Signaling capabilities of a novel H-Ras mutant from the Golgi apparatus

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Signaling capabilities of a novel H-Ras mutant from the Golgi apparatus

by

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MASTER OF SCIENCE

Major: Biochemistry

Program of Study Committee:
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**General Introduction**

**Ras proteins**

The scientific community was first introduced to Ras in 1964. Jennifer Harvey reported that sarcomas formed in new born rodents after exposure to murine leukemogenic virus plasma taken from a leukemic rat [1]. It was found later that the properties that induced the tumor formation was actually part of the rat genome. The gene and its protein product were named H-Ras (Harvey rat sarcoma) after the discoverer and origin [2]. In later years, the Kirsten (K-Ras) and neuroblastoma (N-Ras) isoforms of Ras were discovered [3-5]. Ultimately, the Ras genes were isolated from human cancer cells [6-8]. A mutated form of Ras has been implicated in 35% of lung tumors, 40% of colon tumors, 60% of thyroid tumors and 90% of pancreatic cancers [9].

Four different isoforms of Ras are encoded in the human genome: H-Ras, N-Ras, K-Ras 4A and K-Ras 4B. K-Ras 4B is universally expressed and K-Ras 4A is the alternatively spliced isoform [10]. The four Ras isoforms are highly homologous in amino acid sequence. All four isoforms of Ras are 100% conserved in the first 86 residues and 79% conserved in residues 86-164. The Ras isoforms depart in sequence conservation in the 25 C-terminus amino acids also known as the hypervariable domain. All Ras isoforms have a CAAX box motif at the C-terminus where C is cysteine, A is an aliphatic amino acid and X is any amino acid. The CAAX box motif is responsible for initiating the post-translational modifications that occur on all Ras proteins [10, 11].
Ras the GTPase

Ras is a 21kDa monomeric GTPase that cycles between the active GTP state and inactive GDP state. The intrinsic GTPase activity of Ras is very poor, hydrolyzing GTP at a rate of about $10^{-4}$ s$^{-1}$. The GTPase activity is accelerated $10^5$ fold by the GTPase activating protein (GAP). Because the level of GTP in the cell is nearly 10 times that of GDP, Ras will naturally be bound to GTP. In order to accelerate the exchange of GDP for GTP, guanine nucleotide exchange factor (GEF), is required to dissociate the GDP. The three structural regions involved in GTPase activity are: the P-Loop, switch 1 and switch 2. The amino acid mutations of G12V and Q61L occur in the P-Loop and switch 2 regions, respectively. It is these mutations that hinder the GTPase activity and incur upon Ras its oncogenic properties [12].

Ras effectors

When Ras is bound to GTP it is able to associate with its effectors. The most studied pathway to date has been the mitogen-activated protein kinase/extracellular regulated kinase (MAPK/Erk) cascade. The MAPK, p42 and p44, are known as Erk1 and Erk2 respectively and are so similar that they are considered nearly functionally identical [13]. The main components of the MAPK cascade consist of Raf-1, MAP/Erk kinase (MEK) and Erk1/2. The GTP bound Ras protein binds Raf-1 via its Ras binding domain (RBD). Raf-1, a serine/threonine kinase, then phosphorylates and activates MEK. MEK, a dual specificity tyrosine/threonine kinase, phosphorylates and activates Erk1 and Erk2. Erk1 and Erk2 are serine/threonine kinases that form dimers when phosphorylated and translocate to the nucleus where they phosphorylate the Ets family of transcription factors [14]. Recently, the scaffold
protein kinase suppressor of Ras-1 (KSR-1) was demonstrated to be an important part of MAPK signaling. KSR-1 is able to bind Raf-1, MEK and Erk enabling a more controlled signal simply by having all of the effectors in the same area. More importantly, the scaffold protein, similar expression to FGF (fibroblast growth factor) genes (Sef), was found bound to MEK and Erk on the Golgi [15]. This added to the growing list of evidence supporting the importance of signal transduction from the Golgi.

Three other well characterized effectors of Ras are MEKK, phosphatidyl inositol-3-kinase (PI3K) and Ral GEFs. The MEKK are involved in the phosphorylation c-Jun N-terminal kinase (JNK) which lead to the phosphorylation of the c-Jun transcription factor [16]. PI3K phosphorylates Akt, otherwise known as protein kinase b (PKB), which mediates antiapoptotic signals. PI3K also links the Ras pathway to Rho/Rac proteins by a mechanism that has yet been elucidated [17]. The Ral GEFs interact with Ral GTPases, a relative of Ras that is involved in activating phospholipase D1. Some of the Ral GEFs are: Ral-GDS, RLF and Rgl [18]. A recent report has implicated RalGDS in having a role in human oncogenesis[19].

**Posttranslational processing**

Ras begins its life in the cell by being translated on free ribosomes in the cytosol. The C-terminal CAAX box motif is then recognized by farnesyl transferase (FTPase) where it binds Ras with nanomolar affinity. The FTPase then attaches a 15 carbon isoprenoid, farnesyl diphosphate, to the cysteine of the CAAX box motif via a very stable thioether bond [20]. With a lipid tethered to the C-terminus, Ras becomes a substrate for Ras and a-factor converting enzyme (Rce1) a protein that resides in the ER membrane and cleaves the AAX
amino acids from the C-terminus of Ras [21]. Ras then proceeds to the next enzyme, isoprenylcysteine carboxyl methyltransferase (Icmt), where the α-carboxyl group on the isoprenylated cysteine is methylated with S-adenosylmethione donating the methyl group[22]. At this point the different Ras isoforms diverge. K-Ras, with its polylysine sequence in the HVR, does not require further processing and proceeds to the plasma membrane (PM) via an uncharacterized mechanism[23]. N-Ras and H-Ras are further modified by Ras palmitoyltransferase (RPT) which attaches a palmitoyl from palmitoyl-CoA to C181 on N-Ras and C181 and C184 on H-Ras via a labile thioester bond. Recently the mammalian RPT complex, DHHC9 (Asp-His-His-Cys motif) and GCP16 (Golgi localized protein), was identified as a palmitoyl transferase for H-Ras and N-Ras. Immunofluorescence analysis indicated that this complex resides in the ER and the Golgi [24]. Recent studies report evidence of an acylation cycle that shuffles a depalmitoylated Ras to the Golgi to be repalmitoylated. This substantiates a mechanism of Ras palmitoylation on the Golgi [25, 26].

Ras signaling from endosomes

Since its discovery, it was believed that Ras needs to be at the PM to signal. This paradigm was put into question in 1994. It was demonstrated that, after administering a large dose of epidermal growth factor (EGF) to rat parenchyma, the Ras effectors Shc, Grb2 and mSOS were isolated on early endosomes [27]. This discovery fostered the idea that epidermal growth factor receptor (EGFR) signaling may continue while the EFGFR is on endosomes.
The next step came through the utilization of the K44A dominant negative mutation of dynamin. Dynamin is a GTPase that regulates clathrin mediated endocytosis and the K44A mutation prevents endocytosis. It was found that MAP kinase activity was suppressed in endocytosis deficient cells containing K44A dynamin. The p85 subunit of PI3 kinase also showed a greatly reduced phosphorylation. These data lead to the conclusion that EGFR trafficking via endosomes was important to promote downstream signaling events[28].

There is direct evidence that Ras is bound to endosomes. Ras was found on purified endosomal fractions separated from rat liver [29]. Specific Ras isoform targeting to endosomes was elucidated by Jiang and Sorkin. Both H-Ras and K-Ras were found on endosomes together with their effector proteins using fusion protein based technology and fluorescence resonance energy transfer (FRET). Interestingly, the relative amount of H-Ras was higher than K-Ras on endosomes and GTP loading was not a factor. The most important tool employed was the Ras binding domain(RBD) of Raf-1 fused to YFP. Since the RBD only binds a Ras protein that is GTP loaded, the YFP-RBD was a valuable tool to gauge whether Ras was active in a cellular environment [30].

Because the antibody probe used in the Pol experiment was not specific to H-Ras the question remained which Ras isoform was on endosomes. Hancock’s group used the dynamin K44A mutant along with GFP fusion proteins of H-Ras and K-Ras to demonstrate that H-Ras and not K-Ras was dependant upon endosomal traffic in order to propagate its signal through the Raf/MEK/MAPK cascade. Unlike previous experiments that looked at EGFR stimulation, Hancock used the G12V constitutively active form of Ras to initiate Raf binding to Ras[31]. The scientific community was now fairly convinced that Ras could not
only signal from a platform other than the PM, but the H-Ras isoform actually required endosomes to relay its signal through at least one of the known cascades.

**Ras signaling on the ER**

The C181,C184S double mutant of H-Ras is palmitoylation deficient and is limited to the ER and cytosol[23]. The first transmembrane domain of the avian infectious bronchitis virus M protein (M1) has also been shown to be restricted to the ER because of its transmembrane sequence [32]. H-Ras61L was fused to the C-terminus of the M1 protein and it, as well as, the 61L version of the C181S, C184S double mutant were transfected into NIH-3T3 cells and stained for focus formation. Both constructs were found to have approximately 60% of the transforming activity as H-Ras 61L transfected cells. This was a strong indication of biological activity of H-Ras on the ER. Phosphorylation levels of proteins in Ras signal cascades were also measured from COS cells transiently transfected with H-Ras61L containing the ER localized mutations. The percent of H-Ras61L induced phosphorylation of phospho-Akt, Erk and Jnk was lower in the ER localized mutants than in H-Ras61LWT (H-Ras with only the Q61L mutation) but higher than vector alone. Interestingly, in the M1 mutant, the phospho-Jnk activation was greater than H-Ras61LWT or the C181S,C184S construct [33]. This data seems to point to a difference in signaling capability when H-Ras is in its natural membrane orientation, C181S, C184S mutant, versus an N-terminal tether, M1, where the orientation of H-Ras in regards to the membrane may be completely different.

In order to test whether H-Ras could be GTP loaded while residing on the ER, COS-7 cells were transiently transfected with H-Ras and stimulated with either ionomycin or lysophosphatidyl acid (LPA) and imaged. It was found that the Ras GEFs, RasGRF1 and 2,
both colocalized with H-Ras, indicating GTP loading. GTP loading was also confirmed with the M1 tether and C181S,C184S mutant [34].

These sets of experiments demonstrated that both a constitutively active, as well as, a signal activated H-Ras can signal from the ER.

**H-Ras signaling from the Golgi Apparatus**

With strong evidence for H-Ras signaling from both endosomes and the ER, researchers asked whether Ras could signal from the Golgi. H-Ras and N-Ras had both been shown to accumulate in the Golgi, so the idea of the Golgi as signaling platform was plausible [35].

Golgi localized H-RasWT and H-Ras61L activity was shown in COS-1 cells. First, the cells were cotransfected with H-RasWT and GFP-RBD and stimulated with EGF. There was a distinct difference in GFP-RBD recruitment to the Golgi versus the PM. The PM recruitment was rapid, within 1-3 minutes, with a peak at 10 minutes. The Golgi recruitment was delayed, occurred in 10 minutes, with continued accumulation to 60 minutes. Next phosphorylation levels of downstream effectors were compared in cells expressing either H-Ras61LWT and KDELr-H-Ras61LWT. The KDEL receptor (KDELr) is a resident protein in the Golgi that traffics back to the ER. An N193D mutation in KDELr keeps it in the Golgi. KDELr was tethered to the N-terminus of H-Ras61L. The levels of phosphorylation for the cells expressing the KDELr-H-Ras61L construct were greater in phospho-Akt and Erk when compared to cells expressing H-Ras61LWT. Phosphorylation levels of phospho-Jnk in the KDELr-H-Ras61L cells were, however, much lower than the H-Ras61LWT cells [33]. These assays established the ability of H-Ras to signal from the Golgi.
H-Ras was shown to have a GEF specific to the Golgi in PC12, COS-1 and Jurkat cells. Phospholipase Cγ1 (PLCγ1) was activated in a calcium-dependant manner that recruited RasGRP1, a Ras GEF, to the Golgi upon stimulation [36]. With the confirmation of a Golgi specific GEF for Ras, it seemed clear that Ras could signal from the Golgi in its natural state. Recently, it was reported that a Ras effector, Ras interacting protein (Rain), was Golgi specific [37].

All assays to this point were done with transiently transfected cells, the next step was to use a stable cell line. NIH-3T3 stable cell lines were created with various membrane site-specific proteins fused to H-RasG12V, one of which was the KDELr. Although KDELr-H-RasV12 bound to RBD-GST in vitro, colocalization with endogenous c-Raf in vivo was limited. The phosphorylation levels of phospho-Akt and Erk were diminished in the KDELr-H-RasV12 cells compared to the H-RasV12 cells. Phospho-Jnk and GTP loaded Ral were both comparable in each cell type [38]. As with the M1 constructs on the ER, there seemed to be a distinct preference for signaling pathways from the Golgi.

**Trafficking of C-terminal mutations in H-Ras**

The C-terminus of each of the Ras isoforms is unique and it is this difference that aids in targeting each to a specific pathway and preferred compartment. Single point mutations that remove one or both of the palmitoylation sites give insights into a possible preference in the C-terminus orientation with a membrane, or the lifetime of the Ras molecule on a particular membrane. If there is a C181S mutation, leaving only the C184 palmitoylated, H-Ras will accumulate on the Golgi with very little reaching the PM. The C184S H-Ras protein is much more prevalent on the PM with some Golgi and ER accumulation as well.
Notably, both mutations are equally more soluble than the wild type H-Ras, with approximately 50% of each total protein being in the soluble fraction of the cell lysates. Cells treated with [\(^3\)H]palmitic acid were lysed and blotted and demonstrated that each mutant did indeed have a palmitate [39].

A C-terminus mutation of H-Ras created by Buss and colleagues changed the wild type GCMSCKCLVS to GSDQCECVLS (SDQ). The removal of the lysine and addition of glutamate and aspartate changes the overall charge on the C-terminus from +1 to -2, as well as, removing the site for 181 palmitate attachment. The work of this thesis shows that this protein has strong Golgi accumulation with none of the protein reaching the PM. As with the C181S and C184S mutations, the SDQ mutation renders the H-Ras protein much more soluble than the wild type. Though SDQ transformation was almost zero, activity could be restored by adding a leader sequence to the N-terminus that encoded a myristoylation site. This indicated that the protein was in the correct conformation and could bind its effectors. [\(^3\)H] palmitic acid labeling was also performed and illustrated that the SDQ mutant was palmitoylated [40]. The SDQ mutant therefore seems to be an excellent tool for studying H-Ras signaling from the Golgi.

**Purpose of the SDQ mutant and this thesis**

By studying signaling of the SDQ mutant we hope to ascertain a truer sense of cascades that originate from the Golgi. We believe that SDQ H-Ras presents a more natural orientation of the molecule in the cell. Whereas SDQ has been shown to be palmitoylated and is, therefore, most probably tethered to the membrane via its lipids, the KDELr-H-Ras fusion protein with its N-terminus tethered to a membrane cannot be assured to have the
same natural orientation. Indeed, the KDELr-H-RasV12 mutants showed a lack of biological activity when they were stained for focus formation [38]. Despite the fact that Willumsen was unable to show biological activity with the SDQ61L mutant, the more recent work, with the mutant expressing from a stronger cDNA vector, showed focus formation at about 30% of H-RasWT61L [41]. With the ability to cycle on and off the membrane and a more natural membrane orientation, the hypothesis of this thesis is that the SDQ mutation of H-Ras seems an excellent candidate for testing signaling from the Golgi.

**List of abbreviations**

GEF-guanine nucleotide exchange factor

GAP-GTPase activating protein

RBD-Ras binding domain

EGF-epidermal growth factor

PM-plasma membrane

ERGIC-ER-Golgi intermediate compartment

GTP-guanine nucleotide triphosphate

ER-endoplasmic reticulum

MAPK-mitogen-activated protein kinase

Erk-extracellular regulated kinase

JNK-c-Jun N-terminal kinase
Materials and Methods

Plasmids

H-RasWT and H-RasWT61L were expressed from pcDNA3 (Invitrogen). The SDQCE, CDQSE, C181S and C184S mutants were generated oligonucleotide-directed polymerase chain reaction (PCR). The 5' primer is 5'-GGGGGATCCACCA TGACAA GTACAAGCTT-3', which is fully complimentary to the 5' sequence of human H-Ras. The mutagenic oligonucleotides 5'-ATAGACGTCTTAAGGATTGAATTATGCGTAAGTGAA ACTAGTGACCGCCCG-3' (CDQSE) and 5'-ATAGACGTCTTAAGGATTGAATTATGCG TAAGTGAAACTAGTGTCGGCCCCG-3' (SDQCE) were used as 3' primers for introducing an EcoRI site and Ras carboxyl terminal mutants. The mutagenic oligonucleotides 5'-CCTGCAGCTCATGGAGCCGGGGCCACT-3' (C181S) and 5'-GAGCACACACTTGGAGCT CATGCAGCC-3' (C184S) were used as 3' primers for site directed mutagenesis via PCR.

Cell culture and transfection

NIH3T3 and COS-7 cells were grown in DMEM (GIBCO-Invitrogen) + 10% bovine calf serum. All cells were grown in 10% CO₂. Stable NIH3T3 cells lines were created by transfecting (Effectene) a 100mm cell culture dish 70% confluent with NIH3T3 cells with 1.0 µg of DNA for 24 hours. Cells were then selected with 750 µg/ml of G418 and protein expression was confirmed via immunofluorescence. Briefly, cells from 3 separate experiments were grown on coverslips, stained with anti-H-Ras antibody, mounted on glass slides and imaged with a Leica DMIRE2 fluorescent microscope. 100 cells were counted on each coverslip and each cell expressing an H-Ras construct was scored. The standard
deviation of the 3 separate cells counts was calculated and the SEM reported. Student T-test compared the values of wild type cells versus either the SDQ or CDQ cells. COS-7 cells were plated 24 hours prior to transient transfection (Effectene) in a 12 well tissue culture plate at a density of about $10^5$-$10^6$ cells per well and transfected as described by the Effectene protocol. NIH3T3 and COS-7 cells were plated on pre-washed coverslips (Corning) coated with poly-L-lysine (100 µg/ml) and fibronectin (50 µg/ml).

**Immunofluorescent images**

COS-7 cells were cultured and transfected as described above. NIH-3T3 cells stably expressing H-RasWT, CDQSE and SDQCE were grown on coverslips for 24 hours prior to being treated with 40 ng/ml of EGF for either 5, 30 or 60 minutes. The cells were washed in PBS twice and fixed with 4% paraformaldehyde in PBS 30 minutes at room temperature. The cells were permeabilized with -20°C methanol for 30 seconds and quenched with 50mM NH₄Cl in PBS for 15 minutes at room temperature. Cells were then blocked with blocking buffer (0.4% BSA and 2% horse serum in PBS) for 1 hour at room temperature. Cells were then incubated in primary antibody diluted in blocking buffer for 1 hour at room temperature, rinsed 3 times in TTBS and incubated in secondary antibody another hour at room temperature. Cells were then washed in PBS and distilled water and mounted on a glass slide with mounting media (Vectashield) and adhered with regular nail polish. Untransfected cells were stained as a negative control. Each set of experiments was repeated 3 times.
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**Imaging**

Cells were imaged with a 100x oil lens on a Leica DM IRE2 inverted fluorescent microscope. Images were captured with a Q Imaging Retiga 1300 camera, processed with Openlab 3.1.4 and converted to JPEG files with Adobe Photoshop 7.0. X-ray films of western blots were scanned with an HP 2300 series scanner/printer and densitometry was carried out with ImageJ.
**Immunoblots**

NIH-3T3 cells were cultured in 100mm tissue culture plates and treated with 40 ng/ml EGF for either 10, 30 or 60 minutes. Cells were lysed with 1.0 ml of lysis buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 20 mM MgCl and 0.5% NP-40) on ice and 5X SDS-PAGE loading buffer (10% SDS, 400mM Tris pH 6.8, 500 mM DTT, 10% glycerol, Bromophenol blue 0.04%) was added immediately. Samples were boiled for 10 minutes and proteins were separated by SDS-PAGE. Proteins were transferred by electrophoration to a PVDF membrane and blocked with 5% milk in TTBS at room temperature for 1 hour. Membranes were then probed with primary antibody overnight at 4°C, rinsed 3 times in TTBS and probed in secondary HRP conjugates for 1 hour at room temperature. Membranes were then rinsed 3 times in TTBS and developed with SuperSignal according to the manufacturer’s protocol (Pierce).
Results

H-RasSDQCE is trapped on ERGIC and cis-Golgi

Studies have demonstrated that H-RasWT, H-RasC181S and H-RasC184S accumulate in a perinuclear region. In order to discern which organelle demonstrates H-Ras accumulation, COS-7 cells were transfected with either H-RasWT, C181S, C184S, SDQCE or CDQSE for 24 hours, fixed and stained with H-Ras antibody and either ERGIC-53 or giantin. The ER Golgi intermediate compartment (ERGIC) is an organelle that exists between the ER and the cis-Golgi. H-RasWT, C181S, C184S and SDQCE all showed some colocalization with ERGIC-53 (Figure 1). CDQSE, however, had very limited colocalization with ERGIC-53. Since complete colocalization of the H-Ras proteins with ERGIC-53 was not observed there was the possibility that H-Ras was accumulating on the cis-Golgi as well as the ERGIC. H-RasWT, C181S, C184S and SDQCE all were colocalized to some extent with giantin (Figure 2). Once again CDQSE colocalized very little with giantin. It seems that H-RasWT, C181S, C184S and SDQCE accumulates on both the ERGIC and Golgi, however CDQSE did not.

The SDQCE transfected cells did not display any PM decoration by this Ras construct. H-RasWT, C184S and, to a lesser extent, C181S expressing cells did show Ras accumulation at the PM. The CDQSE expressing cells showed a strong perinuclear and ER staining. SDQ is lacking a potential palmitoylation site like C181S, but is unable to reach the PM. SDQ has the same amino acid charge change like CDQSE, but it is able to accumulate in the ERGIC/cis-Golgi unlike the ER localization of CDQSE. The combination charge
change due to amino acid mutation and lack of palmitoylation site at C181 has incurred upon SDQ its unique qualities of Golgi localization without PM localization.

**H-RasSDQCE can be GTP loaded on the Golgi after EGF stimulation**

The next test was to determine whether SDQ could be GTP loaded on the Golgi. NIH-3T3 cells stably expressing either SDQ, CDQSE or H-RasWT were stimulated with 40 ng/ml of EGF for either 5, 30 or 60 minutes. The cells were fixed and stained with H-Ras and Raf-1 antibodies to determine if endogenous Raf-1 would colocalized with H-Ras upon stimulation.

H-RasWT showed colocalization with Raf-1 at the PM at both 5 minutes and 30 minutes of stimulation (Figure 3B and 3C). H-RasWT also colocalizes with Raf-1 on endosomes (Figure 3B, C and D). Endosomal colocalization begins at 5 minutes and predominates over PM colocalization by 60 minutes.

CDQSE was unable to demonstrate any significant colocalization with Raf-1 upon stimulation (Figure 4). By 60 minutes of stimulation a few areas of weak colocalization are evident (Figure 4D). CDQSE does not appear to have the ability to be GTP loaded via the EGF pathway or cannot interact with Raf-1 from its ER membrane location.

SDQ shows strong colocalization with Raf-1 after 30 minutes of stimulation (Figure 5). After 5 minutes of stimulation SDQ does not colocalize with Raf-1 (Figure 5B). Strong colocalization of SDQ with Raf-1 is evident after 30 minutes of stimulation with very strong colocalization apparent at the 60 minute mark (Figure 5C and D). H-Ras is thus able to be GTP loaded on the Golgi via EGF stimulation and can interact with Raf-1 at this location.
Stable cells were counted and percentage of cells expressing H-RasWT, SDQCE or CDQSE was determined (Figure 6). The number of cells expressing H-RasWT or SDQCE (T-test, P<0.1) and H-RasWT or CDQSE (T-test; P<0.01) were found to be significantly different. The lower percentage of cells expressing H-RasWT versus either SDQCE or CDQSE may be due to the different C-termini which may effect the lifetime of the protein in the cell.

**SDQ elicits a delayed signal cascade on the Golgi**

Previous reports had demonstrated the activation of downstream phosphorylation signals in cells expressing a Golgi localized H-Ras constitutively active, Q61L or G12V, construct. This observation was evident for three of the common signaling cascades associated with H-Ras: Erk, Akt and Jnk. In order to verify if H-Ras could signal on the Golgi via an external stimulus, NIH-3T3 cells stably expressing either H-RasWT, SDQCE or CDQSE were stimulated with 40 ng/ml of EGF for 10, 30 or 60 minutes. Untransfected NIH-3T3 cells were used as a control and stimulated in the same manner. NIH-3T3 cells stably expressing the Q61L constitutively active form of H-Ras, CDQSE or SDQCE were grown to confluence, lysed and loaded as positive controls. Relative activation levels were determined by establishing the phospho-protein signal to total protein signal ratio with the ImageJ densiometry program. H-RasWT, CDQSE and NIH-3T3 control cells showed an increase in phospho-Erk activation after 10 minutes of EGF stimulation with phosphorylation levels decreasing after 30 and 60 minutes of stimulation (Figure 7B-D). The levels of activation after 10 minutes of stimulation were 3-6 fold greater than that of the control Q61L. Activation levels then fell to
Figure 1. SDQ resides on the ERGIC, not on the PM. COS-7 expressing the indicated H-Ras constructs were costained with anti-H-Ras and anti-ERGIC-53 antibodies. (A) H-RasWT shows colocalization with ERGIC-53 and is evident on the PM. (B) C184S colocalized with ERGIC-53 and little PM staining. (C) CDQSE shows minimal ERGIC-53 colocalization. (D) C181S colocalized with ERGIC-53 with very little PM localization. (E) SDQCE colocalized with ERGIC-53 with no PM staining evident. Scale bars represent 10 μm. Insets show ERGIC region.
Figure 2. SDQ accumulates on the Golgi, not on the PM. COS-7 expressing the indicated H-Ras constructs were costained with anti-H-Ras and anti-cis-Golgi antibodies (giantin). (A) H-RasWT shows colocalization with the giantin and is evident on the PM. (B) C184S colocalized with the giantin and little PM staining. (C) CDQSE shows minimal giantin colocalization. (D) C181S colocalized with giantin with very little PM localization. (E) SDQCE colocalized with giantin with no PM staining evident. Scale bars represent 10 μm. Insets show cis-Golgi region.
Figure 3. H-RasWT recruits Raf-1 on PM and endosomes. NIH-3T3 cells stably expressing H-RasWT stimulated with 40 ng/ml of EGF and costained with anti-H-Ras and anti-Raf-1 antibodies. (A) No EGF stimulation shows no colocalization of H-Ras and Raf-1. (B) 5 minutes of EGF stimulation. Raf-1 is recruited to PM and begins to appear on endosomes. (C) 30 minutes of EGF stimulation shows H-Ras and Raf-1 on endosomes. (D) 60 minutes of EGF stimulation shows more colocalization on endosomes and not on PM. Arrows show areas of Raf-1 recruitment. Scale bars represent 10 μm.
Figure 4. CDQSE on endomembranes shows little recruitment of Raf-1. NIH-3T3 cells stably expressing CDQSE stimulated with 40 ng/ml of EGF and costained with anti-H-Ras and anti-Raf-1 antibodies. (A-D) 0, 5, 30 and 60 minutes of EGF stimulation respectively. Limited colocalization occurs at 30 and 60 minutes. Arrows indicate areas of Raf-1 recruitment. Scale bars represent 10 μm.
Figure 5. SDQCE on perinuclear membranes shows strong recruitment of Raf-1 after 30 minutes of stimulation. NIH-3T3 cells stably expressing SDQCE stimulated with 40 ng/ml of EGF and costained with anti-H-Ras and anti-Raf-1 antibodies. (A-D) 0, 5, 30 and 60 minutes of EGF stimulation respectively. Strong colocalization at 30 minutes with very strong colocalization at 60 minutes. Scale bars represent 10 μm.
that of the Q61L after 60 minutes of stimulation. SDQCE showed a delayed response to EGF stimulation. Phosphorylation levels of Erk increased slightly after 10 minutes, peaked after 30 minutes and were sustained after 60 minutes with levels approximately 2.5 fold that of Q61L (Figure 7A). SDQCE is able to signal through the Erk cascade via EGF stimulation in a temporal manner that differs from H-RasWT or CDQSE.

Phospho-Akt activation levels are similar to Q61L in all stable cells lines, however the timing of peak activation differed. H-RasWT, CDQSE and NIH-3T3 control cells demonstrate an increase in phospho-Akt activation after 10 minutes of EGF stimulation with a decrease in signal after 30 and 60 minutes (Figure 8B-D). Phospho-Akt activation in SDQCE cells displayed a deferred response compared to H-RasWT or CDQSE. The greatest activation occurred after 30 minutes of stimulation with little drop off after 60 minutes (Figure 8A). The greatest levels of phospho-Akt activation in all stable cell lines were not significantly different from Q61L.

SDQCE, CDQSE and H-RasWT stable cells demonstrated peak levels of phospho-Jnk activation approximately 2-3 fold greater than the Q61L control (Figure 9A-C).
Figure 7. Phospho-Erk is phosphorylated more slowly and with a greater duration in SDQ cells. NIH-3T3 cells stably expressing either H-RasWT, CDQSE or SDQCE were stimulated with 40 ng/ml of EGF for 10, 30 or 60 minutes, lysed, run on SDS-PAGE, transferred to PVDF and probed with anti-phospho-Erk, anti-Erk1, anti-H-Ras or anti-Caveolin antibodies. (A) SDQCE expressing cells show increased phosphorylation of Erk after 30 minutes of stimulation and remain activated after 60 minutes (B-D) CDQSE, H-RasWT and NIH-3T3 untransfected cells (respectively) demonstrate a strong peak of phospho-Erk after 10 minutes of EGF stimulation and diminishes after 30 minutes of stimulation.
Figure 8. Phospho-Akt activation is distinct in SDQ cells. NIH-3T3 cells stably expressing either H-RasWT, CDQSE or SDQCE were stimulated with 40 ng/ml of EGF for 10, 30 or 60 minutes, lysed, run on SDS-PAGE, transferred to PVDF and probed with anti-phospho-Akt, anti-Akt, anti-H-Ras or anti-Caveolin antibodies. (A) SDQCE expressing cells show strong phospho-Akt signal after 30 minutes of EGF stimulation and continued signal after 60 minutes. (B-D) CDQSE, H-RasWT and NIH-3T3 untransfected cells (respectively) demonstrate an increase in phospho-Akt signal after 10 minutes of EGF stimulation with a gradual decrease in signal after 30 and 60 minutes.
Figure 9. Phospho-Jnk activation differs among cell types. NIH-3T3 cells stably expressing either H-RasWT, CDQSE or SDQCE were stimulated with 40 ng/ml of EGF for 10, 30 or 60 minutes, lysed, run on SDS-PAGE, transferred to PVDF and probed with anti-phosph-Jnk, anti-Jnk, anti-H-Ras or anti-Caveolin antibodies. (A) SDQCE expressing cells show phospho-Jnk activation after 10 minutes of EGF stimulation and continued activation after 30 minutes. (B-D) CDQSE, H-RasWT and NIH-3T3 untransfected cells (respectively) exhibit strong phospho-Jnk activation after 10 minutes of EGF stimulation and diminish after 30 minutes.
H-RasWT, CDQSE and control cells gave a peak of phospho-Jnk activation after 10 minutes of EGF stimulation and decreased after 30 and 60 minutes (Figure 9B-D). SDQCE stable cells displayed a phospho-Jnk activation peak at 10 minutes of EGF stimulation that was matched after 30 minutes of stimulation and decreased after 60 minutes of stimulation (Figure 9A).
**Discussion**

Ras’s link to cancer has made it one of the most studied signaling molecules in cell biology. The very discovery of Ras came from the analysis of carcinoma cells and since then the structure and function of Ras has been dissected in order to understand its role in oncogenicity. The latest findings that Ras is active on the ER and the Golgi have given new light to the function of Ras. Recent evidence has pointed to a role in Ras signaling from the Golgi and a possible link to tumorigenesis.

The data in this thesis have contributed to the overall understanding of Ras signaling from the Golgi. The SDQ mutant appears to be an excellent tool to parse the complex signaling pathways that occur in the cell. Based on the evidence in this thesis as well as other reports, SDQ accumulates in the ERGIC/cis-Golgi region, is palmitoylated and is most probably folded in the correct conformation to be able to initiate signal cascades when bound to GTP [40, 41]. Indeed, SDQ is unique from the CDQCE, C181S and C184S mutations to the tail region of H-Ras in that it does not reach the PM. It seems that the combination of an overall change in charge from +1 to -2, coupled with a C181S mutation that eliminates a palmitoylation site, is enough to allow SDQ to traffic to the cis-Golgi and go no further. More importantly, this thesis as well as other reports have demonstrated that a wild type H-Ras will accumulate on the cis-Golgi [23, 25, 26, 31, 33, 39]. This lends credence to idea that, in a natural setting, H-Ras will spend a great deal of time on the Golgi and the Golgi membrane may provide a suitable platform for signaling.

Further evidence of H-Ras signaling was demonstrated via recruitment of the Ras effector, Raf-1, after stimulation with the growth factor EGF. As illustrated by
immunofluorescence, the SDQ Golgi trapped mutant showed a delayed and sustained recruitment of Raf-1. This was in contrast to H-Ras wild type which is activated very quickly at the PM and shows Raf-1 recruitment. The phenomenon of a delayed recruitment of Ras effectors to the Golgi has been verified by other labs in a variety of cell types. The idea of the Golgi being a bona fide signaling platform has also been enforced by the findings of Golgi specific GEFs for Ras.

The final piece of evidence in this thesis that lends support to the importance of a Golgi initiated signal cascade is established by a temporal difference of activation of downstream effectors of Ras. Whereas the cells stably expressing either H-RasWT, H-RasCDQSE or untransfected cells show an immediate response to EGF stimulation, the SDQ mutant shows a delayed and sustained activation of the downstream effectors Erk and Akt. This sustained activation of two signaling cascades is significant in greater perpetuation of the initial extra cellular stimulation. This may lead the cell to an increased probability of transformation or differentiation or an increase in growth or metabolism. Indeed, the PI3K/Akt pathway has been implicated in glucose uptake and metabolism, so the notion of a molecule that would increase glucose uptake in a time of need does not seem unlikely. Just as important, neuronal cells will differentiate and T-cells will grow when the Ras/Raf/MEK/Erk pathway is stimulated from a Golgi bound Ras [36].

The majority of the focus on Ras signaling looks at its role in cancer. To that end many studies have implemented the constitutively active form of Ras to assess its signaling parameters [31, 33, 36, 38]. Several studies have used NIH-3T3 cells to stably express either the G12V or Q61L active versions of H-Ras with conflicting results [33,38]. The
Willumsen, et al. report [40] used SDQ61L for its transformation focus formation assays with poor results. Transforming activity was restored, however, when a myristoylation leader sequence was attached to the N-terminus. This indicated that the SDQ protein was folded properly and could signal enough to cause transformation. A later study by Zheng [41] demonstrated that SDQ could transform and cause focus formation at a rate of approximately 30% of H-RasWT61L. The discrepancy was most probably due to a more robustly expressing vector having been used by Zheng. Zheng also showed that SDQ61L could phosphorylate Erk and Akt on an order close to that of H-RasWT61L. Matallanas [38], however, showed that with the KDELr tethered H-RasWT12V the level of phosho-Erk activation was one tenth that of H-RasWT12V and the phosho-Akt was about a third of the WT. Matallanas also found no transforming ability when staining for focus formation. The difference in outcomes of the SDQ versus the KDELr mutants may be both in the folding and orientation of the Ras molecule as well as a biological need for Ras to cycle on and off the Golgi. Because the KDELrN193D is a integral membrane protein, it being tethered to the N-terminus of H-Ras may disrupt the normal conformation of H-Ras, reducing its affinity for Raf-1 or PI3K. Matallanas acknowledged that the N193D mutation of KDELr may account for its lack of transforming ability. Since the KDELrN193D can not cycle back to the ER, it may affect the normal sequence of signaling events. The SDQ mutant is tethered to the membrane with the correct conformation and orientation and with a higher amount being in the cytosol compared to H-RasWT, it seems to have the ability to cycle on and off the Golgi. Indeed, studies have shown that H-Ras is deacylated at the PM and becomes reacylated at the
Golgi. The acylation cycle appears to be essential for proper membrane localization of H-Ras as well as promoting a signaling cascade [25,26].

These data in this thesis have demonstrated that SDQ is an excellent tool for examining Golgi based signaling. While the most documented pathways have been studied, there is still a great deal of work to be done where Golgi signaling is considered.
References


