp57 antibodies do not interfere with the trafficking of Kras, it is unlikely that ERp57 has a role in CAAX processing. Therefore, it would be expected that ERp57 plays a role in the vesicular trafficking in the secretory pathway, but this also is unlikely because of the lack of effect of ERp57 antibodies on the trafficking of VSVG protein. Instead, the specificity of inhibition of Hras trafficking compared to that of Kras and VSVG by ERp57 antibodies indicates that ERp57 acts at one or more biochemically distinct steps in the Hras trafficking pathway (Rusinol et al. manuscript under revision). Given the steps that Kras and VSVG have in common with Hras trafficking, the possible site of action for ERp57 should occur after CAAX box processing and before entry into the secretory pathway at the Golgi. Hras trafficking through the secretory pathway requires CAAX processing and palmitoylation, to allow for the specific targeting of Hras to the caveolin-enriched plasma membrane microdomains.
(Li et al. 1996). In contrast, Kras traffics to the plasma membrane using a different route, which as we show does not require ERp57 function, and associates with the non-caveolin regions of the plasma membrane (Choy et al. 1999; Roy et al. 1999; Apolloni et al. 2000). However, because ERp57 has been proven to interact with lipid rafts in the plasma membrane (Guo et al. 2002) we are able to speculate the possibility that ERp57 could mediate the final destination of Hras to the caveolin-enriched plasma membrane microdomains (Rusinol et al. manuscript under revision). The mechanism by which ERp57 participates in Hras function can possibly defined by the isolation and characterization of the large vesicles that contain Hras and are known to accumulate upon ERp57 inhibition (Rusinol et al. manuscript under revision).

**Figure 12:** Model of Ras Trafficking with Potential Erp57 Interaction Sites. Based on our observations the potential sites for ERp57 to interact with Hras have been added to the ras trafficking model. Adapted from Apolloni A, Prior IA, Lindsay M, Parton RG, Hancock JF. H-ras but not K-ras traffics to the plasma membrane through the exocytic pathway. Mol Cell Biol. 2000 Apr; 20(7): 2475-87.


Sinensky M. Recent advances in the study of prenylated proteins. Biochim Biophys Acta. 2000a Apr12; 1484(2-3): 93-106. Review.


APPENDIX
LIST OF ABBREVIATIONS

a.a.     amino acid
BSA     bovine serum albumin
bp      base pair
DNA     deoxyribonucleic acid
DMEM    Dulbecco’s Minimal Essential Medium
dsRNA   double-stranded RNA
ER      endoplasmic reticulum
FBS     fetal bovine serum
FITC    fluorescein isothiocyanate
FTase   farnesyl transferase
GAP     GTPase activating protein
GDP     guanine diphosphate
GTP     guanine triphosphate
HrasCT  Hras 21aa C-terminal fragment
IgG     immunoglobulin G
IP      immunoprecipitate
Kb      kilobase
kDa     kilodaltons
mRNA    messenger RNA
oligo   oligonucleotide
PalmTase palmitoyl transferase
PBS     phosphate buffered saline
pcCMT   prenylcysteine carboxymethyltransferase
<table>
<thead>
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<tr>
<td>PDI</td>
<td>protein disulfide isomerase</td>
</tr>
<tr>
<td>PMCA ATPase</td>
<td>Calcium pump of the plasma membrane</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosyl methionine</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>TCL</td>
<td>total cell lysate</td>
</tr>
<tr>
<td>tsVSVG</td>
<td>temperature sensitive vesicular stomatitis virus glycoprotein</td>
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<td>vesicular stomatitis virus glycoprotein</td>
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VITA

JAIME LYN PARMAN

Personal Data: Date of Birth: August 25, 1975
Place of Birth: Greeneville, Tennessee
Marital Status: Single

Education: Public Schools, Greeneville, Tennessee
Walters State Community College, Morristown, Tennessee;
    General Studies, A.S., 1997
East Tennessee State University, Johnson City, Tennessee;
    Microbiology, B.S., 2001
East Tennessee State University, James H. Quillen College Of Medicine
    Johnson City, Tennessee;
    Biomedical Science with emphasis in Biochemistry and
    Molecular Biology, M.S., 2003

Professional Experience: Graduate Assistant, East Tennessee State University, James H. Quillen College of Medicine, June 2001- December 2003


Honors and Awards: First Place, Student’s Choice Award, Division II-Research Forum
East Tennessee State University, 2002.

Second Place, Division II-Research Forum
East Tennessee State University, 2002.