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Defining the Role of Heterotrimeric G-proteins in EGF Receptor Trafficking and Signaling

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biomedical Sciences

by

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2009
The Dissertation of Anthony O. Beas is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2009
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ABSTRACT OF DISSERTATION

Defining the Role of Heterotrimeric G-proteins in EGFR Trafficking and Signaling

by

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Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2009

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RGS-PX1 functions as a GAP that inactivates Gαs and regulates EGFR trafficking and signaling, while GIV binds Gαi to form a molecular switch that and activates Gαi and promotes efficient receptor signaling. Yet the mechanisms by which Gαs/RGS-PX1 and Gαi/GIV affect EGFR signaling
remained unclear. I set out to further define the roles of Gαs/RGS-PX1 and Gαi/GIV in EGFR trafficking and signaling.

We found that Gαs regulates EGFR degradation. Specifically, Gαs depletion by RNAi delayed and Gαs overexpression promoted EGF-induced degradation of EGFR, a key step in the downregulation of receptor signaling. In addition, Gαs and RGS-PX1 form a complex with Hrs, a component necessary for receptor sorting for degradation. Thus, Gαs promotes EGFR degradation and forms a complex with RGS-PX1 and Hrs that might be required for efficient receptor sorting. Gαs depletion also enhanced ligand-induced EGFR autophosphorylation and ERK 1/2 signaling, and a siRNA-resistant, inactive Gαs-G226A mutant but not an active Gαs-Q227L mutant reversed these effects. Together, these data indicate that activation of Gαs delays and inactivation of Gαs by RGS-PX1 promotes downregulation of EGFR signaling.

We also found that the Gαi-GIV switch reprograms EGFR signaling to influence whether cells migrate or proliferate. Specifically, GIV interacts with EGFR, and GIV’s GEF motif promotes several events including formation of a ligand-induced complex between EGFR, Gαi, and actin which promotes receptor signaling within the plasma membrane (PM)-actin bed, receptor autophosphorylation, PM-based signaling to Akt and PLCγ, and cell migration. Disabling GIV’s GEF motif by mutation (GIV-F1685A) disrupts this complex which promotes receptor signaling trafficking from endosomes, inhibits
receptor autophosphorylation, and enhances endosome-based signaling to ERK 1/2 MAPK and c-Src/STAT5b and cell proliferation. Furthermore, early during progression of colorectal carcinomas, before metastatic invasion the Gαi-GIV switch is disabled due to the loss of GIV’s carboxy-terminus and critical GEF motif by alternative splicing. Later during metastatic invasion the switch is assembled by upregulation of GIV. Therefore, the switch is selectively assembled or not at different stages of oncogenesis leading to pro-migration or proliferative cellular profiles, respectively. These traits might cumulatively influence growth and invasiveness of tumors during oncogenic progression.
Chapter 1

Introduction
SUMMARY

In this chapter, I provide a general overview of the heterotrimeric G-protein activity cycle, the role of G-proteins as intracellular transducers of G-protein coupled receptors (GPCRs), and the different types of post-translational modifications and accessory proteins that can modulate G-protein activity. I then review the classic roles for $\alpha_s$ and $\alpha_i$ in regulating cAMP-based signaling and discuss how these G-proteins are implicated in intracellular trafficking and cell migration. I also discuss two specific $\alpha_s$ and $\alpha_i$ binding proteins, RGS-PX1 and GIV, that modulate the G-protein activity cycle and link these G-proteins to EGFR signaling, intracellular trafficking, cell migration, and cell proliferation. I also highlight specific questions that I set out to help answer in subsequent chapters of this thesis regarding the mechanisms by which $\alpha_s$ and $\alpha_i$ might affect the cellular processes listed above.

Overview of the G-protein Signaling

Heterotrimeric G-proteins are composed of $\alpha$, $\beta$, and $\gamma$ subunits. 21 $\alpha$, 6 $\beta$, and 12 $\gamma$ subunits have been discovered which can couple to one another in various combinations to form a heterotrimer (1). G-proteins are grouped based on the primary sequences of the $\alpha$ subunits into four subfamilies—$\alpha_{12}$, $\alpha_q$, $\alpha_i$, and $\alpha_s$ (1). Classically, they are coupled to GPCRs at the plasma membrane (PM). Ligand binding to a GPCR leads to a change in its conformation that in turn allows its C-terminus to act as a guanine-nucleotide exchange factor (GEF) to trigger the exchange of GDP for GTP on the $\alpha$
subunit (1). Following this exchange, the G-protein dissociates into two active signaling units--active GTP-bound Gα and a Gβγ complex. Each unit can then initiate downstream signaling through effector proteins until the Gα subunit hydrolyzes GTP. Hydrolysis is catalyzed by GTPase activating proteins (GAPs) that belong to the Regulator of G-protein Signaling (RGS) family of proteins (2). GTP hydrolysis inactivates the G-protein and leads to reassembly of the trimeric complex (1) (Fig. 1).

G-protein activity is regulated at other levels including covalent modification. For example, G-protein targeting to membranes by lipid modification plays a critical role in G-protein signaling. Most G-proteins are covalently modified by myristoylation (C-14) of glycine and/or palmitoylation (C-16) of cysteine residues on Gα subunits and geranylgeranylation of Gγ subunits to help direct G-protein localization to membranes where they can interact with GPCRs and effectors (3). In addition to lipid modification, some G-proteins are phosphorylated at serine and tyrosine residues, which can affect interactions with Gβγ, GPCRs, and effector proteins (3); however, the effects of phosphorylation on G-proteins have not been extensively studied.

Gαs and Gαi: Established Modulators of cAMP Signaling

Gαs-based cAMP signaling is the archetypical signaling pathway. Classically, activation of Gαs by a ligand-occupied GPCR initiates binding of the α subunit to adenyl cyclase (AC) and its stimulation to produce the second messenger, cAMP (4). cAMP then binds to and activates cAMP-dependent Protein Kinase (PKA) and Epac, and each can subsequently affect a variety of
downstream signaling pathways (5,6). Gαi can inhibit adenylyl cyclase and thereby attenuate cAMP signaling upon activation (7). Three Nobel prizes (4,8-11) were awarded based on work that led to our understanding of this signaling pathway, which underscores its importance to biomedical science and medicine. In addition, Gαs and Gαi signaling are implicated in numerous, diverse processes, including metabolism, hormonal signaling, cell growth and proliferation, chemotaxis, and intracellular trafficking (12). In fact, one of our group’s initial goals was to further understand the role of Gαs and Gαi in intracellular trafficking. In this thesis, I discuss how our trafficking studies were catalyzed and expanded to include receptor signaling, cell migration, and cell proliferation by the discovery of novel binding proteins for Gαs and Gαi that modulate G-protein activity.

**GAPs Catalyze GTP Hydrolysis to Inactivate G-proteins**

G-protein signaling is turned off by GTP hydrolysis. Although Gα subunits have an intrinsic rate of GTP hydrolysis that can be observed in vitro, inactivation of G-proteins in vivo occurs much faster (13). The discrepancy between in vitro and in vivo rates of inactivation was resolved by the discovery of GAPs that bind GTP-bound Gα subunits and catalyze GTP hydrolysis (13). Most GAPs for G-proteins belong to the RGS family of proteins (13) although a few other proteins i.e., PLCβ and the γ subunit of a retinal cyclic GMP specific phosphodiesterase, can act as GAPs for Gαq and the α subunit of transducin, respectively (14,15).

RGS proteins evolutionarily stem from SST2 in yeast, which desensitizes the yeast pheromone response (16,17) by negatively regulating GPA1 (18). Our
group discovered one of the first mammalian RGS proteins, GAIP (Gα interacting protein) (19) and recognized that GAIP could interact with Gαi subunits through a conserved, ~130 amino acid core RGS domain that was shared with SST2 and other proteins (19). Two other groups identified the RGS family of proteins at about the same time (20,21), and the RGS domain was subsequently discovered to function as a GAP for G-proteins (2,13,22). Since then, over 30 RGS and RGS-like proteins have been identified (13). RGS proteins preferentially bind to G-proteins loaded with AlF₄⁻, which mimics the transition state of GTP hydrolysis (13,23). Although RGS proteins are thought to desensitize GPCR signaling by simply catalyzing GTP hydrolysis and thereby turning off the G-protein, there is evidence indicating that RGS proteins have more complex functions. For example, some smaller RGS proteins (e.g., RGS4) enhance GPCR signaling, potentially by increasing the frequency that the G-proteins can be activated (24,25). Thus, certain smaller RGS proteins are thought of as “modulators” of G-protein signaling (24). Additionally, some RGS proteins (e.g., RGS10 (26)) can discriminate signaling from different GPCRs that are coupled to the same class of G-protein (27). There are also larger RGS proteins that have multiple domains and are considered “integrators” of G-protein signaling since they link G-proteins to additional signaling pathways (24). For example, p115Rho-GEF has an RGS domain that functions as a GAP for Gα12 and a GEF domain that activates the small GTPase, RhoA (28). Overall, a complex interplay exists between G-proteins and RGS proteins that fine-tunes G-protein-based signaling and integrates G-protein signaling with other signaling pathways.
Most RGS proteins identified to date are GAPs for G\textalpha i and G\textalpha q (13). Although RGS2 (29) and Axin (30) possess an RGS domain that can bind to G\textalpha s, no GAP function has been reported (30,31). In fact, our group discovered RGS-PX1, the only RGS domain-containing GAP reported to date for G\textalpha s (32).

**RGS-PX1 Inactivates G\textalpha s and Localizes to Endosomes**

RGS-PX1 was initially discovered through a bioinformatics search for novel RGS domain-containing proteins (32) (Fig. 2). The RGS domain of RGS-PX1 was found to bind specifically to G\textalpha s loaded with AlF$_4^-$ (30,32), inactivate it, and attenuate cAMP production stimulated by a GPCR (32). RGS-PX1 is also known as SNX13 because it contains a Phox-homology (PX) domain (32) that is shared among the Sorting Nexin (SNX) family of proteins (33) (Fig. 2). The PX domain of RGS-PX1, like that of other SNX proteins, binds phosphoinositides, especially PI3P (32,34), and RGS-PX1 localizes to endosomes (32) which are enriched in PI3P (32,35). That RGS-PX1 binds to and inactivates G\textalpha s, binds phosphoinositides, and localizes to endosomes suggested that RGS-PX1 might inactivate G\textalpha s and affect endocytic trafficking.

**G-proteins and Intracellular Trafficking**

Evidence from other groups had previously implicated G-proteins in intracellular trafficking, including endocytic trafficking. For example, G-proteins have been localized to endosomes as well as to the Golgi apparatus, endoplasmic reticulum (ER), and secretion granules, suggesting that G-proteins function at these organelles which are hubs for vesicular transport (36). In
addition, transcytosis and endosome fusion as well as transport from the ER to the Golgi, within the Golgi, and from the Golgi to the PM are affected by treatment with pharmacological agents that modulate G-protein activity including AlF$_4^-$ (generally activates G-proteins (37,38)), mastoparan (activates G$_{ai/o}$ (39)), cholera toxin (activates G$_s$ (40)), and pertussis toxin (inactivates G$_i$ (41)) (36) (Fig. 2). There is also evidence that G$\beta_\gamma$ subunits function in intracellular trafficking. For example, G$\beta_\gamma$ may promote clathrin mediated endocytosis (42). G$\beta_\gamma$ also inhibits the GTPase activity of dynamin (43) which is required for efficient fission of clathrin-coated vesicles from membranes (44). G$\beta_\gamma$ has also been implicated in transport from the trans-Golgi network (TGN) to the cell surface (45). Overall, however, the role of G-proteins in endocytic trafficking remains poorly understood (36,46). Our group’s discovery of RGS-PX1 catalyzed further studies of the role of G$_s$ in endocytic trafficking and led to investigate the affects of RGS-PX1 and G$_s$ on one of the best-characterized model systems for endocytic trafficking, the epidermal growth factor receptor (EGFR).

**EGFR Trafficking and Signaling**

EGFR is a member of the ErbB family of Receptor Tyrosine Kinases (RTKs) (47) and features an extracellular ligand-binding, a transmembrane, and a cytoplasmic domain containing a protein tyrosine kinase domain flanked by regulatory sequences (48). EGFR signaling has been implicated in the regulation of cell proliferation, cell migration, and development (49).
EGFR signaling and trafficking are closely intertwined and involve a series of rapid, successive signaling and trafficking events that are temporally and spatially separated (Fig. 3). For example, ligand-binding induces EGFR dimerization as well as autophosphorylation and ubiquitination of specific tyrosine and lysine residues, respectively, on the receptor’s C-terminus (48). Of the specific tyr residues that are autophosphorylated, pY992 and pY1068 are major sites that induce downstream signaling as they bind and rapidly recruit the SH2 adaptors PLCγ, Grb2, and Gab1 which in turn activate DAG/IP3/Ca^{2+}, Ras/MAPK, and class I PI3K/Akt signaling, respectively (50-53). Ligand-binding simultaneously induces downregulation to shut down EGFR signaling. Downregulation involves EGFR endocytosis, its trafficking to and sorting at endosomes, and subsequent delivery to lysosomes for degradation (47,54,55) (Fig. 3). Mechanistically, downregulation requires the SH2 adaptor c-Cbl to bind pY1045 and ubiquitinate the receptor (56). Hrs then binds the ubiquitin moieties on EGFR and recruits the ESCRT machinery to sort the receptor for degradation and prevent its recycling back to the PM (57). The critical role for downregulation in attenuating EGFR signaling is underscored by the fact that escape of the receptor from this process leads to uncontrolled growth and proliferation (54,55,58,59).

Although one of the main functions of endocytic trafficking of EGFR is rapid downregulation, evidence suggests that receptor trafficking introduces a spatial component to receptor signaling (60). For example, EGF binding induces trafficking of activated EGFR from the PM to a successive series of more
juxtanuclear compartments including early endosomes (~5-15 min), late endosomes (~15-30 min), and lysosomes (>30 min) in HeLa cells (61,62). Activated EGFR can recruit SH2 adaptors (63,64) as well as activated ERK 1/2 MAPK and c-Src (60,65,66) to endosomes. In fact, efficient EGFR endocytosis was found to be necessary to activate specific downstream signaling pathways (e.g., ERK 1/2) (67), suggesting that receptor signaling from the PM vs. endosomes is distinct. That endosome-based EGFR signaling is distinct from PM-based signaling is also supported by the fact that class I PI3K/Akt and PLCγ signaling require phosphoinositide-4,5-phosphate (PIP2) that is generated at the PM but not at endosomes (60). Thus, there is no PIP2-based class I PI3K/Akt and PLCγ signaling that occurs at endosomes (60).

Although the extent and specific role(s) of endosome-based EGFR signaling remain controversial (47), evidence suggests that cells can exploit the spatial aspects of EGFR signaling for different processes. For example, migrating cells seem to preferentially promote PM-based receptor signaling to activate PI3K and actin remodeling proteins required for migration (60,68). EGFR signaling from endosomes can promote cell survival (66) and cell proliferation (60,69). Work discussed in subsequent chapters of this thesis provides further evidence for a specific role for endosome-based EGFR signaling in promoting cell proliferation. Overall, the spatial, temporal, and trafficking components of EGFR trafficking provide multiple levels of complexity and regulation of receptor-initiated signal transduction.
Role of G-proteins in EGFR Signaling

Previous studies have shown a link between G-protein and EGFR signaling. For example, GPCR stimulation was found to transactivate EGFR (70), a finding that led to intense research by numerous groups (71-73). Transactivation of EGFR by GPCRs is in part responsible for promoting proliferation and invasion in certain forms of cancer (72). This process has been shown to occur via GPCR-stimulated cleavage of pro-EGFR ligands (e.g., HB-EGF) from the PM by membrane anchored disintegrin metalloproteases (e.g., ADAM family of proteases) (71,74). This process involves G-proteins, c-Src tyrosine kinases, calcium, and PKC (71). GPCRs have also been shown to transactivate EGFR via GPCR:EGFR complex formation (73,75,76), GPCR-stimulated tyrosine phosphorylation of the C-terminus of EGFR (77), as well as production of reactive oxygen species (ROS) (77). Overall, the exact mechanisms for GPCR transactivation of EGFR are unclear and cell type, GPCR, and G-protein dependent.

There is also evidence that EGFR can signal via G-proteins, but the evidence is fragmentary at best. For example, Go13 was found to promote EGF-induced cell migration (78). EGFR was also reported to directly interact with and phosphorylate, and activate Goαs (79) but the functional consequences of phosphorylation were not investigated. Our group recently found that Goαi3 is required for EGF-induced activation of Akt (80,81). Thus, the exact role of G-proteins in EGFR signaling remains unclear. In this thesis, I provide mechanistic insights into the role of both Goαs and Goαi in EGFR signaling.
RGS-PX1 Regulates EGFR Trafficking and Signaling

The discovery of RGS-PX1 provided an additional avenue to study the role of Gαs in EGFR trafficking and signaling. Indeed, our group found that RGS-PX1 localizes to endosomes, overexpression of RGS-PX1 attenuates Gαs signaling, delays EGF-induced degradation of EGFR, and prolongs ERK 1/2 downstream signaling (32). These results indicate that RGS-PX1 functions both as a RGS protein that attenuates Gαs signaling and as a functional sorting nexin that localizes to endosomes and regulates EGFR degradation and downstream signaling (32). Based on these data, many key questions arose concerning the role of RGS-PX1 and Gαs in EGFR dynamics including: 1) whether Gαs also localizes to endosomes, 2) whether Gαs can affect EGFR trafficking and signaling, 3) the underlying mechanism by which RGS-PX1 and Gαs affect EGFR trafficking, and 4) if the effects of Gαs are activity dependent. The work presented in Chapter 3 and 4 of this thesis helps answer these questions. In addition, I also discuss in Chapter 4 additional studies that could be pursued to further define the role of Gαs in EGFR trafficking and signaling.

Non-receptor GEFs Activate G-proteins

G-protein activity is also modulated by GEFs that accelerate the rate of GDP to GTP exchange. Ligand-occupied GPCRs are the canonical GEFs for G-proteins (1). Over 800 genes for GPCRs have been identified in the human genome, and all share a canonical, 7-transmembrane structure (1). Recently, a few non-receptor GEFs for G-proteins have been described—tubulin, Ric8, presenilin-1, AGS1, CSP, and GiV (81-83). In contrast to canonical GPCR
GEFs, non-receptor GEFs are not structurally related (82,83). In addition, most bind to and activate Gαi/o and no common GEF motif has been identified (81,84,85). Ric-8, the best-studied non-receptor GEF for Gαi, is thought to link Gαi to cell division (86). However, the precise role of Ric-8’s GEF function, much less the role of other non-receptor GEFs, remains poorly understood.

**GIV is a Non-receptor GEF that Activates Gαi**

Our group recently found that Gα-interacting vesicle associated protein (GIV), a protein that we discovered based on its ability to bind to Gαi3 (87), functions as a non-receptor GEF for Gαi through a defined, evolutionarily conserved motif in the carboxy-terminus of GIV (81) (Fig. 4). Our group further modeled the Gαi-GIV interface based on the crystal structure of the KB-752 peptide bound to Gαi (88,89) and identified critical residues that when mutated (F1685A) abolish the ability of GIV’s GEF motif to bind to and activate Gαi (81). Therefore, GIV is the first non-receptor GEF discovered to date that can activate Gαi through a defined motif (81).

**GIV is Required for Cell Migration**

GIV was simultaneously discovered by three other groups and named girdin, APE, and HookRP1 based on its ability to bind actin, Akt, microtubules and dynamin, respectively (90-92). One of these groups found that GIV is required for growth factor (i.e., insulin, EGF, and VEGF)-induced activation of Akt, actin remodeling, and cell migration (92). Based on these findings and our own discovery that GIV binds to and activates Gαi3, we reasoned that GIV may
link G\(\alpha\)i3 signaling to growth factor signaling and actin remodeling during cell migration.

**Overview of Cell Migration**

Cell migration is initiated when a chemoattractant binds to and activates GPCRs (e.g., CXCR4, lysophosphatidic acid receptor-1 (LPAR1) (93)) or Receptor Tyrosine Kinases (RTKs) (e.g., EGFR, insulin receptor (92)) on quiescent cells (94) which triggers polarization, a process whereby the Golgi apparatus, microtubules, and the Microtubule Organizing Complex (MTOC) are positioned in front of the nucleus facing the leading edge of the cell (94) (Fig. 5). Receptor signaling also stimulates PI3K to produce phosphoinositide-3,4,5-triphosphate (PIP3) at the leading edge (94). Meanwhile, PTEN phosphatases remove PIP3 at the trailing edge of the cell (95). The collective actions of PI3K and PTEN lead to the formation of a steep gradient in PIP3 between the leading and trailing edges of the cell (95). As a result, proteins containing a Pleckstrin Homology (PH) domain that bind PIP3 (e.g., Akt and RacGEFs) are preferentially recruited to the leading edge where they promote the dynamic assembly of actin filament protrusions (e.g., filipodia and lamellipodia (94)). Dynamic assembly of integrin-based contact points provides the traction necessary for the cell to move forward (95). Myosin II provides the actin-based mechanical force to pull the trailing edge forward (Fig. 5) (95). In this thesis, I outline our work that shows how that GIV and G\(\alpha\)i cooperate to selectively amplify growth factor receptor initiated pro-migration signaling to promote cell migration.
Role of G-proteins in Cell Migration

Substantial previous work had implicated G-protein signaling in cell migration. For example, many chemoattractants have been shown to signal via their cognate GPCRs to stimulate cell migration during normal (e.g., development) and abnormal (e.g., cancer invasion) processes (93), which indirectly implicates G-protein signaling in migration. Gβγ complexes have been directly implicated in cell migration based on their ability to bind and activate PI3K (94,96) and p-Rex1, a PIP3-dependent Rac GTPase exchange factor (97); PI3K and p-Rex1 are critical components of cell migration induced by chemoattractants and growth factors (94,98). In contrast, much less is known about the mechanisms by which Gα subunits affect cell migration. Chemotactic GPCRs such as CXCR4, LPA1R and PAR1 induce cell migration in a pertussis toxin sensitive manner, which implies that GPCR-Gαi coupling is required to induce cell migration (93). RTKs were also found to induce cell migration in a Gα13-dependent manner (78), but again, the exact mechanisms are not described. Our group was the first to show a direct role for Gαi subunits in cell migration (80,81). Specifically, we found that Gαi3 is required for all of GIV’s pro-migration functions including growth factor (e.g., insulin and EGF)-stimulated activation of Akt, actin remodeling, and cell migration (80). Because GIV is required for cell migration (92) and binds to and activates Gαi subunits (81), our group reasoned that GIV-dependent activation of Gαi may promote cell migration.
GIV-dependent Activation of $\alpha_i$ is Required for Cell Migration

To test this hypothesis, our group expressed a GEF-deficient GIV-F1685A mutant and found that it disrupted GPCR and growth factor induced activation of Akt, actin remodeling, and cell migration (81). Additional studies revealed that GIV-dependent activation of $\alpha_i$ promotes Akt activation via release of $\beta\gamma$ subunits and stimulation of PI3K (81). These findings led to the conclusion that $\alpha_i$3 and GIV form a molecular switch that activates $\alpha_i$ to mediate growth factor pro-migration signaling to PI3K/Akt and actin remodeling during cell migration (81).

A critical question that arises based on the study above is how the $\alpha_i$-GIV switch couples to growth-factor receptors to promote pro-migration signaling and cell migration. Studies outlined in subsequent chapters of this thesis highlight a novel mechanism whereby GIV’s GEF motif forms a critical link between G-protein and EGFR signaling to preferentially amplify pro-migration receptor signaling and remodel actin to promote cell migration.

Cell Migration vs. Cell Proliferation

As mentioned above, EGFR signaling can promote cell migration and cell proliferation (60). Yet, studies by others have reported that migrating epithelial cells do not proliferate (99,100), and similar observations have been made for invading cancer cells, i.e., migrating invading cancer cells do not proliferate and proliferating tumor cells do not migrate, a phenomenon termed migration-proliferation dichotomy (101). Thus, cell migration and cell proliferation are mutually exclusive processes. Based on these observations, a critical question
that arises is how does EGFR signaling selectively promote cell migration vs. proliferation?

In the past, other groups have used EGFR mutants to understand the mechanisms by which EGFR promotes cell proliferation and cell migration. For example, Gill and colleagues found that a kinase active, C-terminally truncated, and endocytosis defective mutant of EGFR (ΔCT-EGFR) missing all the tyrosine autophosphorylation sites supports ligand-induced cell proliferation and transformation of EGFR-deficient cells (54,58). Later, Wells and colleagues found that the same ΔCT-EGFR mutant could promote ligand-induced cell proliferation but not cell migration (102), yet inclusion of autophosphorylation sites (e.g., Y992, Y1068, Y1173) preferentially supported cell migration in response to ligand (102). Based on these findings, Wells and colleagues proposed that autophosphorylation of EGFR was required for cell migration and that an unknown, “post-receptor” mechanism determined whether cells migrate or proliferate (102).

That EGFR signaling is a key component of the decision process to migrate or proliferate is further supported by a bioinformatics study that simulated migration-proliferation dichotomy based solely on the EGFR signaling network (103). Haugh proposed that differential EGFR signaling initiated from the PM (pro-migration) vs. endosomes (proliferative) might be a critical determinant of whether cells migrate or proliferate (60), although the mechanism by which receptor signaling is spatially segregated is unclear. Thus, determination of cell fate by EGFR signaling might involve receptor autophosphorylation, downstream
signaling, spatial distribution, and trafficking and downregulation (i.e., receptor dynamics).

Work from our group and others demonstrated that EGF-induced Akt signaling (80,92), actin remodeling (92), and cell migration (92) require the G\(_{\alpha i}\)-GIV switch. A question that arises is: does the G\(_{\alpha i}\)-GIV switch promote EGF-induced cell migration and concomitantly suppress EGF-induced cell proliferation? If so, does the switch affect the decision process to migrate or proliferate by modulating receptor dynamics? Since expression of the GEF-deficient mutant, GIV-FA, disables the G\(_{\alpha i}\)-GIV switch and inhibits cell migration (81), could disabling the switch enhance cell proliferation? In Chapter 2 of this thesis, I set out to answer these questions. Because cancer cells display migration-proliferation dichotomy (103), we also investigated whether the G\(_{\alpha i}\)-GIV switch could play a key role in oncogenic progression.
Figure 1-1. Overview of the G-protein Cycle. G-protein signaling is initiated when a ligand-occupied GPCR catalyzes the exchange of GDP for GTP on the α-subunit of the G-protein (1,104). This exchange leads to dissociation of the G-protein into two active signaling units, a GTP-bound Gα subunit and a Gβγ complex, and each unit can initiate downstream signaling through its respective effectors (1). Signaling ceases when the Gα subunit hydrolyzes GTP to GDP, which leads to re-association of the trimeric complex with the receptor (1). G-proteins can also be activated by a few newly discovered non-receptor GEFs (Ric-8 (105), AGS1 (106), CSP (83), and GIV (81)), but their functions are less well studied. G-protein signaling is turned off by a large family of GAPs or Regulator of G-protein Signaling (RGS) family of proteins (2). RGS proteins share a conserved, ~130 amino acid RGS domain that binds to GTP-bound Gα and catalyzes hydrolysis (13).
Figure 1-2. Schematic of the domains of RGS-PX1. RGS-PX1 is a 957 a.a. protein that harbors multiple domains including: two potential transmembrane domains (ϕ), coiled-coil (cc) regions, a Regulator of G-protein Signaling (RGS) domain that binds to and inactivates Gαs (30,32), a Phox-homology (PX) domain that binds phosphoinositide lipids, especially PI3P (32,34), and a PX-associated domain (PXA) of unknown function (32).
Figure 1-3. EGFR Trafficking and Signaling. [1] EGF binding to EGFR at the plasma membrane (PM) induces receptor dimerization and autophosphorylation (PO₄⁻) at specific tyr residues within its cytoplasmic domain, many of which bind to and recruit SH2-adaptor proteins from the cytoplasm (49). The SH2-adaptors in turn direct activation of downstream signaling including but not limited to ERK 1/2 MAPK, PLCγ, and PI3K/Akt (107). EGFR signaling is shut down by downregulation. Downregulation [2] involves ubiquitination of the receptor, receptor internalization via clathrin-mediated (CM) as well as non-CM endocytic pathways (108,109), and trafficking to endosomes (47). At endosomes [3], the tyrosine-phosphorylated C-termini continue to recruit SH2 adaptors that can initiate downstream signaling (110). [4] EGFR is then either recycled back to the PM or sorted in an ubiquitin-dependent manner into internal vesicles within endosomes which sequesters it and prevents its recycling and downstream signaling (55). [5] These multivesicular bodies (MVBs) containing intraluminal EGFR then mature into or fuse with lysosomes resulting in receptor degradation (55).
Figure 1-4. Schematic of the domains of GIV. GIV is a large, 1870 a.a. protein with multiple functional domains including: a Gα binding domain (87), a Hook domain that binds microtubules (91), a GEF motif that activates Gαi (81), a phosphoinositide (PI) binding domain (81,92), an actin binding domain (92), and an Akt binding domain (90).
Figure 1-5. Overview of Cell Migration. A quiescent cell senses a gradient in chemoattractant and then signals through GPCRs and/or RTKs that in turn initiates polarization, a process whereby the Golgi apparatus and the Microtubule Organizing Center (MTOC) are repositioned to the front of the nucleus and a leading edge is formed (indicated in red) that is enriched in PIP3 binding proteins required for actin remodeling (e.g., PI3K/Akt and GIV) and pseudopod formation (92,94). Arrow, direction of migration; N, nucleus; G, Golgi; red dot, Microtubule organizing center, MTOC; -----, microtubule tracks.
REFERENCES


Chapter 2

A $G_{\alpha i}$-GIV Molecular Switch Decides whether Cells Migrate or Proliferate in Response to Growth Factors
SUMMARY

Cells responding to growth factors either migrate or proliferate, but not both at the same time, a phenomenon termed migration-proliferation dichotomy. The mechanism that underlies this cellular decision-making remains unknown. We demonstrate that a Gαi-GIV switch, comprised of the trimeric G protein, Gαi and GIV, its non-receptor GEF, reprograms epidermal growth factor receptor (EGFR)-signaling and orchestrates migration-proliferation-dichotomy by modulating Gαi activity. This switch interacts with and regulates EGFR phosphorylation, thereby affecting SH2-adaptor recruitment, receptor distribution between the PM and endosomes, and the rate of receptor degradation. Consequently, an intact switch triggers motility by selectively amplifying EGFR-initiated motogenic signals (PI3K-Akt and PLCγ1) from the PM, while a disabled switch triggers mitosis by selectively propagating mitogenic signals (ERK1/2 and Src-STAT5) from endosomes. In colorectal carcinomas, the Gαi-GIV switch is disabled during early stages of tumor growth by downregulation of GIV and is assembled later during metastatic invasion by upregulation of GIV. This selective assembly of the switch in tumor cells generates distinct epigenetic traits with respect to proliferation vs. migration, which could cumulatively influence growth and invasiveness of tumors during oncogenic progression.

INTRODUCTION

Cancer cells within a tumor either migrate or proliferate, but not both at the same time, a phenomenon termed migration-proliferation dichotomy (1,2).
Although this term was initially coined in the context of invading cancer cells, similar observations were made during epithelial wound healing where migration and proliferation are compartmentalized events (3-5). These observations reinforce the concept that migrating cells must halt mitosis. Recently, computational modeling of the epidermal growth factor receptor (EGFR)-dependent gene-protein network predicted that differential signaling downstream of EGFR is sufficient to ‘simulate’ the migration-proliferation dichotomy (6).

Although EGFR can trigger both motility and mitosis (7,8), the type/concentration of the EGFR-activating ligand (9), abundance of EGFR (10) or its spatial distribution (11) influences whether cells migrate or divide. In migrating cells, a distinct set of receptor-initiated signals (e.g., PLCγ1 and PI3K-Akt) are amplified and coupled to actin remodeling within pseudopods at the leading edge (11). In proliferating cells, another set of receptor-initiated signals (e.g., MAPK/ERK1/2, c-Src/STAT5b) is amplified and propagated which leads to activation of nuclear transcription factors that drive DNA synthesis during mitosis (11,12). Ligand-stimulation initiates both sets of signals, which are rapidly modulated (either attenuated or amplified) in the immediate post-receptor phase such that migration and mitosis are executed in a mutually exclusive manner (8). Little is known about how receptor-initiated signals are refined to deliver one predominant message to the cell and how such post-receptor decision making is formulated.

We recently discovered (13) a molecular switch comprised of a trimeric G protein subunit, Gαi, and a non-receptor Guanine-nucleotide Exchange Factor
(GEF), GIV (\(G_\alpha\)-Interacting, Vesicle-associated protein; aka, Girdin) that is required for growth factors (EGF, VEGF, Insulin) to enhance Akt, remodel actin within pseudopods, and trigger cell migration (14-16). We showed that the \(G_\alpha i\)-GIV switch is necessary for triggering motility during epithelial wound healing, macrophage chemotaxis and tumor cell migration (15), and others implicated GIV in cancer invasion/metastasis (17) by virtue of its pro-migratory features. We defined the most critical component of the \(G_\alpha i\)-GIV switch as the regulatory ‘GEF motif’ within GIV’s C-terminus (CT) that forms a unique interface with the \(G_\alpha i\)-subunit (13), which we henceforth refer to as the \(G_\alpha i\)-GIV interface. By assembling this critical interface with \(G_\alpha i\), GIV’s GEF motif activates the \(G_\alpha\)-subunit and releases ‘free’ \(G_\beta \gamma\), thereby amplifying Akt signaling via the \(G_\beta \gamma\)-PI3K pathway. Since the \(G_\alpha i\)-GIV switch operates as a signal amplifier within the growth factor-signaling network through activation of \(G_\alpha i\), we sought to investigate if the presence or absence of an intact switch broadly affected EGFR signaling, and whether this switch could dictate cellular decision making with respect to migration vs. proliferation.

MATERIALS AND METHODS

Reagents and antibodies

Unless otherwise indicated all reagents were of analytical grade obtained from Sigma-Aldrich (St. Louis, MO) and cell culture media were purchased from Invitrogen (Carlsbad, CA). Silencer Negative Control #1 siRNA and siRNA \(G_\alpha i3\) were purchased from Ambion and Santa Cruz, respectively. Streptavidin-
horseradish peroxidase (HRP), biotinylated EGF and unconjugted mouse submaxillary EGF were purchased from Invitrogen and Rhodamine Red X-anti-HRP was from Jackson ImmunoResearch. Antibodies against GIV that were used in this work include rabbit serum and affinity purified anti-GIV coiled-coil IgG (GIV-ccAb) raised against the coiled coil domain of GIV (15,18), and affinity purified anti-Girdin C-terminus (GIV-CTAb) raised against last 18 aa of GIV’s C-term (IBL America, Minnesota)(14). The mAb #225 used to visualize EGFR by immunoblotting or immunofluorescence and for immunoprecipitating the receptor was a generous gift from Gordon N. Gill, UCSD (19). Other antibodies used in this work include: pAb against Gαs and Gαi3 (Calbiochem, CA); Gα (M-14), EGFR and Gαi3 used for immunoblotting (SCBT, CA); mAb EEA1 (BD Biosciences), pAb STAT3 (Cell Signaling), pAb STAT5b and mAb phospho-STAT3 (Santa Cruz, CA), mAb phospho-(Y694/Y699)-STAT5 (Millipore), pAb phospho-Y527-Src (Cell Signaling), mAb c-Src (Santa Cruz), pAb PLCγ1 (Cell Signaling), pAb phospho-Y783-PLCγ1 (Cell Signaling), mAb c-Cbl (BD Biosciences), mAb Akt (BD Biosciences), pAb phosho-Akt (Ser 473, Cell Signaling), mAb ERK 1/2 (Cell Signaling), mAb phospho-ERK 1/2 (Cell Signaling), mAb actin (Sigma), mAb pY845-EGFR (Millipore), and polyclonal EGFR antibodies (total, pY992, pY1045, pY1068) (Cell Signaling). Anti-mouse and anti-rabbit Alexa-594- and Alexa-488-coupled goat secondary antibodies for IF, phalloidin-Texas Red and anti-V5 were purchased from Invitrogen. Goat anti-rabbit and goat anti-mouse Alexa Fluoro 680 or IRDye 800 F(ab’)2 for immunoblotting were from Li-Cor Biosciences (Lincoln, NE). Control Mouse and
rabbit IgGs for immunoprecipitations was purchased from BioRad (Hercules, CA) and Sigma (St. Louis, MO) respectively.

**Plasmid constructs, mutagenesis and protein expression**

Flag-EGFR was a generous gift from Dr. Howard A. Rockman (Duke University Medical Center, Durham, North Carolina)(20). Cloning of G\(\alpha_i3\) and GIV into pGEX-4T-1 or pET28b were described previously (14). To transiently express N- and C-terminal Flag-tagged G\(\alpha_i3\) protein in Cos7 cells, G\(\alpha_i3\) was cloned into p3XFlag-CMVTM-10 or p3XFlag-CMVTM-14 expression vectors, respectively, using BamH I and Hind III restriction enzymes. Expression and purification of His-GIV-CT (1623-1870) was done as previously (Garcia-Marcos et al). GIV and G\(\alpha_i3\) mutants were generated using specific primers (sequences available upon request) following the manufacturer’s instructions (QuickChange II, Stratagene, San Diego, CA). The truncated GIV\(\Delta\)CT construct was generated by creating a stop codon using a similar protocol to that above. RNAi-resistant GIV was generated by the silent mutations as described (14). All constructs were checked by DNA sequencing.

Plasmids encoding GST-G\(\alpha_i3\), His-G\(\alpha_i3\) or His-GIV-CT fusion constructs were used to express these proteins in E. coli as described (15,21). For the His-tagged G\(\alpha_i3\) or GIV-CT, a similar procedure was followed using His-lysis buffer [50 mM Na\(\text{H}_2\)PO\(_4\) pH 7.4, 300 mM NaCl, 10 mM imidazole, 1% (v:v) Triton X-100, 2X protease inhibitor cocktail (Complete EDTA-free, Roche Diagnostics)], and cobalt resin for purification (HisPur Cobalt Resin, Pierce). His-G\(\alpha_i3\) used for the GTPase assays were buffer exchanged into G protein storage buffer (20 mM...
Tris-HCl pH 7.4, 200 mM NaCl, 1 mM MgCl₂, 1mM DTT, 10 μM GDP, 5% (v:v) glycerol) prior to storage at -80 °C. The purified protein (95%) was properly folded as assessed by a protease protection assay (Garcia-Marcos et al. 2008).

**Cell culture, transfection and lysis**

Unless mentioned otherwise, all cell lines used in this work were propagated as per ATCC guidelines. The 21T breast cell lines (16N, NT and MT2) were obtained from Arthur Pardee (Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA) and maintained as described previously (22,23). Ls-174T and Ls-LiM6 were obtained from Robert Bresalier (MD Anderson Cancer Center, TX) and grown as described previously (24). Ls-174T is a poorly metastatic colon cancer cell line harvested from a Duke’s clinical stage B tumor. Ls-LiM6 is a highly metastatic sub-clone of Ls-174T cells that was selected by serial passage of Ls-174T through a murine cecum-to-liver metastasis model. Transfection was carried out using FUGENE 6 (Roche Diagnostics) or Oligofectamine (Invitrogen) following the manufacturers’ protocols, and stable cell lines were selected using the neomycin analogue, G418 (Cellgro).

Transfections to transiently overexpress proteins using Flag-EGFR, GIV or Gαi3 plasmids or protein silencing using siRNA Gαi3 were carried out exactly as described (25). HeLa cell lines stably expressing vector control (HeLa-V), GIV (HeLa-GIV-wt), GIV-F1685A mutant (HeLa-GIV-FA) or GIV 1-1453 (GIVΔCT) were selected after transfection in the presence of G418 (500 μg/ml) for 6 weeks, followed by clonal selection. Clones were chosen for each construct that had
relatively low expression levels of GIV (~3 times higher than endogenous levels). For each construct, two separate clones were investigated and similar results were obtained.

For growth factor stimulation assays involving serum starvation, the serum concentration was reduced to 0.2% for 12 hr, and if indicated, cells were then stimulated with 50 nM EGF for indicated times. Whole cell lysates were prepared after washing cells with cold PBS by resuspending cells in sample buffer and boiling immediately.

Lysates used as a source for EGFR or GIV in pulldown or immunoprecipitation assays were prepared by resuspending cells in lysis buffer (20 mM HEPES, pH 7.2, 5 mM Mg-acetate, 125 mM K-acetate, 0.4% Triton X-100, 1 mM DTT, supplemented with phosphatase (Sigma) and protease (Roche) inhibitor cocktails, passing them through a 28G needle at 4 °C, and clearing (10,000g for 5 min) before use in subsequent experiments.

**Measurement of Mitotic Index**

Mitotic index, as determined by the abundance of the marker for mitosis, phosphorylated histone H3 (26), was estimated by a combination of immunofluorescence and immunoblotting as described previously (27,28).

**Scratch-wound Assays**

Scratch wound assays were carried out as described previously (15). To quantify cell migration (expressed as % wound area covered), images were analyzed using Image J (NIH) software to calculate the difference between the
wound area at 0 min and at the end of the migration assay divided by the area at 0 min x 100.

**Immunoprecipitations and binding assays**

Cell lysates (~1-2 mg protein) were incubated 4 hr at 4°C with either 2 μl of mAb anti-Flag (Sigma) or pAb GIV (ccAb) or their respective pre-immune control IgGs. Protein G agarose beads (Invitrogen) were added and incubated at 4°C for an additional 60 min. Beads were washed then either resuspended and boiled in SDS sample buffer, or beads were incubated with purified His-GIV-CT (a.a. 1623-1870) overnight, washed, and then resuspended and boiled in SDS sample buffer. The in-vitro binding assays using GST-fused proteins were carried out as previously described (15). Briefly, purified GST-fusion proteins (15-20 μg) or GST alone (30 μg) were immobilized on glutathione-Sepharose beads and resuspended in binding buffer supplemented with nucleotides (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.4% (v:v) NP-40, 10 mM MgCl₂, 5 mM EDTA, 2 mM DTT, protease inhibitor cocktail supplemented with either 30 μM GDP or 30 μM GDP, 30 μM AlCl₃, 10 mM NaF) (15). Thereafter, [³⁵S]Met- (GE Healthcare) labeled GIV prepared using the TnT Quick Coupled Transcription/Translation System (Promega) was added to the binding buffer and binding was carried out overnight at 4°C with constant tumbling. The following day the beads were washed (4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% (v:v) Tween 20, 10 mM MgCl₂, 5 mM EDTA, 2 mM DTT), and boiled in sample buffer for SDS-PAGE. The washing buffer was supplemented with GDP or GDP, AlCl₃ and NaF as during binding.
**Immunofluorescence**

All of the steps for IF were carried out at room temperature as described previously (15). Briefly, cells were fixed with 3% paraformaldehyde for 30 min, permeabilized (0.2% Triton X-100) for 45 min and incubated for 1 h with first primary and then secondary antibodies. Antibody dilutions were as follows: EEA1, 1:250; EGFR (Santa Cruz), 1:100; EGFR #225 mAb, 1:500; Phalloidin-Texas Red, 1:1000; secondary Alexa-conjugated antibodies, 1:500, and DAPI, 1:2000 (Molecular Probes). For surface labeling of EGFR, HRP-conjugated EGF was generated by incubating 5 μg of Strep-HRP with 0.3 μg of biotinylated-EGF in 300 μl of PBS overnight at 4C as described previously (29). Ligand-bound receptor was then visualized using Rhodamine- Red X anti-HRP antibody (1:100, Jackson ImmunoResearch). Images were acquired with a Zeiss Axioimager M1 microscope using a 100x aperture (Zeiss Plan Neofluar, 1.30 NA), Hamamatsu Orca-ER camera, and Openlab software (Improvision). All individual images were processed using Image J software (NIH) and assembled for presentation using Photoshop and Illustrator software (both Adobe).

For EGFR-GIV co-localization studies (**Fig. 2-4**) wide-field microscopy was performed using the 100x Neofluor objective lenses (N.A. 1.30, oil immersion) on an inverted Olympus (Melville, NY) IX-70 DeltaVision Restoration microscope. The microscope was equipped with DAPI (360/40 excitation, 457/50 emission), FITC (490/20 excitation, 528/38 emission), and tetramethylrhodamine B isothiocyanate (TRITC; 555/28 excitation, 617/73 emission) band pass filter sets, a Bioptechs (Butler, PA) FCS2 motorized stage, and a Photometrics
(Tucson, AZ) CH350 CCD camera (Hamamatsu). Optical sections were collected at 0.2-μm intervals along the apical-basal axis. Applied Precision software (DeltaVision) was used to deconvolve z-section series of images. All individual images were processed using Image J software and assembled for presentation using Photoshop and Illustrator software (both Adobe).

**Growth curves**

Confluent monolayers of HeLa cells (untransfected controls, vector transfected, stably expressing GIV-wt or GIVF1685A (~3 fold normal) or GIVΔCT (levels similar to those observed in poorly metastatic MCF7 cells) were maintained in 10% FBS supplemented with penicillin, streptomycin and 500 μg of G418. Each cell line was seeded at a density of 2 x 10^4 cells per well (6-well plate), cultured in the presence of the indicated amounts of serum (10% or 1%), and harvested every day for up to 5 d. After harvesting, the cells were stained with Trypan blue, and only the total number of live cells as determined by Trypan blue dye exclusion were counted using a hemocytometer (Y axis) and plotted against the number of days (X axis). Prolonged incubation in 1% serum beyond day 5 resulted in extensive cell death in the vector control and GIV-wt expressing cell line, but not in those expressing GIVF1685A or GIVΔCT, thus limiting the overall duration of the assay to 5 d.

**Immunohistochemistry**

Immunohistochemistry was performed as previously described (30). Briefly, slides containing colon cancer tissues were deparaffinized in xylene and rehydrated in graded alcohols to water. Slides were immersed in sodium citrate
buffer (pH 6.0), and heated in a microwave 4 times, 4 min each, for antigen retrieval. Slides were then processed using a DAKO® Signal Catalyzed Amplification (CSA) System (DAKO Corporation, Carpinteria, CA). Endogenous peroxidase activity was blocked by incubation with H₂O₂. Ten percent goat serum was added for 15 min to block nonspecific protein binding. Rabbit GIV-CTAb 6 µg/ml (a.k.a., anti-Girdin; Immuno-Biological laboratories) was incubated overnight and then rinsed with PBS. Biotinylated anti-rabbit IgG were added for 15 min followed by incubation with peroxidase-labeled streptavidin for 15 min at room temperature. Sections were washed with PBS, incubated with DAB and H₂O₂ for 1 min, lightly counterstained with hematoxylin, dehydrated in graded alcohols, cleared in xylene, and coverslipped. Staining was scored as negative or positive by three independent observers blinded to patient outcome and stage.

**Live Cell imaging**

HeLa cells were grown to confluency in DMEM with 10% serum. For study of cells expressing Gαi3-YFP proteins, experiments were performed as described (15). For study of GIV-wt and GIV-FA HeLa cells, serum was reduced to 0.2% and after 6-12 hrs cells were scratch wounded with a 20 µL pipette tip and 0.1 nM EGF was added. Cells were immediately transferred to an incubated (37°C, 5% CO₂) Olympus X81 inverted microscope with ASI x-y-z moving stage (UCSD Neuroscience Microscopy Facility) to capture many fields simultaneously. Cells were imaged at 20x magnification every 10 min for up to 16 h. The extent of cell migration or division at the wound edge was assessed by counting the number of cells in each field that displayed polarized motility or
successful cell divisions divided by the total number of cells originating within 2-3 cells (about 2.5 cm on the 20x image) of the wound edge in each field.

**RT-PCR**

Total RNA was isolated from each cell line using the RNeasy Mini Kit (Qiagen). First strand cDNA was generated by RT-PCR using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen). Primers used in this work were designed using Invitrogen’s Oligo Perfect™ Designer and evaluated with NetPrimer from Premierbiosoft. Positioning of primers was dependent on the target domains of GIV we intended to amplify (Fig. 2-S7, S10), and GAPDH (Allele Biotechnology and Pharmaceuticals) mRNA amplified from the same samples served as an internal control. Thirty-five cycles of PCR were carried out across all experiments using JumpStart Taq DNA Polymerase (Sigma) to determine the presence of these regions of interest. The amplified PCR products were visualized, photographed (Chemi Doc XRS; Bio-Rad Laboratories, Hercules, CA) and quantified using Quantity One® SW software (Bio-Rad). For sequence analysis, PCR products were purified using “Qiaquick” (Qiagen) as per the manufacturer’s protocol and sequenced (DNA sequencing facility, UCSD Moore’s Cancer Center). To rule out contamination due to genomic DNA, RT-PCR-minus reactions were run similarly as a one step reaction using total RNA for PCR, eliminating the step of cDNA synthesis using reverse transcriptase. To rule out gene fusion events at the C-terminus of GIV, rapid amplification of cDNA ends (3’RACE kit, Invitrogen) reactions were carried out on some of the cell lines (HT29, Ls174T and MCF7).
Statistical Analysis

Each experiment presented in the figures is representative of at least three independent experiments. Statistical significance (p value) between various conditions where obtained was assessed with the Student’s t-test. All graphical data presented in this work was prepared using GraphPad Software, Inc., San Diego, CA.

RESULTS

GIV-dependent Gαi Activity Dictates the Cellular Choice to Migrate or Proliferate

To investigate how the Gαi-GIV switch affects cellular preference for migration vs. proliferation, we used live cell imaging to simultaneously monitor both processes in HeLa epithelial cell lines stably expressing either wild-type GIV (GIV-wt cells) or a dominant negative, GEF-deficient F1685A mutant of GIV (GIV-FA cells) incapable of interacting with or activating the G protein. Both cell lines expressed GIV 3-fold over endogenous (Fig. 2-S1, (13)). To stimulate the cells we used EGF in combination with scratch-wounding and found that motile GIV-wt cells typically migrated into the wound, whereas GIV-FA cells showed reduced migration but ~50% more mitotic events at the wound edge (Fig. 2-1A). The mitotic index, as determined by the levels of phospho-Histone H3, an indicator of mitosis (26) was consistently high in GIV-FA cells, intermediate in untransfected cells and low in GIV-wt cells following scratch wounding (Fig. 2-1B, C). Analysis of growth curves of each cell line at steady-state in the presence of either 10% or 1% serum confirmed that expression of GIV-wt suppressed
whereas expression of GIV-F1685A enhanced cell proliferation (Fig. 2-S2A, B). Thus, GIV-FA cells possess a proliferative advantage that persists across a range of stimuli. Overall, these results demonstrate that upon stimulation, cells with an intact Gαi-GIV switch preferentially migrate and suppress proliferation, while cells with a disabled switch preferentially proliferate and suppress migration.

Since GIV-wt and GIV-FA cells differ by a single point mutation that specifically affects GIV’s GEF activity and prevents activation of Gαi (13), we asked if the dichotomy between migration and proliferation is a direct consequence of GIV’s ability to modulate the activity of Gαi in response to GFs. To investigate this, we overexpressed YFP-tagged Gαi3-wt, or constitutively active (GTPase deficient) Gαi3-Q204L (QL) or inactive Gαi3-G203A (GA) mutants (31) and monitored cell motility and division simultaneously by live cell imaging (Fig. 2-1D). The proportion of cells that displayed polarized migration, as determined by directional motility into the wound was similar for Gαi3wt and Gαi3QL but was reduced by ~50% for Gαi3GA. Mitosis was infrequent (~ 3%) among cells expressing Gαi3-wt-YFP and Gαi3QL-YFP but was increased four-fold among cells expressing Gαi3GA-YFP (Fig. 2-1D). These results, together with our findings in GIV-wt vs. GIV-FA cells, demonstrate that the activity status of Gαi3 can determine if cells migrate or proliferate and that modulation of Gαi activity by GIV’s GEF motif serves as a critical determinant of cellular preference for migration or proliferation.
The $G_{\alpha i}$-GIV Switch Reprograms EGFR Signaling

Because previous work demonstrates the importance of EGFR-signaling in influencing whether cells migrate or proliferate (6-8), we asked if cells expressing an intact vs. a disabled $G_{\alpha i}$-GIV switch had distinctive effects on EGFR signaling. GIV-wt, GIV-FA and untransfected HeLa cells were treated with EGF and analyzed for activation of four major signals initiated by EGFR--Akt, PLC$_{\gamma 1}$, ERK1/2 MAPK and c-Src-STAT5b--by immunoblotting (11,12). We found that GIV-wt cells showed enhanced Akt and PLC$_{\gamma 1}$ but attenuated STAT5b and ERK1/2 signaling (Fig. 2-1E, F). By contrast, GIV-FA cells showed enhanced ERK1/2 and STAT5b (Fig. 2-1E, F, Fig. 2-S3) but attenuated Akt and PLC$_{\gamma 1}$ signaling. c-Src activity was also decreased in GIV-wt and increased in GIV-FA cells compared to controls (Fig. 2-1E, F), as determined by dephosphorylation of pY527 on Src, which coincides with entry into mitosis (32). Similar contrasting profiles of ERK1/2 activation were also evident when EGFR was stimulated in GIV-wt and GIV-FA cells by scratch-wounding (data not shown), indicating that the $G_{\alpha i}$-GIV interface plays a decisive role in selective signal amplification irrespective of the mode of receptor activation. Thus, in GIV-wt cells, Akt and PLC$_{\gamma 1}$ signals that promote motility (motogenic) are preferentially amplified and c-Src/STAT5b and ERK1/2 signals that promote mitosis (mitogenic) are suppressed, whereas GIV-FA cells display a mirror-image response (Fig. 2-1G). These results demonstrate that the integrity of the $G_{\alpha i}$-GIV switch, the key function of which is to modulate $G_{\alpha i}$ activity (13), is a critical component of the
decision process by which EGFR-initiated signals are selectively enhanced or suppressed.

**The G\(\alpha\)i-GIV Switch Regulates EGFR Phosphorylation and Recruitment of SH2-Adaptors**

Next we asked if the G\(\alpha\)i-GIV switch alters ligand-induced EGFR phosphorylation or adaptor-recruitment, two early and decisive events in EGFR-signaling (33). Serum starved GIV-wt and GIV-FA cells were stimulated with EGF and analyzed for EGFR autophosphorylation (as determined by phosphorylation at Y992, Y1045, and Y1068, which serve as the docking sites for major SH2-adaptors (Fig. 2-2A)) or Src-dependent EGFR phosphorylation (at Y845) using site-specific phospho-Tyr antibodies by immunoblotting. Promigratory GIV-wt cells displayed enhanced EGFR autophosphorylation but normal phosphorylation at the c-Src substrate, Y845 (Fig 2-2B, C) compared to controls. By contrast, proproliferative GIV-FA cells displayed attenuated EGFR autophosphorylation but sustained phosphorylation at Y845 (Fig. 2-2B, C). These results demonstrate that the presence of an intact G\(\alpha\)i-GIV interface enhances receptor autophosphorylation, whereas a disrupted interface enhances c-Src-mediated receptor phosphorylation.

SH2-adaptor recruitment to the above sites of autophosphorylation on EGFR tail (Fig. 2-2A) in GIV-wt vs. GIV-FA cells was evaluated by immunoprecipitating the receptor and analyzing the receptor-bound complexes for phospho-PLC\(\gamma\)1, c-Cbl, and Grb2 as well as total and phospho-Y845 EGFR by immunoblotting (Fig. 2-2D-H). Enhanced EGFR autophosphorylation in GIV-
wt cells coincided with increased, while attenuated EGFR autophosphorylation in GIV-FA cells coincided with decreased receptor-SH2 adaptor association compared to untransfected controls (Fig. 2-2B, D-H). These results demonstrate that the Gαi-GIV switch affects two of the earliest events in EGFR signaling— the pattern of receptor phosphorylation and SH2-adaptor recruitment.

The Gαi-GIV Switch is a Key Determinant of EGFR Localization and Degradation

Because the Gαi-GIV switch affects EGFR signaling (Fig. 1, 2) and EGFR-signaling is regulated by receptor internalization and its compartmentalization in endosomes (11), we next compared the localization of EGFR in GIV-wt and GIV-FA cells by immunofluorescence. Upon stimulation with HRP-conjugated EGF, in GIV-wt cells a larger pool of ligand-bound EGFR appeared as punctate staining on the PM at 15 min compared to controls (Fig. 2-3A, e), where the receptor co-localized with cortical actin (Fig. 2-3B, d-f). By contrast, in GIV-FA cells most of the EGFR was internalized into EEA1-positive early endosomal compartments (Fig. 2-3A, i, l) and was virtually undetectable at the PM (Fig. 2-3A, f). These data indicate that ligand-induced receptor internalization was delayed in GIV-wt and accelerated in GIV-FA cells. An identical pattern was detected when total EGFR was analyzed using a mAb (34), regardless of whether high (50 nM, Fig. 2-S4A) or low (0.1 nM, data not shown) concentrations of EGF was used. Enhanced EGFR-association with the PM-actin bed was also seen in GIV-wt, but not GIV-FA cells in wounding assays (Fig. 2-
S4B), indicating that an intact Gαi-GIV switch enhances receptor-association with the PM during cell migration.

At 60 min (Fig. 2-3C), a significant amount of EGFR was still detectable in GIV-FA cells but was undetectable in GIV-wt cells. Similar results were obtained by immunoblotting, in that ~20% of the receptor remained in GIV-wt cells at 30 min after ligand stimulation and ~40% in controls vs. ~74% in GIV-FA cells (Fig. 2-3D), even though all these cell lines had equal amounts of EGFR at steady-state (Fig. 2-S1). Thus, GIV-wt promotes and GIV-FA inhibits EGFR degradation. Cumulatively, these results indicate that the Gαi-GIV switch regulates spatiotemporal aspects of EGFR signaling by affecting the distribution of EGFR between PM and endosomes and the rate of EGFR degradation.

**Gαi, GIV, Actin and EGFR Interact within a Signaling Complex**

Since the Gαi-GIV interface affected some of the earliest events in EGFR signaling (Fig. 2-2) and its association with the cortical actin bed (Fig. 2-3B-D), we reasoned that GIV and/or Gαi might interact with EGFR. To investigate this possibility, we carried out immunoprecipitation assays from Cos7 cells expressing FLAG-tagged EGFR and analyzed the receptor-bound complexes for the presence of GIV by immunoblotting. We found that GIV co-immunoprecipitates with EGFR (and *vice versa*) under both starved and EGF-stimulated conditions (Fig. 2-4A, Fig. 2-S5A) and receptor activation was associated with increased GIV-EGFR association. Additionally, we identified Gαi3, GIV and EGFR within the same complex from Cos7 cells co-expressing FLAG-Gαi3 and EGFR (Fig. 2-4B), indicating that a ternary complex can form *in*
vivo. The GIV-EGFR interaction was confirmed for the endogenous proteins using A431 cells (that express high levels of EGFR (35)) (Fig. 2-S5B). Additionally, patchy colocalization of endogenous GIV and EGFR was seen at the PM in HeLa cells by confocal microscopy (Fig. 4C). These results indicate that the EGFR-GIV interaction occurs at the PM.

Because the G\(\alpha\)i-GIV switch alters EGFR colocalization with cortical actin (Fig. 2-1E, 2-2, 2-3B) and actin-EGFR interactions are known to affect receptor autophosphorylation and retention at the PM (36,37), we investigated whether the switch could modulate receptor binding to actin. We immunoprecipitated EGFR from GIV-wt and GIV-FA cells and analyzed the receptor-bound proteins for actin and G\(\alpha\)i3 by immunoblotting (Fig. 2-4D). In GIV-wt cells EGFR transiently and maximally associated with actin and G\(\alpha\)i3 at 15 min after ligand stimulation, whereas in GIV-FA cells EGFR-actin association and G\(\alpha\)i3 recruitment to the receptor were virtually abolished. These results indicate that the interaction between G\(\alpha\)i3 and GIV is critical for effective formation/stabilization of an EGFR-actin scaffold complex and that EGFR, actin and G protein are linked via GIV.

Based on these results we propose (Fig. 2-4E) that GIV promotes EGFR-actin association, receptor autophosphorylation and retention of the receptor at the PM by coupling G\(\alpha\)i-dependent actin remodeling (13) to ligand-activated EGFR in the immediate post-receptor phase. As a consequence of effective coupling of GIV-dependent G\(\alpha\)i activity to actin and EGFR in GIV-wt cells, or uncoupling in GIV-FA cells, the receptor is differentially retained at the PM-actin
bed and receptor-initiated signals are enhanced or suppressed to trigger either motility or mitosis (see legend for Fig. 2-4E).

**Activity of the G\textsubscript{ai}-GIV Switch is Enhanced or Suppressed in Cancer Cells by Up- or Downregulation of GIV Expression, Respectively**

Next we investigated if the integrity of the G\textsubscript{ai}-GIV switch is dysregulated during tumor progression, a GF-driven process in which cells with a proliferative advantage initially dominate the tumor and promote growth, whereas cells with promigratory/invasive characteristics are preferentially enriched later and drive tumor cell invasion during metastasis (38,39). The first clue that such might be the case came from our finding, using an antibody against the C-terminal 19 aa of GIV, that full length GIV (GIV-fl, \(\sim 250\) kD) was detected in highly invasive but not in highly proliferative colon cancer cells, whereas G\textsubscript{ai}3 levels were similar (15). Therefore, we looked for GIV-fl mRNA by RT-PCR using two C-terminal GIV-domain specific primers (C-terminus, CT, and G-binding domain, GBD) (Fig. 2-S6A) and found that wherever GIV-fl protein was undetectable the transcript was also virtually abolished (Fig. 2-5A). Similar analysis of three breast cancer cell lines (36) revealed that GIV-fl (protein and mRNA) was expressed only in slow growing, highly motile/invasive cells and undetectable in fast growing, poorly motile cells (Fig. 2-5B). In contrast to cancer cells, a GIV-fl transcript was always detected in normal colon and breast epithelia (Fig. 2-S6B, C). Further comparison of the abundance of PCR products (GBD through C-terminus) in an extended set of cancer vs. normal cells revealed that across 3 different carcinomas, GIV-fl was consistently upregulated \(\sim 3-30\) fold in highly invasive
cells and downregulated ~5-12 fold in poorly invasive cells (Fig. 2-5C). In highly invasive cells, GIV selectively bound Gαi3 in the presence of GDP, but not GDP•AlF₄⁻ (data not shown) indicating that GIV retains its characteristics as a non-receptor GEF (13). These results indicate that altered expression of GIV, but not Gαi, is the key determinant of whether Gαi is activated via the assembly of a functional Gαi-GIV switch. Consequently, in slow growing, highly invasive cells that overexpress GIV the Gαi-GIV switch is hyperactivated, whereas in fast growing, poorly invasive cells that suppress GIV, the Gαi-GIV switch is inactivated and Gαi activity is suppressed.

**In Poorly Invasive Cells, GIV is Replaced by GIVΔCT, a C-terminally Truncated Variant that Fails to Assemble a Functional Switch with Gαi**

Since metastasis-related genes typically show aberrant expression by splicing (40) and the delicate equilibrium of antagonistic splice variants is often perturbed in tumors (41), we asked if dysregulation of GIV-fl expression occurs due to alternative splicing. Using RT-PCR and a series of oligonucleotide primers (Fig. 2-S7) we investigated the N-terminus of GIV and detected transcripts of predicted size and sequence in highly invasive cancer cells and their respective normal controls (Fig. 2-6A, 2-S8). In the poorly invasive colon (Ls174T and HT29) and breast (MCF7) cancer cells, this product was either accompanied by or virtually replaced by an additional, larger PCR product (Fig. 2-6A, 2-S8). Based on the positioning of the primer pairs, transcript size and sequence analysis, we deduced that the amplified RNA transcript is the product of an alternative splicing event due to failure of processing and resultant retention of
the 1,000 bp-long 19\textsuperscript{th} intron which we named GIV-Intron Retention 19 (GIV-IR19). In the poorly invasive cells, the abundance of GIV-IR19 was inversely proportional to that of GIV-fl, i.e., the higher the percentage of intron retention (IR), the less GIV-fl mRNA or protein (Fig. 2-5, 2-6B). These results indicate that in poorly invasive cells, IR effectively disrupted GIV-fl translation and downregulated GIV-fl expression, as is known to occur in other instances (42).

The retained 19\textsuperscript{th} intron within GIV-IR19 contained an in-frame stop codon (Fig. 2-6C) and we asked if this aberrant splice variant is translated into protein. Although GIV-fl was undetectable in poorly invasive cells using GIV-CTAb (Fig. 2-5, 2-6D), a $\sim$135 KDa protein was detected using an affinity purified GIV-ccAb antibody, raised against aa 1174–1499 which includes the coiled-coil but excludes the C-terminus (18). Based on the sequence of GIV-IR19, this translated protein was predicted to exclude the entire C-terminus (Fig. 2-6C) and thus, we named it GIV\textDelta CT. As anticipated, in the absence of both G\alpha i-interacting domains, GBD (18) and the critical GEF motif (13), GIV\textDelta CT did not bind G\alpha i3 in \textit{in vitro} binding assays (Fig. 2-6E), and therefore is incapable of assembling a functional switch with G\alpha i \textit{in vivo}.

**GIV\textDelta CT Inhibits Cell Migration but Enhances Proliferation by Modulating EGFR-Signaling**

To investigate the cellular consequences of replacing GIV-fl with GIV\textDelta CT, we generated a HeLa cell line expressing GIV\textDelta CT such that the ratio of GIV-fl:GIV\textDelta CT was identical to that found in poorly invasive MCF7 cells (percent intron retention = $\sim$65%) (Fig. 2-6A, B). We found that cells expressing GIV\textDelta CT
migrated poorly, failed to enhance Akt in scratch-wound assays (Fig. 2-6F, G) but grew rapidly (Fig. 2-6H, I) compared to GIV-wt and controls, indicating that lack of GIV’s C-terminus rendered GIVΔCT cells immotile but highly proliferative, much like the GIV-F1685A mutant (Fig. 2-1). Cells expressing GIVΔCT suppressed their motogenic response to EGF (as determined by Akt and PLCγ1 activities) and concomitantly enhanced mitogenic (ERK1/2 and STAT5b) signals (Fig. 2-6J), suppressed EGFR-autophosphorylation (Fig. 2-6L) and delayed receptor degradation (Fig. 2-6K), thereby mimicking the signaling profile displayed by GIV-FA cells. These results indicate that poorly invasive tumor cells that downregulate GIV-fl and replace it with GIVΔCT, disable the Gαi-GIV switch and thereby, preferentially propagate mitogenic signals downstream of EGFR with a resultant gain of proliferative advantage.

**During Cancer Progression Tumor Cells that Suppress GIV-fl Predominate Early and those that Induce GIV-fl are Enriched Later**

To investigate the pattern of GIV expression during the course of oncogenic progression, we analyzed paraffin-embedded human colorectal cancers representing various stages of invasive disease (Duke’s stages A through D, where ‘A’ is locally restricted tumor without spread and ‘D’ indicates the presence of distant macroscopic metastases) for GIV by immunohistochemistry (IHC). In normal colon, moderate amounts of GIV-fl (as determined by GIV-CTAb) were detected in epithelial cells lining the crypt bases (Fig. 2-7A, a, b). The stroma stained very strongly in normal tissue and cancers alike (Fig. 2-7A, 2-S9b), in keeping with the previously reported high GIV-fl
expression in mesenchymal cells (14-17). However, in the epithelia of adenomatous polyps (Fig. 2-7A, c, d) and in non-invasive (Duke’s A) tumors (Fig. 2-7A, e, f, 7B) GIV-fl was virtually undetectable. Among tumors of intermediate stages (Duke’s B and C) GIV-fl expression was heterogeneous and could be detected in some, but not others (Fig. 2-7B). Pockets of positively-stained tumor cells were often found within the stromal tissue at the invading margin of the tumor (Fig. 2-7A, g, h). All complex tumors of advanced stage (Duke’s D) stained positive for GIV-fl (Fig. 2-7B), and with increasing stage of invasiveness the intensity of GIV-fl staining in tumor epithelium approached that of the surrounding stroma (Fig. 2-7A, i, j). Both normal and tumor tissue stained homogeneously positive for GIV’s N-terminus (using GIV-ccAb) irrespective of their clinical stage (data not shown). Thus, tumors of early stages stained positive for GIV’s N-terminus, but not for GIV’s C-terminus, and the late stages stained positive for both. These results validate that the phenomenon of alternative splicing observed in cancer cell lines (Fig. 2-6A) also occurs in tumors in situ. As a result, during early stages of tumor growth, cells in which GIV is alternatively spliced to generate GIVΔCT predominate. During later stages of metastatic progression, cells with upregulated GIV-fl expression dominate the tumor. These results indicate that the Gi-GIV switch is disabled first and enabled later by up- or downregulation of GIV-fl during cancer progression (see legend for Fig 2-7C).
DISCUSSION

A Gαi-GIV Switch Establishes Migration-Proliferation Dichotomy

We describe a novel role for GIV-dependent G protein activation in dictating whether cells migrate or proliferate. GIV-wt cells that assemble a functional Gαi-GIV switch frequently migrate but rarely divide in response to EGF (Fig. 2-1). By contrast, GIV-FA cells which express a mutant that is specifically defective in its ability to interact with or activate Gαi (13), frequently divide but rarely migrate. Cells expressing active or inactive Gαi3 mutants also display preferential motility or mitosis, respectively, indicating that Gαi activity is sufficient for dictating cellular decision making. Overall, these results indicate that modulation of Gαi activity via the Gαi-GIV switch is the key to establishing this striking phenotypic dichotomy upon growth factor stimulation.

Mechanistically, we demonstrated that the phenotypic dichotomy imparted by the Gαi-GIV switch stems from differential amplification or attenuation of signals initiated by EGFR (Fig. 2-1). In response to EGF, promigratory GIV-wt cells enhanced motogenic signals and concomitantly suppress mitogenic ones, whereas proproliferative GIV-FA cells enhance mitogenic signals and concomitantly suppress motogenic ones. These results are in keeping with previous work that portrays a central role for EGFR (Athale et al., 2005) and an unknown post-receptor event in establishing migration-proliferation dichotomy (8). Our finding that G protein activity plays a key role in orchestrating this dichotomy is also consistent with previous work demonstrating that migration is
triggered by active G\(\alpha i3\) (15), but mitosis is enhanced by inactive G\(\alpha i\) (43). We conclude that two major signaling pathways, G proteins and growth factor receptors that converge upon GIV, participate in establishing this migration-proliferation dichotomy and that GIV-dependent Gi activity imparts this phenotypic dichotomy. Without GIV (14) or Gi (15,43) cells neither migrate nor gain proliferative advantage, suggesting that both G\(\alpha i\) and GIV are required to create the dichotomy.

**GIV Links G proteins to a Growth Factor Receptor**

Regarding how growth factor and G protein-signaling are linked, we demonstrate that GIV colocalizes and interacts with EGFR and that G\(\alpha i\) and GIV can exist as a complex with the receptor (Fig. 2-4). This G\(\alpha i\)-GIV-EGFR ternary complex is formed in GIV-wt, but not in GIV-FA cells where the G\(\alpha i\)-GIV interaction is disrupted. Consequently, efficient coupling of receptor signaling to Gi-dependent Akt enhancement and actin remodeling in response to growth factors occurs exclusively in GIV-wt cells, but not in GIV-FA cells (13). Indirect stimulation of growth factor receptors by G protein-dependent intermediates is well established (44); however, there is little mechanistic insight into how growth factor receptors signal via G proteins (45,46). We provide evidence that GIV can serve as a common platform for growth factor and G protein signaling by linking EGFR to G\(\alpha i\). Whether GIV interacts with the receptor indirectly via SH-2 adaptors, or directly by binding to phospho-tyrosine(s) on the receptor tail, is unknown. Regardless of how the interaction is mediated, since a variety of
growth factor receptors (VEGF, Insulin and IGF1) also utilize GIV to trigger cell migration (47) we speculate that they share a common mechanism.

**The G\(\alpha\)-GIV Switch Exerts Spatiotemporal Control on EGFR Signaling**

We demonstrate that EGFR autophosphorylation, SH2-adaptor recruitment and EGFR-actin association at the PM are enhanced and prolonged upon ligand stimulation in motile cells featuring an active G\(\alpha\)-GIV switch, but these events are suppressed in poorly motile cells featuring a disabled switch, (Fig. 2-2, 2-3). These results are consistent with previous reports that EGFR autophosphorylation is essential for motility but is dispensable for mitosis (7). Because an intact G\(\alpha\)-GIV interface is essential for GIV-dependent actin remodeling (13) and EGFR-association with actin promotes formation of signaling complexes (37), we propose a model in which the G\(\alpha\)-GIV switch modulates EGFR-signaling by regulating EGFR-actin dynamics on the PM (see legend, Fig. 2-4E). We also show that in the absence of the G\(\alpha\)-GIV switch, EGFR phosphorylation at Y845, a c-Src substrate site, is sustained compared to controls. Phosphorylation at Y845 is consistent with elevated mitogenic c-Src activity found in these cells and likely to be responsible for the observed sustained STAT5b phosphorylation, the key downstream signaling intermediate of c-Src-Y845 that drives EGF-induced DNA synthesis during mitosis (12).

We demonstrate that the G\(\alpha\)-GIV switch dictates trafficking of the activated receptor (Fig. 2-3). In motile GIV-wt cells, an intact switch enhanced and prolonged EGFR association with the PM-actin bed, but promoted rapid degradation of the receptor upon internalization. In these cells, receptor signaling
from the PM is enhanced but attenuated from internal membranes. In promitotic GIV-FA cells, a disabled switch virtually abolished EGFR association with the PM-actin bed, but delayed receptor degradation upon internalization with resultant retention of receptor within juxtanuclear endosomes. In these cells, receptor signaling from the PM is abbreviated and signaling from internal membranes is prolonged. That motogenic signals are enhanced only in the presence of an intact Gαi-GIV switch is likely due to enrichment of PI4,5P2 restrictively at the PM (11), a key substrate for two EGFR-recruited motogenic enzymes, PI3K and PLCγ1. That the activity of the mitogenic c-Src-Y845-STAT5b pathway is sustained in the absence of a Gi-GIV switch even at ~30 min after ligand-stimulated receptor internalization (Fig. 2-1E, 2-2B, C) is likely due to persistence of activated EGFR on internal membranes and delayed receptor degradation. Concomitant with the enhanced c-Src activity, hyperactivation of ERK1/2 was also observed in these cells and is in keeping with the role of c-Src in activating ERK (48). The accelerated rates of receptor degradation in GIV-wt cells and delayed rates in GIV-FA cells could either be a consequence of differential recruitment of c-Cbl (Fig. 2-2E, G), the ubiquitin ligase that promotes endolysosomal degradation of EGFR (49), or reflect a function of GIV in endosomal trafficking of EGFR (50). Our findings are in keeping with prior observations that receptors at the PM initiate motogenic and mitogenic signals and couple them to actin remodeling to preferentially trigger motility, and internalized receptors propagate mitogenic signals instead (11,36). Based on above, we conclude that the Gαi-GIV switch triggers motility by enhancing
motogenic signals from the PM and inhibits mitosis by accelerating receptor degradation, thereby restricting mitogenic signals from internalized receptors. A disabled switch inhibits motility by restricting motogenic signals from PM and triggers mitosis by delaying receptor degradation, thereby propagating mitogenic signals from internalized receptors.

**The $G_{\alpha i}$-GIV Switch Imparts Migration-Proliferation Dichotomy during Oncogenesis**

We demonstrate that during cancer progression, a process that heavily relies on growth factor signaling (39), the activity of the $G_{\alpha i}$-GIV switch is altered by dysregulation of GIV expression in heterogeneous subsets of tumor cells (**Fig. 2-7C**). In fast-growing, poorly-invasive cells GIV-fl is downregulated and replaced by a GIVΔCT splice variant, which is incapable of interacting with $G_{\alpha i}$ (**Fig. 2-6A-C**). These cells behave like the “switch-disabled” GIV-FA cells in that they selectively propagate mitogenic signals in response to EGF and proliferate rapidly despite nutrient deprivation (**Fig. 2-1, 2-6E-I**), features which enable early tumor growth (38). In slow growing, highly invasive cells GIV-fl expression is upregulated, either by mRNA stabilization or transcriptional induction (**Fig. 2-5**). These cells behave like GIV-wt cells where increased levels of GIV’s C-terminal “non-receptor GEF domain”, results in hyperactivation of the $G_{\alpha i}$-GIV switch (13). A hyperactivated switch selectively enhances motogenic signals, triggers motility and inhibits proliferation (**Fig. 2-1**), a set of features that hinder early tumor growth (38), but implicate GIV in metastatic invasion (17). Consistent with the phenotype we observed in GIV-wt and GIVΔCT HeLa cells, others (36)
reported that in response to EGF, MCF7 cells (that express GIVΔCT) grow faster and migrate slower compared to MDA-MB231 cells (that overexpress GIV-fl).

We also demonstrate that fast-growing cells featuring GIVΔCT, which do not assemble a Gαi-GIV switch, predominate early during oncogenesis, whereas highly invasive cells that overexpress GIV-fl and display a hyperactivated Gαi-GIV switch predominate later during metastatic progression. To gain insight into the factors predisposing GIV to alternative splicing, we analyzed the splice site and found it to be ‘weak’ based on the lack of homology to consensus mammalian splice signals (Fig. 2-S8D). However, this suboptimal splice site alone is not enough to explain the missplicing event observed in poorly invasive breast and colonic cancer cells, because the sequence is recognized and this intron is efficiently excised in corresponding healthy cells or their highly invasive variants (Fig. 2-6A). Additionally, no mutations were found within the 19th intron or the flanking exons that could affect pre-mRNA splicing, suggesting that other mechanisms may restrict GIVΔCT expression to poorly invasive cells, e.g., differential expression of splicing factors during cancer progression (51). Regardless of the mechanism, splicing-mediated exclusion of the C-terminus and downregulation of GIV-fl was a strikingly conserved theme in cells populating the early tumor. To the best of our knowledge, this is the first example of a protein that undergoes bipartite dysregulation during oncogenesis, i.e., induced or suppressed by splicing in two subsets of cancer cells imparting promigratory or proproliferative advantages, respectively (Fig. 2-7C). This is also in keeping with
studies demonstrating that phenotypic heterogeneity exists among cells within the same tumor (1,2).

We conclude that the presence or absence of a G\(_\alpha\text{i}-\text{GIV}\) switch, which activates G\(_\alpha\text{i}\), dictates how growth factor receptor-signaling is reprogrammed and whether cells migrate or proliferate. Mechanistic insights gained, sheds light on the enigmatic origin of the migration-proliferation dichotomy during oncogenesis.
Figure 2-1. A G\(\alpha_{i}\)-GIV switch imparts migration-proliferation dichotomy by modulating motogenic and mitogenic signals initiated by EGFR. (A) GIV-wt cells preferentially migrate while GIV-FA cells preferentially proliferate. Confluent monolayers of HeLa cells stably expressing either wild-type GIV (GIV-wt) or the GEF-deficient GIV (GIV-FA) were starved overnight, scratched, treated with 0.1 nM EGF, and the edge of the wound was monitored by live-cell imaging up to 16 hr. Total number of cells along the edge of the wound that either migrated into the wound or underwent mitosis in response to EGF were quantified and expressed as a % of total cells analyzed. Results are shown as mean +/- S.E.M (n=3). (B, C) Mitotic index is low in GIV-wt whereas high in GIV-FA cells. (B). Lysates of GIV-wt and GIV-FA cells before and after scratch-wounding were analyzed for phosphohistone-H3 (pH3, Ser 28) and total H3 by immunoblotting (IB) and compared to controls. At 2 h after scratch-wounding, pH3:H3 ratio is reduced by 80% in GIV-wt cells and 50% in controls, but remains virtually unchanged in GIV-FA cells. (C) Bar graphs (left panel) comparing the mitotic index 8 h after scratch-wounding, as determined by the number of cells at the edge of the wound (demarcated by a white line) displaying intense pH3 (red) signals associated with condensed chromatin by immunofluorescence (right panel, a-c), and expressed as the percent of total cells counted (DAPI-positive, blue). Results are shown as mean +/- S.E.M (n=3). (D) Active G\(\alpha_{i3}\) (Q204L) promotes migration while inactive G\(\alpha_{i3}\) (G203A) promotes mitosis. Bar graphs comparing the proportion of HeLa cells expressing G\(\alpha_{i3}\)wt-YFP, G\(\alpha_{i3}\)(QL)-YFP or G\(\alpha_{i3}\)(GA)-YFP at the edge of a freshly made wound that display polarized migration or mitosis, visualized by live cell imaging.
Figure 2-1 (continued). (E, F) GIV-wt cells enhanced motogenic and suppressed mitogenic signals in response to EGF whereas GIV-FA cells enhanced mitogenic and suppressed motogenic signals. (E) Starved GIV-wt and GIV-FA HeLa cells were stimulated with 50 nM EGF and whole cell lysates were analyzed for total (t) and phospho (p): PLCγ1, c-Src, Akt, ERK 1/2, pSTAT5 and actin by immunoblotting (IB). (F) Bar graphs indicating phospho-protein:actin ratios at the selected time points after EGF stimulation when GIV-wt and GIV-FA cells displayed maximum deviation in signaling profiles compared to each other and untransfected controls. Results are shown as mean +/- S.E.M (n=4); p<0.001 for all comparisons between GIV-wt and GIV-FA. (G) Summary of the effects of an intact vs. disabled Gαi-GIV switch on EGF-intiated signaling pathways and the phenotypic outcome. An intact Gαi-GIV switch preferentially enhances motogenic but attenuates mitogenic cascades and consequently favors motility over mitosis. Disassembling the Gαi-GIV switch preferentially propagates mitogenic but attenuates motogenic cascades and consequently favors mitosis over motility.
Figure 2-2. The Gαi-GIV switch is required for efficient ligand-induced EGFR autophosphorylation and SH2-adaptor recruitment. (A) Schematic representation of EGFR displaying Tyr phosphorylation sites on the cytoplasmic tail and the corresponding SH2-adaptors (PLCγ1, c-Cbl, Grb2) that are specifically recruited to these sites. (B, C). GIV-wt and GIV-FA cells display distinct profiles of EGFR phosphorylation. (B) Untransfected, GIV-wt and GIV-FA HeLa cells were stimulated with EGF as in Fig. 2-1E and analyzed for phospho-EGFR using phospho-Tyr-site specific antibodies to Y845, Y992, Y1045, and Y1068 by immunoblotting (IB). (C) Receptor activation was quantified as in Fig. 2-1F and expressed as fold increase in activation normalized to t=0 min. Results are shown as mean +/- S.E.M (n=3).
Figure 2-2 (continued). (D-H) SH2 adaptors are differentially recruited to EGFR in GIV-wt vs. GIV-FA cells. Lysates prepared from EGF-stimulated GIV-wt and GIV-FA cells (as in Fig. 2-1E) were incubated with anti-EGFR antibodies and immunoprecipitated complexes were analyzed for pPLC_1 (D), c-Cbl (E), Grb2 (F), and tEGFR and pY845 EGFR (G) by immunoblotting (IB). (H) The ratio of SH2 adaptor:tEGFR (y axis) is plotted over time (x axis). Results are representative of 3 separate experiments.
Figure 2-3. The G\textsubscript{q}\textsubscript{i}-GIV switch regulates EGFR localization and rates of receptor degradation. **(A)** In GIV-wt cells ligand-bound EGFR is retained on PM, whereas in GIV-FA cells the receptor maximally colocalizes with EEA1-positive compartments at 15 min. GIV-wt and GIV-FA cells were stimulated with 50 nM HRP-conjugated EGF for 15 min and co-stained for HRP (red), EEA1 (green) and nucleus/DAPI (blue). While permeabilization allows visualization of total receptor, staining without permeabilization selectively detects receptor on the PM. Bar = 10 \textmu M. **(B)** Ligand-stimulated EGFR transiently colocalizes with the PM-associated actin bed in controls (a-c) and GIV-wt (d-f), but not in GIV-FA cells (g-i). GIV-wt and GIV-FA cells were stimulated with 50 nM EGF for 5 min, fixed, co-stained for actin/phalloidin (red), tEGFR (green) and nucleus/DAPI (blue). Bar = 10 \textmu M.
Figure 2-3 (continued). (C) EGFR is virtually undetectable in GIV-wt cells (e, h) but significantly retained in GIV-FA cells (f, i) compared to controls (d, g), 60 min after ligand stimulation, indicating differential rates of receptor degradation within endolysosomal compartments. GIV-wt and GIV-FA cells were stimulated with 50 nM EGF for 60 min and co-stained for EEA1 (green), tEGFR (red) and the nucleus/DAPI (blue). Bar = 10 μM. (D) The rate of EGFR degradation is accelerated in GIV-wt but delayed in GIV-FA cells. Untransfected, GIV-wt and GIV-FA HeLa cells were stimulated with 50 nM EGF as in Fig. 2-1E and analyzed for total EGFR (tEGFR) and actin by immunoblotting (IB, top panel). Band-shifts and doublets were consistently detected at various time points which correlate with phosphorylation of EGFR. The amount of receptor (180 kD, full length) at 30 min was quantified using Odyssey, normalized to actin and expressed as % decrease compared to time 0 min (bottom panel). Results are shown as mean +/- S.E.M.
Figure 2-4. The Gαi-GIV switch interacts with EGFR via GIV. (A) GIV coimmunoprecipitates with FLAG-tagged EGFR. Starved Cos7 cells transfected with FLAG-EGFR or vector alone were stimulated with 100 nM EGF for 5 min prior to lysis. Equal aliquots of lysates (i.e., INPUTS; left panel) were subjected to immunoprecipitation using anti-FLAG antibody and the bound immune complexes were analyzed for EGFR and GIV by immunoblotting (IB, right panel). (B) Gαi3 coimmunoprecipitates with EGFR and GIV. Cos7 cells were transiently transfected with EGFR, FLAG- Gαi3 or vector alone, lysed, and equal aliquots of lysates (i.e., INPUTS; left panel) were subjected to immunoprecipitation with anti-FLAG IgG as in Fig. 2-4A. Gαi3-bound immune complexes were analyzed for EGFR and GIV by immunoblotting (IB, right panel). (C) Endogenous GIV and EGFR partially colocalize at the cell periphery upon ligand stimulation. Starved HeLa cells were stimulated with 50 nM EGF for 5 min, fixed, stained for tEGFR (a, green), GIV (b, red) and the nucleus/DAPI (blue) and analyzed by confocal microscopy. Merged image (c) shows patches at the PM where GIV and EGFR co-localize (arrowheads, yellow). Bar = 10 μM.
Figure 2-4 (continued). (D) Gαi3 and actin are simultaneously recruited to EGFR in GIV-wt, but not in GIV-FA cells upon ligand stimulation. Lysates prepared from EGF-stimulated GIV-wt and GIV-FA cells were incubated with anti-EGFR antibodies (as in Fig. 2-2D-G) and immunoprecipitated complexes were analyzed for tEGFR, pY845 EGFR, actin, GIV and Gαi3 by immunoblotting (IB). (E) Schematic Illustration of Working Model. Enhancement of Gi activity via the Gi-GIV switch is a key determinant of actin remodeling at the PM and PM-based EGFR-signaling. An intact switch (GIV-wt) increases receptor association with the PM-actin bed, prolongs receptor lifespan on the PM and delays internalization, but later shortens the time spent in endosomes by accelerating receptor degradation. A disabled switch (GIV-FA) decreases receptor-actin associations and accelerates internalization, thereby shortens receptor lifespan at the PM, but later prolongs time spent in endosomes due to delayed receptor degradation. As a direct consequence of differential receptor association with the PM-actin bed, GIV-wt, but not GIV-FA cells enhance motogenic (PI3K, PLCγ1) signals from the PM to trigger migration, whereas GIV-FA cells propagate mitogenic (c-Src-STAT5b/ERK1/2,) signals from intracellular membranes to trigger proliferation.
Figure 2-5. Cancer cells induce or suppress the Gαi-GIV switch by upregulating or downregulating GIV expression, respectively. (A, B). In breast and colon carcinoma cells, GIV is detectable only in highly invasive variants. Lysates of colon (A) and breast (B) cancer cell lines with low (L) or high (H) invasiveness were analyzed for expression of GIV (by GIV-CTAb), Gαi3 and actin by immunoblotting (IB) and GIV and GAPDH mRNA by RT-PCR. The domain organization of GIV is illustrated in Fig. 2-S7. CC, coiled-coil; CT, C-terminus; GBD, Gα-binding domain; Fw, forward; Rev, reverse. (C) GIV transcript is upregulated in highly invasive and downregulated in poorly invasive cancer cells. The relative abundance of amplified cDNA products spanning the GBD through the C-terminus in RT-PCR assays (35 cycles) on several cancer cells (colon, breast and skin) is expressed as fold change compared to their respective normal controls. Upregulation (Green bars, highly invasive) = +Y axis; Downregulation (Red bars, poorly invasive) = -Y axis.
Figure 2-6. Poorly invasive cancer cells express an alternatively spliced C-terminal truncated GIV protein, GIVΔCT which fails to assemble a Goi-GIV switch and enhances mitogenic response to EGF. (A) Retention of intron 19 in GIV mRNA occurs exclusively in poorly invasive cancer cells. mRNA isolated from normal (N) and cancer cells with variable invasiveness (H= high; L=low) were subjected to RT-PCR using Exon 19 forward and Exon 20 reverse primers (primer sequences illustrated in Fig. S10). While normal and highly metastatic cells yielded the expected ~250bp PCR product, in poorly invasive cells a larger ~1250bp long product was amplified. (B) Percent intron retention varies among poorly invasive cancer cells. The percent intron retention (IR) (calculated as mRNA retaining intron 19 / mRNA retaining intron 19 + mRNA with intron 19 processed x 100) was variable across cell lines: ~95%, ~85% and ~50-60% in Ls-174T, HT29 and MCF7 cells, respectively.
Figure 2-6 (continued). (C) Alternative splicing of GIV pre-mRNA generates GIV-IR19 isoform with an in-frame stop codon. Schematic illustration of the constitutive and alternative pre-mRNA splicing events, the corresponding GIV mRNA isoforms (GIV and GIV-IR19), the translated aa sequences, and the corresponding predicted protein products (GIV and GIVΔCT). PTC = Premature Stop Codon. (D) A C-terminal truncated protein (GIVΔCT) is expressed exclusively in poorly invasive cells. Lysates of normal (N) or cancer cells with variable metastatic potentials (H= high; L=low) were analyzed for expression of GIV using GIV-CTAb (against C-term 18 aa of GIV) and GIV-ccAb (against the coiled-coil domain of GIV) and actin by immunoblotting (IB). (E) GIVΔCT fails to interact with Gαi3. In-vitro translated (TnT) [35S]Met-labeled full length GIV (aa1-1870, top panel) and GIVΔCT (aa1-1354, bottom panel) constructs were incubated with ~15 µg GST-Gαi3 or GST immobilized on Glutathione beads in the presence of GDP or GDP•AlF₄⁻. Bound GIV was quantified by autoradiography. (F) GIVΔCT cells proliferate but do not migrate. Confluent HeLa cell monolayers expressing GIVΔCT or vector alone, were induced to migrate by scratch-wounding (15), and the area of wound covered at 16 h was quantified as described in Experimental Procedures. Results are expressed as mean +/- SEM. n=3. (G) Lysates prepared from cells in ‘F’ were analyzed for GIV, pAkt, tAkt, Gαi3 and actin by immunoblotting (IB).
Figure 2-6 (continued). (H, I) Growth curves were analyzed for HeLa cells lines stably expressing GIV-wt, GIVΔCT, empty vector, or untransfected control as indicated in media supplemented with either 10% (H) or 1% (I) serum. (J) GIVΔCT selectively enhances mitogenic but suppresses motogenic signals in response to EGF. GIVΔCT, GIV-wt and untransfected HeLa cells were stimulated with 50 nM EGF for the indicated durations, and analyzed as in Fig. 2-1E, F. EGFR-initiated enzyme/kinase activities were quantified across various time points by Odyssey Infrared Imager, normalized to actin and expressed as fold change compared to controls. Results are shown as mean +/- S.E.M (n=3). (K, L) GIVΔCT suppresses EGFR autophosphorylation and delays receptor downregulation in response to EGF. (K) GIVΔCT, GIV-wt and untransfected HeLa cells were stimulated with 50 nM EGF as above, and analyzed as Fig. 2-2B, 2-3D. (L) Extent of receptor tyrosine phosphorylation at specific sites was measured as in Fig. 2-2C and expressed as fold increase in activation normalized to time 0 min. Results are shown as mean +/- S.E.M (n=4).
Figure 2-7. Activity of the Gαi-GIV switch is altered during oncogenic progression. (A) Cells deficient in GIV dominate the tumor early and those that induce GIV appear later during metastatic progression of colorectal adenocarcinomas. Paraffin embedded human colon samples (normal, adenoma or colon cancers) were analyzed for GIV by immunohistochemistry using GIV-CTAb. Stromal tissue (S) consistently stained strongly positive whereas tumor tissue (T) showed variable staining. Representative field from normal colon crypts (a, b), adenomas (c, d), tumors negative for GIV (e, f), positively stained infiltrating tumor pockets (g, h) and homogeneously positively stained tumors (i, j). Panels on right are a higher magnification of the boxed regions in the images on the left. C = crypt; CB = Crypt base. (B) Abundance of GIV-positive cells within a tumor increases with increasing clinical stage of colon carcinoma. GIV expression was analyzed in tumors (as in Fig 2-7A) of variable clinical stages by three independent observers blinded to the disease stage with >95% congruency. Percent of tumors that were scored as positive for full length GIV expression within each clinical stage is displayed as bar graphs, and the total number of tumors examined within each clinical stage is indicated in parenthesis.
Figure 2-7 (continued). (C) Working Model. Early during oncogenesis, normal epithelial cells either downregulate GIV or upregulate GIV to generate two heterogeneous subsets of tumor cells. In cells that downregulate, GIV is replaced with GIVΔCT precluding assembly of a functional Gαi-GIV switch. In the absence of a functional Gαi-GIV switch, these cells enhance mitogenic and suppress motogenic signals downstream of EGFR and are rendered pro-proliferative and poorly metastatic. By contrast, in cells that upregulate GIV ~30-50 fold, the C-terminal GEF domain of GIV assembles and activates the Gαi-GIV–switch with enhanced efficiency. Hyperactivation of the Gαi-GIV switch in these cells preferentially amplifies motogenic and suppresses mitogenic signals in response to EGF rendering these highly-invasive and poorly-proliferative. During tumor growth, the subset of highly proliferative cells in which GIV is downregulated and the Gαi-GIV switch is inactivated dominate the tumor first, and highly invasive cells in which GIV is upregulated and the switch is hyperactivated are enriched later during metastatic invasion.
Figure 2-S1. Comparison of steady-state levels of proteins expressed in HeLa epithelial cell lines used in the current work. Equal aliquots of lysates (~40 µg protein) from HeLa epithelial cell lines stably expressing either wild-type GIV (GIV-wt) or a dominant negative, GEF-deficient F1685A mutant of GIV and the parental cells (untransfected HeLa) were analyzed for GIV, total EGFR, G\_i3, actin and total and phosphorylated Akt (tAkt and pAkt) by immunoblotting (IB). Levels of GIV are ~3 fold in GIV-wt and GIV-FA cells compared to untransfected controls. However, levels of EGFR and G\_i3 are equal among the 3 cells lines at steady-state. As demonstrated previously (21), GIV-wt, but not GIV-FA display enhanced Akt activity at steady-state vs. untransfected controls.
Figure 2-S2. Proliferation is enhanced in GIV-FA cells and suppressed in GIV-wt cells when compared to controls at basal, serum-starved state as well as upon stimulation with growth factor. (A, B) Epithelial cells expressing GIV-F1685A grow faster and those expressing GIV-wt grow slower than controls under basal and serum starved conditions. HeLa cells lines stably expressing GIV-F1685A (solid red circles and lines), GIV-wt (blue triangles and lines), empty vector (discontinuous black line) or untransfected control (continuous black line) were analyzed for rates of proliferation in media supplemented with either 10% (A) or 1% (B) serum. Total number of live cells as detected by Trypan blue dye exclusion were counted using a hemocytometer (Y axis) and plotted against the number of days (X axis).
Figure 2-S3. Activated STAT5b transcription factor is rapidly translocated to the nucleus in GIV-FA cells upon stimulation with EGF. (A) Activation of STAT5b is enhanced in GIV-FA cells but not in controls or GIV-wt and this activated pool is found predominantly in crude nuclear fractions. Serum starved control (top panels), GIV-wt (middle panels), or GIV-FA (bottom panels) HeLa cells were stimulated with 50 nM EGF for 10 min. Cells were lysed in homogenization buffer and crude nuclear, membrane (P100), and cytosolic (S100) fractions were prepared as described previously. 10% PNS, 50% P100, and 10% S100 were analyzed by immunoblotting for tSTAT5b, pSTAT5b, and actin. (B, C) GIV-FA cells, but not GIV-wt or controls display transient and intense co-localization of STAT5b with DAPI-stained nuclear compartment in response to EGF. (B) Serum starved control (left panels), GIV-wt (middle panels), and GIV-FA (right panels) were stimulated with 50 nM EGF for indicated times, fixed, stained for pSTAT5b, and analyzed by light microscopy. (C) pSTAT5b (red) colocalizes with the nucleus (DAPI, blue) in GIV-FA cells following 15 min EGF stimulation. Bars = 10 μm.
Figure 2-S4. EGFR transiently associates with PM-actin patches in GIV-wt, but not in GIV-FA cells. (A) At 15 min after ligand stimulation GIV-wt cells retain a larger proportion of the EGFR on the PM (white arrowheads) (e) compared to controls (d) whereas in GIV-FA cells (f) the receptor is largely internalized within EEA-1 positive endosomes (i, yellow indicates colocalization of EGFR and EEA1). GIV-wt and GIV-FA cells were stimulated with 50 nM EGF for 15 min, fixed, and co-stained for EEA1 (green), tEGFR (red) and the nucleus/DAPI (blue). Bar = 10 μM. (B) EGFR colocalizes with actin patches in GIV-wt, but not GIV-FA cells after scratch-wounding. GIV-wt and GIV-FA HeLa cells were grown in the presence of 0.2% FBS on cover slips to confluence followed by scratch-wounding. Cells were fixed at 30 min after wounding and co-stained for EGFR (green), actin/phalloidin (red) and nucleus/DAPI (blue). Prominent colocalization of EGFR with actin patches (arrowheads) was seen in GIV-wt (a-c), but not in GIV-FA cells (d-f), indicating that the Goi-GIV interface regulates EGFR association with PM-actin bed during cell migration. Arrows denote direction of migration. Bar = 10 μM.
Figure 2-S5. GIV interacts with EGFR. (A) Endogenous GIV co-immunoprecipitates with exogenously expressed FLAG-tagged EGFR. Cos7 cells transiently transfected with FLAG-EGFR or vector alone were serum starved overnight in 0.2% FBS followed by stimulation with 100 nM EGF for 5 min prior to lysis in RIPA buffer. These lysates were subsequently incubated with anti-GIV antibodies for 4 hr, immobilized on Protein A agarose beads and the bound immunocomplexes were analyzed for FLAG-EGFR and GIV by immunoblotting (left panel). Equal aliquots of the lysates used as a source of EGFR and/or GIV for the above co-IP assays (i.e., INPUTS; right panel) were analyzed for expression of FLAG-EGFR, GIV and actin by immunoblotting. (B) Endogenous GIV and EGFR interact weakly in serum starved cells and this interaction is stronger upon ligand stimulation. Serum starved A431 cells were stimulated with 100 nM EGF for 5 min, lysed in RIPA buffer, and then GIV was immunoprecipitated as in Fig. 2-4A. Inputs and bound proteins were analyzed for tEGFR and pY845-EGFR and GIV by immunoblotting (IB).
Figure 2-S6. Analysis of GIV expression by RT-PCR. (A) Schematic illustration of the domain organization of GIV and the domain-specific oligonucleotide primer pairs used for detection of GIV. The N-terminal Hook domain interacts with microtubules, coiled-coil domain mediates homodimerization, Gα-binding domain (GBD) interacts with α-subunits of Gαi and Gαs subfamilies and the extreme C-terminus harbors the GEF motif which activates the Gi-GIV-switch and interacts with Akt kinase and actin. The numbers corresponding to the amino acids forming the boundaries of GIV’s various domains are also displayed. The regions within the coding sequence that were used to design various domain-specific oligonucleotide primer pairs are illustrated. As illustrated, the primers span the central and 3’ coding frame covering the C-terminal half of the protein: the coiled coil domain (CC), the Gα-binding domain (GBD) and the extreme C-terminus (CT) of hGIV. The corresponding coding exon numbers are indicated. Fw, forward; Rev, reverse. Table: Expected sizes (bp) of PCR products for a given combination of forward and reverse oligonucleotide primer pair. cc, Coiled-coil; GBD, Gα-binding domain; ct, Carboxyl terminus; Fwd, forward; Rev, reverse. (B, C) A full length GIV transcript is expressed in human breast and colon epithelia. The GIV transcript in normal colon (B) and breast (C) epithelia was analyzed by RT-PCR using primers (illustrated in Fig. 2-S6A) to amplify consecutive, overlapping sequences encompassing the central and 3’ coding region of the GIV gene. Arrowheads denote the products of expected sizes obtained using the various primer combinations tabulated in Fig. 2-S6A. Sequence analysis confirmed that a full length normal transcript (Reference Sequence, NM_018084) is indeed present in normal adult breast and colon epithelia.
Figure 2-S7. Alignment of GIV mRNA (coding Ref Seq) and protein sequence showing the primers used to map the site of alternative splicing. The mRNA and amino acid sequences (Gene Ccdc88A, a.k.a., KIAA1212; Reference Sequence: NM_018084) of GIV at the interface of the coiled-coil and G-binding domains are aligned. Exons 19-25 are boxed and each coding exon has been color coded to indicate the exon-exon borders. **Bold** underlined regions indicate the sequences of the exon-specific primers used in this study. Star (*) indicates a naturally occurring SNP (Single nucleotide polymorphism) and the affected residue is highlighted in red.
Figure 2-S8. Alternative splicing by intron retention effectively downregulates mature GIV transcript in poorly metastatic cancer cells. (A) Validation of detected intron retention using RT-minus (RT-) control. RNA isolated from the indicated cancer cell lines were subjected to RT-plus and RT-minus assays using exon #19 forward and #20 reverse primer pairs (see methods). The absence of an amplified product under RT-minus conditions indicates that the isolated RNA was free of genomic DNA contaminants. The doublets observed in a 2% agarose gel represent a previously identified (Simpson et al., 2005) isoform in all tissues. (B) The abundance of GIV mRNA in poorly metastatic cells is inversely proportional to the % intron retention (Fig. 2-6B). RNA isolated from cancer cells of variable metastatic potential (N, normal; H, high; L, low) was analyzed for abundance of the translated mature full length GIV RNA using different oligonucleotide primer pairs spanning the C-terminal region of the gene that contains G-binding and GEF domains (illustrated in C). In MCF7, HT29p and Ls174T cells, an inverse correlation was seen between the percent intron retention (Fig. 2-6B), and the amount of full length GIV transcript. In HT29p and Ls174T cells, where % intron retention is high, GIV mRNA is virtually undetectable.
Figure 2-S8 (continued). (C) Schematic illustration of the functional domains of GIV and the corresponding coding exons. Each blue box represents coding exons that are numbered 5' to 3'. Exons encoding specific functional domains are indicated using brackets. The GEF motif is located within exon #29. (D) The splice site flanking intron 19 is weak. Yellow numbered boxes represent coding exonic sequences. Intrinsic sequence with highlighted 5' (donor) and 3' splice sites (branch point, red; poly (Y) track, green; and acceptor) and corresponding optimal mammalian splicing site consensus is illustrated. Sequence of the 19th intron was analyzed to determine its strength as a splice site. The donor and acceptor splice site strengths were scored by the Splice Site Prediction program by Neural Networks (SSPNN, http://www.fruitfly.org/seq_tools/splice.html) which takes other known sequences into account. Although the 5' and 3' boundaries of this intron had a 5' donor splice site beginning with GU dinucleotide and a 3' acceptor site ending with an AG dinucleotide (the GU-AG rule: Shapiro and Senapathy, 1987), the sequence analysis of the flanking regions rated this intron as a ‘weak’ site lacking homology to the consensus mammalian splice signals. Optimum mammalian 5' and 3' splice site sequences are listed.
Figure 2-S9. GIV is highly expressed in stromal tissue but only in modest amounts in the normal colonic epithelium. Paraffin embedded normal human colonic tissue was analyzed for full length GIV by IHC using GIV-CTAb. Panel (a, Neg ctrl) confirms specificity of staining where the step of incubation with primary Ab was excluded. (b, c) Stromal tissue (S) stained strongly positive while the epithelial lining of colonic crypts (arrowheads) displayed mild to moderate staining.
REFERENCES


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Chapter 3

Regulation of Epidermal Growth Factor Receptor Degradation by Heterotrimeric $G_{\alpha}$ Protein
SUMMARY

Heterotrimeric G proteins have been implicated in the regulation of membrane trafficking, but the mechanisms involved are not well understood. Here, we report that overexpression of the stimulatory G protein subunit (Gαs) promotes ligand-dependent degradation of epidermal growth factor (EGF) receptors and Texas Red EGF, and knock-down of Gαs expression by RNA interference (RNAi) delays receptor degradation. We also show that Gαs and its GTPase activating protein (GAP), RGS-PX1, interact with hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), a critical component of the endosomal sorting machinery. Gαs coimmunoprecipitates with Hrs and binds Hrs in pull-down assays. By immunofluorescence, exogenously expressed Gαs colocalizes with myc-Hrs and GFP-RGS-PX1 on early endosomes, and expression of either Hrs or RGS-PX1 increases the localization of Gαs on endosomes. Furthermore, knock-down of both Hrs and Gαs by double RNAi causes greater inhibition of EGF receptor degradation than knock-down of either protein alone, suggesting that Gαs and Hrs have cooperative effects on regulating EGF receptor degradation. These observations define a novel regulatory role for Gαs in EGF receptor degradation and provide mechanistic insights into the function of Gαs in endocytic sorting.
INTRODUCTION

Heterotrimeric G proteins serve as important molecular switches that relay extracellular signals from G protein-coupled receptors (GPCRs) on the cell membrane to downstream effectors (1,2). Besides their plasma membrane location, heterotrimeric G proteins also are found on membranes of intracellular compartments along both the endocytic and secretory pathways where indirect evidence suggests they play several roles in membrane trafficking (3-6). One of the prototypical heterotrimeric G proteins, \( G\alpha_s \), the stimulatory subunit of heterotrimeric G proteins, has been suggested to regulate endocytic trafficking. Reagents that activate \( G\alpha_s \), e.g., cholera toxin and a peptide mimicking the interacting region of \( G\alpha_s \) with the \( \beta\gamma \)-adrenergic receptor, block endosome-endosome and phagosome-endosome fusion in vitro (7-9). Cholera toxin and recombinant \( G\alpha_s \) proteins also have been found to promote transcytosis of the polymeric IgA receptor through endosomes in polarized epithelial cells (10).

Although the molecular basis for the function of \( G\alpha_s \) in signal transduction at the plasma membrane has been well characterized, little is known about the mechanisms whereby \( G\alpha_s \) influences endocytic trafficking. Our recent discovery of RGS-PX1 has provided a putative link between \( G\alpha_s \) and endocytic trafficking (11). RGS-PX1, a member of the regulator of G protein signaling (RGS) protein family (12,13), functions as a GTPase activating protein (GAP) for \( G\alpha_s \) through its conserved RGS domain that
interacts specifically with Gαs, but no other Gα protein (11). RGS-PX1 is also known as sorting nexin 13 (SNX13) and serves as an SNX protein, through its phosphatidylinositol-binding phoX (PX) domain. This domain is shared by SNX proteins that are involved in protein sorting in endosomes (14,15). We showed previously that RGS-PX1 is a functional SNX protein that is localized on endosomes and delays epidermal growth factor (EGF) receptor degradation, probably at the steps of endosome sorting and lysosome targeting (11). The fact that RGS-PX1 can bind Gαs and affect EGF receptor trafficking suggested that Gαs also might be involved in regulating of EGF receptor endocytosis and downregulation.

The EGF receptor represents the classical model system to study mechanisms of ligand-induced receptor endocytosis and down-regulation in mammalian cells (16-18). On ligand binding, EGF receptors are rapidly internalized via clathrin-coated pits and delivered to early endosomes where the majority of the receptors are sorted into the luminal vesicles of late endosomes or multivesicular bodies (MVBs) and targeted for degradation in lysosomes (18-22). Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) has been demonstrated to be a central player in this sorting pathway (22-24). Hrs is found in specialized clathrin- coated microdomains of early endosomes that are enriched in mono-ubiquitinated receptors targeted for lysosomal degradation (25,26). Hrs also recruits the endosomal sorting complexes required for transport (ESCRT) complexes to endosomes through its interaction with Tsg101 in ESCRT complex I and regulates the formation of
MVBs (17, 27, 28). In this study, we have investigated the effects of $G_\alpha$s on EGF receptor degradation. Our findings establish a novel role for $G_\alpha$s in regulating endocytic trafficking and sorting.

**MATERIALS AND METHODS**

**Materials**

Mammalian expression vector pcDNA3.1 containing $G_\alpha$s long (L) and short (S) splicing variants were obtained from Guthrie cDNA Resource Center (Sayre, PA). Mammalian expression vector pXER-EGFR encoding the EGF receptor was obtained from Dr. Gordon Gill (University of California, San Diego, CA). $pG_\alpha$s-green fluorescent protein (GFP) construct, expressing a $G_\alpha$s-GFP fusion protein with GFP inserted between the helical and GTPase domains, was obtained from Dr. Mark Rasenick (University of Illinois, Chicago, IL) (29). The pcDNA3-myc-Hrs construct was obtained from Dr. A Beans (University of Texas Medical School, Houston, TX). The GFP-tagged RGS-PX1 construct containing residues 257–957 of human RGS-PX1 was described previously (11). The FLAGtagged RGS-PX1 construct was prepared by inserting the cDNA encoding human RGS-PX1 (residues 51–957) into p3XFLAG-CMV-10 (Sigma-Aldrich, St. Louis, MO).

**Antibodies**

Affinity-purified rabbit IgG against $G_\alpha$s used for immunoblotting was obtained from Calbiochem (San Diego, CA). Rabbit antibodies against Rab5 were provided by Dr. Angela Wandinger-Ness (University of New Mexico,
Albuquerque, NM). Other antibodies were obtained from the following sources: monoclonal antibodies (mAbs) against actin and FLAG (M2) (Sigma- Aldrich), myc (Cell Signaling Technology, Beverly, MA), and GFP (BD Biosciences Clontech, Palo Alto, CA), and polyclonal antibodies against EGF receptor (Santa Cruz Biotechnology, Santa Cruz, CA) and GFP and Hrs (Alexis Biochemicals, San Diego, CA).

Cell Culture and Transfection

Human embryonic kidney (HEK)293T cells (obtained from Dr. Alexandra Newton, University of California, San Diego, CA), and Cos7 cells were maintained in Dulbecco’s modified Eagle’s high glucose medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT), penicillin, and streptomycin. HEK293 cells were transfected using calcium phosphate as described previously (11). Cos7 cells were transfected using FuGENE 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s instructions.

Epidermal Growth Factor Receptor (EGFR) Degradation Assays

HEK293 cells in six-well plates were transfected with pXER-EGFR together with pcDNA3.1- Gαs-L and pcDNA3.1-Gαs-S (1:1), or pcDNA3.1 empty vector. Twenty-four hours after transfection, cells were serum starved overnight in DMEM with 0.5% fetal bovine serum (FBS) and then incubated in the presence or absence of 100 nM EGF (Molecular Probes, Eugene, OR) at 37°C. Cells were lysed in Laemmli sample buffer or in 50 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton, and Complete protease inhibitor (Roche Diagnostics)
followed by immunoblotting with antibodies to EGFR, Gαs, and actin. EGF receptor degradation was quantified by densitometry (three independent experiments) by using Quantity One software (Bio-Rad, Hercules, CA).

**Immunofluorescence**

Cos7 cells were fixed in 3% paraformaldehyde (PFA) in 100 mM phosphate buffer, pH 7.4, for 30 min, permeabilized with 0.1% Triton X-100 for 10 min, blocked with 10% fetal calf serum for 30 min, and incubated with primary antibodies for 1 h at 25°C, followed by Alexa Fluor 594-conjugated goat anti-mouse F(ab’)2 and/or Alexa 488 goat anti-rabbit F(ab’)2 (Molecular Probes) for 1 h. Some specimens were permeabilized with 0.05% saponin for 1 min at 4°C before fixation. Specimens were analyzed using a Zeiss Axiophot equipped with a Hamamatsu Orca ER charge-coupled device (CCD) or by deconvolution microscopy by using an Applied Precision (Issaquah, WA) Delta Vision imaging system coupled to an S100 fluorescence microscope (Carl Zeiss, Thornwood, NY). For cross-sectional images of cells, stacks were obtained with 200-nm step width. Deconvolution was done on an SGI workstation (Mountain View, CA) by using Delta Vision reconstitution software, and images were processed as Tiff files by using Photoshop 7.0 (Adobe Systems, San Jose, CA).

**Uptake of Texas Red EGF**

Cos7 cells were transfected with pCDNA3, Gαs-GFP, or myc-Hrs for 12 h. After serum starvation for 3 h, cells were incubated in DMEM containing 0.4 µg/ml Texas Red EGF (Molecular Probes) in 0.5% FBS for 10 min at 37°C and
washed and incubated in DMEM containing 0.5% FBS for up to 1 h at 37°C. For semiquantitative analysis of bound and internalized Texas Red EGF, all mages were captured with the exact same settings. Control cells and cells expressing the GFP constructs were traced using Photoshop. For each cell, the number and intensity of positive pixels (pixels with grayscale values between 75 and 255) was determined using Image J software (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij). Results were calculated as the total number of positive pixels per condition multiplied by the cumulative pixel intensity divided by the number of cells. Ten to 30 cell profiles were measured for each condition, and the results are displayed as the mean of three separate experiments.

**Immunogold Labeling**

HEK293 cells were fixed in 4% PFA alone or 4% PFA containing 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, overnight, pelleted in 10% gelatin in phosphate buffer, cryoprotected, and snap frozen in liquid nitrogen. Ultrathin cryosections (70–90 nm) were cut at −100°C on a Leica Ultracut UCT with an EM FCS cryoattachment (Leica, Bannockburn, IL) by using a Diatome diamond knife (Diatome US, Fort Washington, PA), picked up with a 1:1 mixture of 2.3 M sucrose and 2% methyl cellulose (15 cp), and transferred onto Formvar- and carbon-coated copper grids. Sections were blocked and incubated with primary antibodies for 2 h at room temperature, followed by gold conjugated goat anti-rabbit IgG and gold conjugated goat anti-mouse IgG (Amersham Biosciences, Piscataway, NJ) for 1 h. Sections were contrasted
for 10 min with 2% neutral uranyl acetate and stained for 10 min with 0.2% uranyl acetate in 1.8% methyl cellulose on ice. Grids were viewed and photographed using a Philips CM-10 transmission electron microscope (FEI, Hilsboro, OR) equipped with a 794 Multiscan CCD camera (Gatan, Pleasanton, CA).

**Coimmunoprecipitation**

HEK293 cells were plated in 60-mm plates and transfected with various constructs. After 48 h, cells were lysed in 1% Triton X-100 in phosphate-buffered saline (PBS) buffer containing protease inhibitors (0.12 mg/ml phenylmethylsulfonyl fluoride, 2 mg/ml leupeptin, and 1 mg/ml aprotinin) at 4°C for 30 min and centrifuged at 15,000 X g for 10 min. Cell lysates were incubated with primary antibodies overnight at 4°C, followed by incubation with protein A- or G-Sepharose (Oncogene, San Diego, CA) for an additional 1 h at 4°C. Beads were washed (three times) with lysis buffer and boiled in Laemmli sample buffer, and bound immune complexes were analyzed by SDS-PAGE and immunoblotting.

For coimmunoprecipitation of Hrs and Gαs from cytosolic and membrane fractions, HEK293 cells were scraped into cold PBS containing protease inhibitors and homogenized by 10 passages through a 28 1/2-gauge needle. Nuclei and unbroken cells were removed by centrifugation, and postnuclear supernatants were centrifuged at 100,000 X g for 1 h at 4°C to prepare cytosolic (supernatant, S100) and membrane (pellet, P100) fractions (11). Membrane pellets were lysed in 1% Triton X-100 in PBS containing...
protease inhibitors for 1 h, centrifuged (15,000 X g for 10 min), and the membrane lysates and cytosolic fractions were used for immunoprecipitation.

**Immunoblotting**

Protein samples were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked in Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 0.1% Tween 20 and 5% nonfat milk and incubated with primary antibodies for 2 h at room temperature or overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit or antimouse IgG (Bio-Rad) and enhanced chemiluminescence detection (Pierce Chemical, Rockford, IL).

**In Vitro Glutathione S-Transferase (GST) Pull-Down Assays**

Full-length rat Hrs cDNA and a human RGS-PX1 fragment (PXC) containing the PX domain and the C-terminus (residues 526–957) were amplified by polymerase chain reaction (PCR) and subcloned into pGEX-KG (Amersham Biosciences). GST fusion proteins were expressed in *Escherichia coli* BL21 and purified on glutathione-Sepharose 4B (Pharmacia, Piscataway, NJ) beads according to the manufacturer’s instructions. 35S-labeled, in vitro translation products of Gαs or Hrs were prepared by using the TNT T7 rabbit reticulocyte Quick Coupled Transcription/Translation system (Promega, San Luis Obispo, CA) in the presence of [35S]methionine (1000 Ci/mmol, in vivo cell labeling grade; Amersham Biosciences), pcDNA3.1-Gαs-L and pcDNA3.1-Gαs-S (1:1 ratio) or pCDNA3-myc-Hrs. For pull-down assays, GST fusion
proteins (~75 μg) immobilized on beads were incubated with in vitro-translated products in 20 mM Tris-HCl, pH 8.0, 2 mM MgSO₄, 6 mM β-mercaptoethanol, 5% glycerol, and 0.01% C12E10, in the presence of protease inhibitors for 2 h at 4°C, and washed four times with the same buffer. GST pull-down assays on brain lysates (5 mg) were performed using a lysis buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 300 mM sucrose, 1% Triton X-100, and 0.01% C12E10 as described previously (11). Bound proteins were eluted with Laemmli sample buffer, resolved by SDS-PAGE, and visualized by autoradiography.

RNA Interference

The following small-interfering RNA (siRNA) oligos synthesized by Dharmacon Research (Lafayette, CO) were used for RNAi knock-down of Gαs and Hrs (30): Gαs-sense, 5’-GGC GCA GCG UGA GGC CAA CdTdT; Gαs-antisense, 5-GUU GGC CUC ACG CUG CGC CdTdT; and Hrs-sense, 5’ CGA CAA GAA CCC ACA CGU CdTdT; Hrs-antisense, 5’ GAC GUG UGG GUU CUU GUC GdTdT. All oligos were designed based on human sequences. Scrambled RNA oligos (scramble II duplex; Dharmacon Research) were used as controls. Cos7 cells in six-well plates (30% confluent; 1.5 ml of normal culture medium without antibiotics per well) were transfected with 1 μl of 75 μM siRNA duplex and 8 μl of Oligofectamine reagent (Invitrogen) according to the manufacturer’s instructions. The cells were nalyzed 72 h after transfection. For double RNAi experiments, the total RNAi oligos were kept the same among different wells by addition of scrambled RNAi oligos.
RESULTS

Gαs Overexpression Promotes Degradation of EGF Receptors and Texas Red EGF

RGS-PX1 acts both as a GAP that regulates the activity of Gαs and as a SNX involved in the down-regulation of the EGF receptor (11). These dual activities suggested that RGS-PX1 could link Gαs to EGF receptor sorting at endosomes and that Gαs also might be involved in EGF receptor down-regulation. To find out whether this is the case, we transiently transfected HEK293 cells with EGF receptor and either Gαs or control vector and determined the kinetics of EGF receptor degradation. As shown in Fig. 3-1A, B, cells transfected with Gαs contained less EGF receptors (~20%) at steady state than control cells, suggesting Gαs expression enhances basal turnover of EGFR. Similarly, ligand-induced degradation of the receptor was enhanced in cells transfected with Gαs, because 80% of the receptors were degraded by 60 min after adding EGF (Fig. 3-1A, B), whereas in cells transfected with control vector, only 50% of the receptors had been degraded.

Next, we used immunofluorescence to evaluate the effects of overexpressing Gαs on the uptake and degradation of Texas Red EGF. Cos7 cells transfected with Gαs-GFP (29) or empty vector were incubated with Texas Red EGF for 10 min followed by incubation in the absence of ligand for 30 or 60 min. As shown in Fig. 3-2A, the levels of Texas Red EGF were similar in cells transfected with Gαs-GFP and control vector after 10-min
incubation with Texas Red EGF, suggesting that G\(\alpha\)s overexpression does not impair internalization of EGF. However, at 30 and 60 min “chase,” cells expressing G\(\alpha\)s-GFP contained significantly less Texas Red EGF than those transfected with empty vector (Fig. 3-2A) or GFP alone (our unpublished data). Semiquantitative analysis of the amount of Texas Red EGF remaining (Fig. 3-2B) revealed that in cells transfected with control vector, 66% remained at 30 min and 20% at 60 min, whereas only 30 and 7% remained at the same times in cell expressing G\(\alpha\)s-GFP. As a control, we also transfected Hrs into Cos7 cells and found, consistent with previous reports (25,31,32), that overexpression of Hrs strongly inhibited Texas Red EGF degradation (our unpublished data). We also examined the effect of G\(\alpha\)s overexpression on the uptake of transferrin-Alexa594 in Cos7 cells and found no difference in transferrin uptake between cells transfected with G\(\alpha\)s-GFP and GFP alone (our unpublished data). Together, these results indicate that overexpression of G\(\alpha\)s promotes specific degradation of EGF receptors and their ligands.

**Depletion of G\(\alpha\)s Delays Degradation of EGF Receptors**

We further evaluated the effects of knocking down endogenous G\(\alpha\)s protein levels in Cos7 cells on ligand-induced degradation of EGF receptors. We found that siRNA oligos designed specifically for G\(\alpha\)s blocked EGF-dependent receptor degradation (Fig. 3-3A, B). In cells transfected with scrambled siRNA, 70% of the receptors were degraded after 60-min stimulation with EGF, whereas in cells transfected with G\(\alpha\)s siRNA
degradation was delayed as only 34% of the EGFR had been degraded (Fig. 3-3A, B). In addition, cells transfected with Gαs siRNA had higher levels (140%) of EGF receptor at steady state than cells transfected with scrambled siRNA (Fig. 3-3A, B), suggesting the basal level of EGF receptor turnover is delayed by knockdown of Gαs. Thus, we have shown by three different approaches that Gαs regulates EGF receptor degradation.

RGS-PX1 Interacts with Hrs In Vivo and In Vitro

Next, we investigated whether RGS-PX1 or Gαs delays EGF receptor degradation by interacting with components of the endosomal sorting machinery. We reasoned that RGS-PX1 might bind Hrs, an endosomal protein required for efficient degradation of EGF receptors, because Hrs has been shown to interact with SNX1 (33), the founding member of the SNX protein family that shares strong sequence homology with the C-terminal PX domain and coiled-coil region of RGS-PX1 (11,34).

When we incubated 35S-labeled, in vitro-translated Hrs with GST-RGS-PX1(PXC), a GST fusion protein containing the PX domain and C-terminal coiled-coil region of RGS-PX1 that is homologous to SNX1, Hrs bound to GST-RGS-PX1(PXC), but not to GST alone (Fig. 3-4A). We further tested whether RGS-PX1 coimmunoprecipitates with Hrs in HEK293 cells transfected with myc-tagged Hrs and FLAG-tagged RGS-PX1. We found that when immunoprecipitation was carried out with anti-FLAG IgG, myc-Hrs coprecipitated with FLAG-RGS-PX1 (Fig. 3-4B). Similarly, when anti-myc IgG was used, myc-Hrs coprecipitated with FLAG-RGS-PX1 in cells cotransfected
with both proteins (Fig. 3-4C). These findings support the conclusion that RGS-PX1 interacts with Hrs both in vitro and in vivo.

**Gαs Interacts with Hrs In Vivo and In Vitro**

Given that we have previously shown that RGS-PX1 binds to and serves as a GAP for Gαs (11), we next asked whether Gαs also interacts with Hrs in pull-down and immunoprecipitation assays. We found that 35S-labeled, in vitro-translated Gαs bound to GST-Hrs, but not to GST alone (Figure 5A) and that GST-Hrs, but not GST alone, was able to pull-down endogenous Gαs from brain lysates (Fig. 3-5B). Similarly, when we transfected myc-tagged Hrs together with Gαs-GFP into HEK293 cells and carried out immunoprecipitation with anti-GFP IgG, myc-Hrs coprecipitated with Gαs-GFP (Fig. 3-5C). Because Hrs and Gαs have been found in both membrane and cytosolic fractions, we investigated where they interact. We found that Gαs and Hrs were equally distributed between membrane (P100) and cytosolic fractions (S100) in HEK293 cells expressing myctagged Hrs together with pcDNA3.1-Gαs (Figure 5D, lanes 1 and 2). However, the majority of the Gαs (~95%) coimmunoprecipitated with myc-Hrs from membrane fractions (Fig. 3-5D, lane 4). These results indicate that Gαs interacts with Hrs and that the interaction takes place largely on membranes, presumably on endosomal membranes as both Hrs (35,36) and RGS-PX1 (11) are localized on early endosomes.

**Gαs, RGS-PX1, and Hrs Colocalize on Early Endosomes**

To determine the localization of Gαs and whether it colocalizes with Hrs
and RGS-PX1 on endosomes, we carried out indirect immunofluorescence and deconvolution analysis on Cos7 cells expressing Gαs-GFP alone or Gαs together with RGS-PX1 and Hrs. Because roughly 50% of both Gαs and Hrs are found in cytosolic fractions (Fig. 3-5D), we permeabilized the cells before fixation to release cytosolic proteins and facilitate the detection of membrane-associated pools of Gαs and Hrs. In cells transfected with Gαs-GFP alone, Gαs-GFP showed fine, punctate staining throughout the cytoplasm that partially overlapped with the early endosome markers Hrs (Fig. 3-6-AC) and Rab5 (our unpublished data).

In cells expressing both Gαs-GFP and myc-Hrs, these two proteins strongly colocalized in endosomes (Fig. 3-6D–F). In agreement with previous reports (25,31,32), overexpression of Hrs resulted in enlarged, clustered endosomes. Furthermore, Gαs-GFP and Rab5 colocalized on these enlarged endosomes (Fig. 3-6G–I). More Gαs colocalized with Hrs on these endosomes (Fig. 3-6D–F) compared with cells transfected with Gαs-GFP alone (Fig. 3-6A–C), suggesting expression of Hrs causes more Gαs to translocate to early endosomes. By immunogold labeling, Gαs-GFP and myc-
Hrs colocalized in coated microdomains of these enlarged endosomes (Fig. 3-7). Thus, the immunofluorescence results and the coimmunoprecipitation assays together indicate the Gαs binds Hrs on early endosomes.

In cells cotransfected with untagged Gαs and GFP-RGSPX1, GFP-
RGS-PX1 and Gαs colocalized on endosomes (Fig. 3-8A–C) that also were
labeled with Texas Red EGF after 15 min uptake (**Fig. 3-8D–F**). This is consistent with its early endosome localization reported previously. Again, compared with cells expressing Gαs-GFP alone (**Fig. 3-6A–C**), more Gαs seemed to be localized on early endosomes (**Fig. 3-8A–C**), suggesting expression of RGS-PX1, as well as Hrs, causes more Gαs to translocate to early endosomes.

**Gαs, RGS-PX1, and Hrs Form a Coprecipitatable Complex**

The ability of both RGS-PX1 and Gαs to interact with Hrs and the colocalization of these three proteins on early endosomes suggested that they might be present in the same protein complex. To test this possibility, we performed coimmunoprecipitation experiments by using HEK293 cells transfected with myc-Hrs, FLAG-RGS-PX1, and Gαs. When immunoprecipitation was carried out with an anti-myc IgG, both FLAG-RGS-PX1 and Gαs coprecipitated with myc-Hrs (**Fig. 3-9, lane 3**). Similarly, anti-FLAG IgG was able to bring down both myc-Hrs and Gαs (**Fig. 3-9, lane 2**). These results suggest that Gαs, RGS-PX1, and Hrs form a coprecipitatable protein complex.

**Knockdown of Both Gαs and Hrs Further Delays EGF Receptor Degradation**

The interaction between Gαs and Hrs suggests Gαs may function together with Hrs in the endosomal sorting and down-regulation of the EGF receptor. To test this hypothesis, we performed double RNAi experiment to
knock-down the expression of both Gαs and Hrs (Fig. 3-10A, B). In cells transfected with both Gαs and Hrs RNAi oligos, ~25% of the EGFR was degraded at 30 min after adding EGF, whereas in cells transfected with either Gαs or Hrs RNAi oligos alone, ~50% of the EGF receptors had been degraded at 30 min. These results together with the interaction between Hrs and Gαs strongly suggest that Gαs cooperates with Hrs in regulating EGF receptor degradation.

**DISCUSSION**

Our work presented here demonstrates a regulatory role for the heterotrimeric Gαs protein in EGF receptor trafficking and down-regulation. We find that expression of Gαs accelerates degradation of both EGF receptors and Texas Red EGF, whereas depletion of Gαs by RNAi delays their degradation. We also show that Gαs forms a complex with RGS-PX1 and Hrs that seems to cause more Gαs to translocate to early endosomes. Based on these findings, we propose the following model (Fig. 3-11) for the function of Gαs on endosomes: 1) in the presence of RGS-PX1, Gαs translocates from the plasma membrane or cytoplasm to early endosomes after EGF binding, where it forms a complex with RGS-PX1 and Hrs; and 2) through interaction with Hrs, Gαs regulates endosomal sorting and hence modulates down-regulation of the EGF receptor.

Traditionally, heterotrimeric G proteins have been considered to be largely associated with the cell membrane. Our study indicates early
endosomes represent a novel intracellular location for Gαs. This is in keeping with previous implications that Gαs plays a role in endosomal functions, such as early endosome fusion, phagosome-endosome fusion, and transcytosis of pIgR (7-10). Consistent with our localization data, more recently it was shown that endogenous Gαs also can be found in rat liver endosomes based on cell fractionation and immunofluorescence studies (37).

How translocation of Gαs to endosomes is triggered is still an open question. There are two possible scenarios. First, activation of a GPCR linked to Gαs could stimulate translocation. It has been reported that activation of β-adrenergic receptors or cholera toxin treatment, Gαs dissociates from the cell membrane into the cytoplasm (29,38-41). Alternatively, activation of the EGF receptor by EGF could trigger the translocation of Gαs. It has been shown that Gαs is tyrosine phosphorylated by the EGF receptor in vitro and in response to EGF stimulation in vivo (42,43). Conceivably, this phosphorylation event might be related to the change in the subcellular localization of Gαs. Furthermore, Gαs has been shown to interact directly with the juxtamembrane region (50 aa) of the EGF receptor in both yeast two-hybrid and coimmunoprecipitation assays; this interaction was suggested to be responsible for the activation of adenylyl cyclase by EGF stimulation in cardiomyocytes (44,45). Intriguingly, the juxtamembrane region of the EGF receptor contains a dileucine motif that is required for efficient sorting of receptors to lysosomes (46-48). The juxtamembrane region also includes a
protein kinase C phosphorylation site, and phosphorylation of the EGF receptor by protein kinase C has been shown to switch receptors from the degradation to recycling pathways (46-48). It would be interesting to know whether these sorting motifs are involved in the binding of Gαs to the EGF receptor.

In this work, we have used both overexpression and RNAi knock-down approaches to demonstrate the role of Gαs in EGF receptor degradation. Previously, it has been shown that overexpression of Gαi1 inhibited internalization of low density lipoprotein and transferring, possibly by binding free Gβγ subunits and forming inactive heterotrimers (49). Although overexpression of Gαs may cause similar sequestration of Gβγ subunits, our RNAi knock-down results strongly suggest that Gαs plays a direct role in regulating EGF receptor degradation. Whether free Gβγ also is involved in the degradation of EGF receptor directly remains to be investigated.

As a core component of the endosome sorting machinery, Hrs is evolutionarily conserved in eukaryotes. In budding yeast Saccharomyces cerevisiae, the Hrs homolog Vps27 is one of the “class E” vacuolar protein sorting (Vps) proteins required for formation of MVBs, sorting of membrane proteins into MVBs, or budding into MVBs (17,50). It is noteworthy that S. cerevisiae does not seem to have a Gαs homolog. The two heterotrimeric G proteins encoded in S. cerevisiae, GPA1 and GPA2, are closer to the Gαi rather than the Gαs subfamily of mammalian G proteins in amino acid
sequence. As for RGS-PX1, its putative homolog in *S. cerevisiae*, Mdm1, contains a PX-associated (PXA) domain of unknown function and a PX domain that binds to phosphatidylinositol-3-phosphate (51,52). However, Mdm1 does not have a homologous RGS domain, and no functional link between Mdm1 and MVB sorting has been reported to date. The absence of a Gαs homolog in *S. cerevisiae* and the lack of an RGS domain in Mdm1 lead us to propose that Gαs serves as a regulatory module in the endosome sorting machinery in higher organisms, rather than a evolutionarily conserved core component like Hrs.

Depletion of Hrs by RNAi in mammalian cells was shown to decrease the membrane association of the ESCRT complex, reduce the number of MVBs, and disrupt lysosomal targeting of EGF receptors, leading to impaired EGF receptor down-regulation (27,30). We report here that depletion of Gαs by RNAi, similar to Hrs, delays degradation of EGF receptors. Moreover, simultaneous depletion of Gαs and Hrs by double RNAi further inhibited EGF receptor degradation compared with depletion of Gαs or Hrs alone. These results, together with our observation that Gαs interacts with Hrs, suggest that Gαs and Hrs act together to promote ligand-dependent degradation of EGF receptors. We have previously found that overexpression of RGS-PX1 slowed EGF receptor degradation (11), an effect of RGS-PX1 that could be explained by its GAP activity on Gαs. Alternatively, overexpression of RGSPX1 might have a dominant-negative effect through its interaction with Hrs. Although we have shown that Hrs can form a coimmunoprecipitable complex with RGS-
PX1 and Gαs, there is also the possibility that some complexes may contain Hrs and RGS-PX1 only, or Hrs and Gαs only, and that Gαs may promote EGF receptor degradation by competing RGS-PX1 from Hrs.

Hrs has more recently been shown to regulate degradation of other receptors, including the G protein-coupled receptors CXCR4 (53) and DOR (54) and Drosophila Notch and Patched receptors (55), supporting a general role of Hrs in regulating endosomal sorting and degradation of cell surface receptors. It would be of interest to investigate whether the regulatory function of Gαs in sorting EGF receptors can be extended to other receptors, especially those coupled to heterotrimeric G proteins.

Unlike its positive role in endosomal sorting, Hrs has recently been suggested to prevent endosome fusion. Recombinant Hrs proteins were found to inhibit homotypic fusion of early endosomes, probably by binding to SNAP-25, thereby inhibiting the formation of a SNARE protein complex containing syntaxin 13, SNAP-25, and VAMP2 (56,57). Gαs has similarly been suggested to negatively regulate endosomal fusion based on the observation that activation of Gαs by either cholera toxin or a Gαs stimulatory peptide blocked endosomal fusion in vitro (9).

In summary, our findings support a previously unappreciated role of Gαs in endocytic trafficking and down-regulation of the EGF receptor. Further studies are required to define the precise role of Gαs in endosomal sorting in general, to understand the mechanisms involved in the translocation of Gαs to
early endosomes, and to unravel the differences in the regulation of Gαs functions at the plasma membrane and early endosomes.
Figure 3-1. Overexpression of G\(\alpha\)s promotes degradation of EGFR in HEK293 cells. (A) HEK293 cells were transfected with pXER-EGFR together with pcDNA3.1-G\(\alpha\)s (G\(\alpha\)s) or control vector (control) for 24 h, serum starved overnight, and then treated with 100 nM EGF for 0 or 60 min, followed by immunoblotting with antibodies against EGF receptor, actin, or G\(\alpha\)s. G\(\alpha\)s is seen as two bands representing the long and short forms of G\(\alpha\)s. Data shown are representative of at least three independent experiments. (B) Quantification of EGF receptor degradation. Results from three independent experiments were analyzed by Quantity One software (Bio-Rad). When cells transfected with control vectors are stimulated with EGF, \(~50\%\) of the receptors seen at 0 min are degraded by 60 min after adding EGF. In cells transfected with G\(\alpha\)s, degradation is enhanced as \(~80\%\) of the receptors are degraded by 60 min. Data presented as percentage of total EGF receptor at 0 min in control cells.
Figure 3-2. Overexpression of G\textalpha{s}-GFP promotes degradation of Texas Red-EGF. (A) Cos7 cells transfected with pG\textalpha{s}-GFP or control vector were incubated with Texas Red EGF for 10 min and chased for 30 or 60 min. Cells expressing G\textalpha{s}-GFP (traced in white) and those expressing control vector showed similar levels of Texas Red EGF at 0-min chase. However, after 30- or 60-min chase there is considerably less Texas Red EGF remaining in cells expressing G\textalpha{s}-GFP. (B) Semiquantitative representation of the data shown in A. In cells transfected with control vector ~30\% of the Texas Red EGF is degraded at 30 min and 80\% by 60 min, whereas in cells expressing G\textalpha{s}-GFP ~70\% are degraded at 30 min and ~95\% at 60 min. Average integrated intensity of Texas Red EGF pixels per cell were measured as described in Materials and Methods. Data are expressed as the mean ± SE of three experiments.
Figure 3-3. Depletion of Gαs expression by RNAi delays degradation of the EGF receptor. (A) Cos7 cells were transfected with 37.5 nM control or Gαs-specific siRNA oligos by using Oligofectamine. After 3 d, cells were treated with 100 nM EGF for 60 min, lysed, and analyzed by immunoblotting with antibodies against EGF receptor, Gαs, and actin. The level of Gαs is reduced to ~5% of control levels in cells transfected with oligos specific for Gαs. In cells transfected with control siRNA, 70% of the EGF receptors are degraded after 60-min stimulation with EGF. In cells transfected with Gαs siRNA, degradation is delayed as only 34% of the receptors are degraded after 60 min. Data presented as percentages of the amount of EGFR at 0 min in each group of cells. Data shown are representative of at least three independent experiments. (B) Quantification of EGF receptor degradation. Results from three independent experiments were analyzed by Quantity One software. Data presented as percent of total EGFR present at 0 min in control cells.
**Figure 3-4. RGS-PX1 interacts with Hrs.** (A) In vitro translated, 35S-labeled Hrs binds to GST-RGS-PX1(PXC) but not to GST alone. GST-RGS-PX1(PXC) and GST alone (~75 µg each) immobilized on glutathione beads were incubated with in vitro-translated Hrs. Bound proteins were separated by SDS-PAGE and detected by autoradiography. Input equals 3% of total in vitro translation product. (B) Myc-Hrs (top, lane 4) coimmunoprecipitates with FLAG-RGS-PX1 in cells transfected with both proteins but not in those transfected with myc-Hrs alone (top, lane 3). HEK293 cells were transfected with FLAG-RGS-PX1 and myc-Hrs or myc-Hrs alone, and immunoprecipitation (IP) was carried out on lysates (lanes 1 and 2) with anti-FLAG mouse IgG, followed by immunoblotting (IB) of immunoprecipitates with anti-myc. (C) FLAG-RGS-PX1 coimmunoprecipitates with myc-Hrs. Immunoprecipitation was carried out with anti-myc on lysates from HEK293 cells transfected with myc-Hrs alone (lane 1), FLAG-RGS-PX1 alone (lane 3), or both FLAG-RGS-PX1 and myc-Hrs (lane 2), followed by immunoblotting with anti-FLAG IgG.
Figure 3-5. Interaction between Hrs and Gαs. (A) In vitro-translated, $^{35}$S-labeled Gαs binds to GST-Hrs but not to GST alone. GST-Hrs and GST proteins (~75 µg each) immobilized on glutathione beads were incubated with in vitro-translated, $[^{35}$S]Gαs as in Figure 4. Input equals 3% of total in vitro translation product. (B) Endogenous Gαs from rat brain lysates binds to GST-Hrs but not to GST. GST-Hrs and GST immobilized on glutathione beads were incubated with rat brain lysates (~5 mg). Bound proteins were immunoblotted with anti-Gαs IgG. Input equals 3% of total brain lysate. (C) Myc-Hrs coimmunoprecipitates with Gαs-GFP (lane 4). Lysates (lanes 1 and 2) from HEK293 cells transfected with Gαs-GFP or GFP together with myc-Hrs were immunoprecipitated with anti-GFP, followed by immunoblotting with anti-myc and anti-GFP antibodies. (D) Gαs and Hrs are found in approximately equal amounts in both membrane (P100, lane 2) and cytosolic (S100, lane 1) fractions. Gαs coimmunoprecipitates with myc-Hrs predominantly (~95%) from membrane fractions (lane 4, bottom). Very little Gαs is coprecipitated with myc-Hrs from the cytosolic fraction (lane 3, bottom). Cytosolic (S100, lane 1) and membrane (P100, lane 2) fractions prepared from HEK293 cells transfected with Gαs and myc-Hrs were immunoprecipitated with anti-myc (myc, lanes 3 and 4) or control (ctrl, lanes 5 and 6) mouse IgGs, followed by immunoblotting with anti-Gαs and anti-myc antibodies.
Figure 3-6. Colocalization of Gαs-GFP with myc-Hrs on early endosomes. 
(A–C) In Cos7 cells transfected with Gαs-GFP alone, Gαs is distributed on the 
plasma membrane (arrow, A) and on small vesicular structures (arrowheads, A). Hrs is distributed on early endosomes throughout the cell (B). Merged 
image (yellow) shows occasional overlap in the vesicular distribution of Gαs-
GFP and Hrs (arrowheads and inset, C). (D–I) In cells transfected with Myc-
Hrs, which promotes formation of large, clustered endosomes Gαs-GFP is 
distributed on the plasma membrane (arrow, D) and on the enlarged 
endosomes (arrowheads and inset, D and G). Myc-Hrs (arrowheads and inset, 
E) and Rab5 (arrowheads and inset, H) are also present on these endosomes. 
Gαs-GFP colocalizes (yellow) with Myc-Hrs (arrowheads and inset, F) and 
Rab5 (arrowhead and inset, I). Cos7 cells were transfected with Gαs-GFP 
alone (A–C) or together with Myc-Hrs (D–I) and permeabilized with saponin 
before fixation to release the cytosolic proteins and facilitate the detection of 
membrane-associated pools of Gαs and Hrs. Cells were then fixed with 3% 
PFA, permeabilized, and double labeled with mouse anti-GFP (A, D, and G), 
anti-Hrs (B), or anti-myc (E) IgG or rabbit anti-rab5 (H) IgG and analyzed by 
deconvolution immunofluorescence microscopy. Bar, 2 µm.
Figure 3-7. Immunogold localization of Hrs and G\(\alpha\)s in HEK293 cells. (A). G\(\alpha\)s-GFP (10-nm gold) is localized to numerous coated tubules (arrowheads) and endosomes (asterisks). (B, C). Myc-Hrs (5-nm gold, arrows) and G\(\alpha\)s-GFP (10-nm gold) colocalize in coated domains of early endosomes (asterisks). HEK293 cells transfected with myc-Hrs and G\(\alpha\)s-GFP were fixed either in 4% PFA (A) or a mixture of 4% PFA and 0.2% glutaraldehyde (B, C) and prepared for ultrathin cryosectioning as described in Materials and Methods. Ultrathin cryosections were labeled with anti-myc mAb (5-nm gold) and polyclonal anti-GFP (10-nm gold). Bar, 100 nm.
Figure 3-8. Colocalization of Gαs with RGS-PX1 on early endosomes. GFP-RGS-PX1 is found on endosomes (arrowheads and insets, A, D) and partially colocalizes with Gαs-WT (arrowheads and inset, B, C) on endosomes loaded with Texas Red EGF (arrowheads and inset, E, F). Cos7 cells were transfected with GFP-RGS-PX1 and Gαs-WT. In D–F, cells also were incubated with Texas Red EGF for 15 min at 37°C. Cells were permeabilized with saponin, fixed with 3% PFA, double labeled with mouse anti-GFP mAb (A, D), and rabbit anti-Gαs (B) IgG, and analyzed as described in Figure 3-6. Bar, 2 μm.
Figure 3-9. Gαs, FLAG-RGS-PX1, and myc-Hrs form a coprecipitable complex. HEK293 cells were transfected with pcDNA3-Gαs, FLAG-RGS-PX1, and myc-Hrs. Lysates were immunoprecipitated with anti-FLAG (lane 2), anti-myc (lane 3), or control (ctrl) (lane 4) mouse IgGs, followed by immunoblotting with anti-Gαs (top), anti-FLAG (middle), or anti-myc (bottom) IgG. Gαs (top) coprecipitates with both FLAG-RGS-PX1 (lane 2) and myc-Hrs (lane 3).
Figure 3-10. Simultaneous knockdown of both Gαs and Hrs causes a delay in EGF receptor degradation greater than knock-down of either Gαs or Hrs alone. (A) Cos7 cells were transfected with 75 nM, Gαs siRNA alone, Hrs siRNA alone, both Gαs and Hrs siRNA, or with control siRNA oligos by using Oligofectamine. After 3 d, cells were treated with 100 nM EGF for 30 min, lysed, and analyzed by immunoblotting with antibodies against EGF receptor, Gαs, Hrs, and actin. In cells transfected with Hrs or Gαs RNAi alone, 50–55% of the EGF receptors have been degraded after 30 min, whereas in those transfected with both Gαs and Hrs siRNA, degradation is delayed as only 25% of the receptors have been degraded. Data shown are representative of at least three independent experiments. (B) Quantification of EGF receptor degradation. Results from three independent experiments were analyzed by Quantity One software. Data presented as percent of total EGFR at 0 min in each group of cells.
Figure 3-11. Model of the proposed function of Gαs in EGF receptor degradation. After EGF stimulation Gαs is recruited to early endosomes where it forms a complex with RGS-PX1 and Hrs on the endosomal membrane. Together with Hrs, Gαs promotes the sorting of ubiquitinated (Ub) EGFRs into the luminal vesicles of MVBs and hence facilitates their degradation.
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Chapter 4

Summary, Perspectives, and
Future Directions
SUMMARY AND PERSPECTIVES

G\(_\alpha\)s and RGS-PX1 Regulate EGFR Trafficking and Signaling

When I joined Dr. Farquhar’s laboratory, we had just discovered that RGS-PX1 inactivates G\(_\alpha\)s and modulates EGFR trafficking and signaling (1) which suggested that RGS-PX1 might link G\(_\alpha\)s signaling to EGFR. At the time, work by other groups as well as our own had indirectly implicated G\(_\alpha\)s signaling in EGFR trafficking and signaling. For example, Stahl and colleagues had found that pharmacological agents that activate G\(_\alpha\)s (e.g., cholera toxin) could inhibit endosome fusion (2). Overall, the role of G\(_\alpha\)s in EGFR trafficking and signaling was unclear, and I set out to help define the effects of G\(_\alpha\)s in these processes.

G\(_\alpha\)s is Required for Efficient Ligand-induced EGFR Degradation

In Chapter 3 (3) we discovered that G\(_\alpha\)s was required for efficient ligand-induced degradation of EGFR (Fig. 3-3), a key step in the downregulation of EGFR signaling (4). These findings were the first to implicate any G-protein in the termination of EGFR signaling. We were also the first to localize G\(_\alpha\)s to early endosomes with RGS-PX1 (Fig. 3-8). Since RGS-PX1 inactivates G\(_\alpha\)s (1), the latter finding implies that G\(_\alpha\)s has a function on endosomes that is activation-dependent and requires its specific GAP to inactivate it. Furthermore, the data suggest that inactivation of G\(_\alpha\)s by RGS-PX1 at endosomes might be required for efficient downregulation of EGFR signaling. We also observed that G\(_\alpha\)s and RGS-PX1 interact with Hrs both in vivo and in vitro (Fig. 3-4, 5, 9). Since Hrs is a key component of the endosomal sorting machinery (5), our work implies that
Gαs promotes efficient sorting of EGFR for degradation by forming a complex with Hrs and RGS-PX1 (Fig. 3-11).

About the time that work in Chapter 3 was wrapping up, a previous post-doc had accumulated evidence suggesting that Gαs might promote endosome maturation and, consequently, EGFR degradation. Specifically, she found that Gαs depletion led to enlarged early endosomes and GST-Gαs interacted with Rab5 (a small GTPase that regulates early endosome fusion (6)) and p38 MAPK which phosphorylates and activates a RabGDI to inhibit endosome fusion by extracting Rab5 from endosomes (7)). In fact, she also found that Gαs depletion inhibited p38 activation. I investigated if Gαs promotes p38 activation, phosphorylation and activation of RabGDI, and Rab5 extraction to inhibit further fusion of early endosomes and promote maturation of early endosomes into late endosomes. I set out to reverse the observed changes in endosome morphology and p38 activity following Gαs depletion, to investigate the activity state-dependence of Gαs interactions with Rab5 and p38, and to use in vitro extraction assays (8) to test if Gαs is required for efficient Rab5 extraction.

When optimizing the reversal of siRNA phenotypes, I found that lower siRNA concentrations were as effective at depleting Gαs but they no longer led to enlarged early endosomes or inhibited p38 activity, suggesting that prior observations were due to off-target effects of high siRNA concentrations. After I optimized the in vitro binding assays between GST-Gαs and Rab5/p38 derived from cell lysates based on positive controls (which were not included initially), I
was unable to reproduce the observed interactions. Finally, I successfully developed an *in vitro* Rab5 extraction assay to test whether Gαs depletion might inhibit Rab5 extraction from membranes by RabGDI, but Gαs depletion had no effect. At this point, I stopped pursuing this line of investigation and instead focused on two areas of research: 1) defining the state-dependent effects of Gαs on EGFR signaling, and 2) another ongoing project investigating the role of GIV and Gαi in EGFR signaling, cell migration, and cell proliferation (see below).

**State-dependent Effects of Gαs on EGFR Signaling**

From our work in Chapter 3 (3), I knew that Gαs was required for efficient ligand-induced degradation of EGFR, a key step in signal downregulation, but whether Gαs is required to downregulate EGFR signaling had not been investigated. To answer this question, I first investigated the effects of Gαs on autophosphorylation of EGFR at Y1068 (pY1068), the binding site for Grb2 which mediates ERK 1/2 and Akt signaling, and Y1045 (pY1045), the binding site for c-Cbl which mediates receptor ubiquitination (see Chapter 1 for review) by stimulating Gαs-depleted or control HeLa cells with EGF and monitoring autophosphorylation as in **Fig 2-2**. Although I used Gαs siRNA as my primary tool, I made sure that transfection of Gαs depleted cells with siRNA resistant Gαs (srGαs-WT) reversed any observed effects.

In control and Gαs depleted HeLa cells transfected with vector, I found that pY1045 and pY1068 peaked at 5 min after EGF stimulation with some phosphorylation observed after 15 min (**Fig. 4-1A, C-F**). However, there was ~3
fold more phosphorylation at both these sites at 5 min and ~3.4 and ~2.5 fold more at 15 min after EGF stimulation in Gαs depleted cells transfected with vector compared to controls (Fig. 4-1A, C-F). Transient transfection of Gαs depleted cells with srGαs-WT partially reversed the effects of Gαs depletion (Fig. 4-1A, C-F), indicating that Gαs is required to efficiently downregulate pY1045 and pY1068 signaling. Furthermore, Gαs depletion and reversal studies showed a similar trend at pY992-EGFR, the binding site for PLCγ (data not shown). I next asked if the activity status of Gαs affects EGFR autophosphorylation by similarly assessing the ability of siRNA resistant (sr) active Gαs-Q227L (QL) (GTPase-deficient and constitutively binds GTP (9)) or inactive srGαs-G226A (GA) (constitutively binds Gβγ like inactive, GDP-bound Gαs (10)) mutants to reverse the effects of Gαs depletion. I found that inactive srGαs-GA but not empty vector or active srGαs-QL had less autophosphorylation at Y1045 and Y1068 at 5 and 15 min after EGF stimulation (Fig. 4-1B-F). Thus, the inactive but not the active Gαs mutant can reverse the effects of Gαs depletion which indicates that Gαs activation enhances and inactivation inhibits EGFR autophosphorylation. Whether or not the observed changes in autophosphorylation affect SH2 adaptor recruitment and downstream signaling will be investigated as outlined in future directions.

Based on our previous work in Chapter 3 (3) and my recent findings above (Fig. 4-1), I reason that RGS-PX1 might first bind and inactivate Gαs (1), then inactive Gαs may preferentially form a complex with Hrs that facilitates receptor
sorting and consequently, signal downregulation. Based on this model (Fig. 4-2), overexpressed srGαs-QL would not be expected to facilitate downregulation because it would not form a complex with Hrs which is required for receptor sorting, whereas srGαsGA would facilitate downregulation by forming the complex. Furthermore, Gβγ is a classic GDI for Gαs that is released upon G-protein activation and is known to promote EGFR signaling (11), Akt activation (12), dynamin GTPase (13), affect clathrin-mediated endocytosis (14), and mediate growth factor stimulated actin remodeling (15), which is a critical component of PM-based signaling by EGFR (16,17). Since the levels of free Gβγ complexes are expected to be higher following depletion or activation of Gαs, Gβγ might mediate some of the effects of Gαs on EGFR signaling (Fig 4-2). Experiments outlined in future directions are designed to shed light on these possibilities.

The Gαi-GIV switch is required for cell migration

As mentioned above, I also had the opportunity to work on another ongoing project investigating the effects of Gαi and GIV on EGFR signaling, cell migration, and cell proliferation. When I joined the project, we knew that GIV is a GEF that specifically binds and activates Gαi (12,18,19). In addition, we had demonstrated that GIV and Gαi form a molecular switch that promotes growth factor-induced actin remodeling, activation of Akt, and cell migration (12,19,20), features that implicate GIV in the metastatic progression of breast cancer (21). In addition, it was also known that migrating metastatic cells cannot proliferate
and proliferating tumor cells cannot migrate, a phenomena termed migration-proliferation dichotomy (22), and that these mutually exclusive processes are influenced by EGFR signaling via an unknown post-receptor mechanism (23,24). Others had proposed that activated EGFR might induce either cell migration or proliferation based on whether the receptor signaled from the PM or endosomes, respectively (see Chapter 1 and (25) for review), but how the distribution of the receptor is regulated remained unknown. That the $G_\alpha_i$-GIV switch is required for EGF-induced Akt signaling, actin remodeling, and cell migration (12,19,20) led us to investigate whether the $G_\alpha_i$-GIV switch influences EGFR signaling and preferentially promotes cell migration.

**The $G_\alpha_i$-GIV switch determines whether cells migrate or proliferate by programming EGFR signaling**

The work outlined in Chapter 2 is the first to describe that the $G_\alpha_i$-GIV switch determines cell fate by influencing EGFR signaling. Specifically, we discovered that the $G_\alpha_i$-GIV switch dictates critical components of EGFR signaling that determine whether cells migrate or proliferate including receptor distribution (PM vs. endosome) (Fig. 2-3), downregulation (Fig. 2-2, 3), autophosphorylation (Fig. 2-2), and downstream signaling (Fig. 2-1) (see Chapter 1, Fig. 1-3, and (23,24) for review of EGFR trafficking). Mechanistically, my work revealed that GIV’s GEF motif forms a critical link between EGFR, $G_\alpha_i$, and actin (Fig. 2-3, 4). Since EGFR:actin association enhances receptor autophosphorylation, retention at the PM, and cell migration (16,17,26), we concluded that the $G_\alpha_i$-GIV switch dictates the critical
components of receptor signaling that determine cell fate by regulating receptor:actin interactions. In addition, we also provide the first example of a protein (e.g., GIV) that is suppressed by alternative splicing or induced by upregulation to impart early proliferative or later migratory advantages, respectively, during oncogenesis (Fig. 2-6, 7).

Overall, our findings provide mechanistic insights into how EGFR signaling is linked to $G_{\alpha i}$ signaling and programmed to determine cell fate, they lend further credence to the importance and specificity of receptor signaling from endosomes in cell proliferation, and they elucidate the potential origin of migration-proliferation dichotomy. Experiments outlined in future directions will further investigate important questions that arise based on our work, including whether the $G_{\alpha i}$-GIV switch directly interacts with EGFR and/or affects additional steps (e.g., recycling, sorting for degradation) in receptor trafficking.

**FUTURE DIRECTIONS**

The work outlined in this thesis has elucidated some of the mechanisms by which G-proteins can regulate EGFR signaling, yet many critical questions arise based on this work as well as the models described above. For example, could $G_{\alpha s}$ inactivation by RGS-PX1 and complex formation between inactive $G_{\alpha s}$ and Hrs promote efficient downregulation of EGFR signaling? Does $G_{\alpha s}$ affect multiple steps of EGFR trafficking (internalization, recycling, and/or sorting for degradation)? Could the effects of $G_{\alpha s}$ on EGFR autophosphorylation (Fig. 4-2) lead to changes in SH2 adaptor recruitment and downstream signaling?
Furthermore, is the Gαi-GIV switch required for efficient EGFR internalization and/or recycling? Could disruption of the Gαi-GIV switch disrupt ubiquitination and delay sorting of EGFR for degradation? Does the Gαi-GIV switch interact directly with EGFR? I have designed the follow experiments to gain deeper insights into the mechanisms involved as well as further elucidate the similarities and/or differences by which Gαs and Gαi affect EGFR trafficking and signaling.

I. What are the Mechanisms by which Gαs Promotes Downregulation of EGFR Signaling?

A. Does inactive Gαs preferentially bind Hrs? Our group has previously used in vitro binding assays with purified recombinant GST-tagged G-proteins, cell or tissue lysates, or in vitro translated proteins to investigate state-dependent interactions with G-proteins (1,12,19). I will immobilize purified GST (negative control) or GST-Gαs on glutathione beads, pre-load these proteins with nucleotide by incubation with GDP or GDP/AlF₄⁻ (the latter mimics the γ phosphate of GTP (1,12,19,27)), incubate with detergent extracts (rat brain or HeLa cells) or in vitro translated Hrs, and then analyze bound proteins by immunoblotting for Hrs, Gβ (positive control for binding inactive, GDP-bound GST-Gαs), or RGS-PX1 (positive control for binding active, GDP/AlF₄⁻ bound GST-Gαs or autoradiography, respectively (1,19). To investigate state-dependent complex formation in vivo, I will express internally-tagged, Gαs-GFP (3) WT, active QL, or inactive GA mutant, immunoprecipitate using anti-GFP or anti-Hrs antibody, then analyze bound proteins for Hrs, GFP, Gβ, and RGS-PX1.
In vivo interaction might require EGF stimulation, so I will stimulate cells with EGF before immunoprecipitation. Using the above methods, I should be able to determine whether Hrs preferentially interacts with inactive Gαs and confirm that RGS-PX1 preferentially binds active Gαs.

**B) Does RGS-PX1 facilitate downregulation of EGFR signaling by inactivating Gαs?** Our lab recently made an RGS-PX1 antibody and successfully used siRNA oligos to specifically deplete endogenous RGS-PX1. Therefore, I will deplete endogenous RGS-PX1 from HeLa cells, stimulate with EGF, and analyze EGFR signaling as in Fig. 2-1, 2-2, and 4-1, and siRNA-resistant RGS-PX1 will be used to reverse any effects of depletion.

I will then test the effects of specifically disrupting the RGS-PX1:Gαs interaction on EGFR signaling. The RGS domain of RGS-PX1 binds directly to Gαs in a state-dependent manner (1,28). Based on our alignment of RGS domains from RGS-PX1 and other RGS proteins (1), RGS-PX1 shares three highly conserved residues within the RGS domain (D461, L492, and D496) that serve a critical role in the interaction between RGS4 and Gαi (1,29,30) and could similarly direct binding of RGS-PX1 to Gαs. These sites will be mutated to alanine (29,30), and then pull-down assays carried out to test state-dependent interaction between GST-Gαs and in vitro translated RGS-PX1-WT or mutants as above. If RGS-PX1:Gαs binding is not abolished by a single mutation, I will try double or triple mutants. To verify that expression of RGS-PX1-WT but not the RGS-PX1 mutant can attenuate Gαs-based signaling, I will test whether RGS-
PX1-WT can selectively attenuate isoproterenol stimulated cAMP-production using cAMP assays as in (1). Based on my model (Fig. 4-2), I predict that depleting RGS-PX1 or abolishing the RGS-PX1:Gαs interaction would both be expected to prolong activation of Gαs and in turn, enhance EGFR signaling.

D) Does Gβγ mediate the effects of Gαs activation on EGFR signaling? Since cells express many Gβ and Gγ isoforms (31), it would be difficult to test whether Gβγ depletion disrupts EGFR signaling. An alternative, established method is to express βARK-ct, a C-terminal fragment of the G-protein Coupled Receptor Kinase 2 (GRK2) that binds, sequesters, and thereby inhibits Gβγ signaling (32). Gαs depleted cells will be transfected with βARK-ct, Gαs-GA, or vector alone; EGF stimulated; and then EGFR signaling analyzed as in Fig. 2-1, 2 and 4-1. If free Gβγ enhances EGFR signaling in Gαs depleted cells, then transfection of βARK-ct would be expected to reverse this effect compared to controls.

II. What steps in EGFR trafficking are affected by Gαs?

We know now that Gαs depletion is required for efficient ligand-induced degradation of EGFR (Fig. 3-3) and downregulation of EGFR signaling (Fig. 4-1). Our findings could be due to delayed ligand-induced receptor internalization, enhanced receptor recycling from endosomes back to the PM, and/or delayed receptor sorting at endosomes (5,33,34). Studies outlined below can be used to pinpoint the effect of Gαs on EGFR trafficking.
A. Is Gαs required for efficient ligand-induced EGFR internalization and/or recycling? Internalization can be monitored by following 125I-EGF labeled EGFR as in (33,35,36). Control or Gαs depleted HeLa cells will first be incubated for 1 hr at 4 °C with 5 ng/mL 125I-EGF to label surface receptors; then incubated at 37 °C (0, 5, or 15 min); and finally stripped with an acidic buffer that removes surface-bound ligand (fraction A). Stripped cells are solubilized in NaOH containing 0.1% SDS to measure the acid-inaccessible, internalized ligand (fraction B). Fractions are then counted in a γ-counter, and the cell-surface associated ligand calculated (A/(A+B)) (33,35,36). If Gαs depletion delays EGFR internalization, the fraction of 125I-EGF remaining at the cell surface would be expected to be greater than in controls.

To follow EGFR recycling and degradation (33,35,36), I will similarly surface label control or Gαs depleted HeLa cells as above but then allow ligand uptake for 5 min at 37° C before stripping as above. Cells are then incubated an additional 0, 5, 15, 30, 60 min at 37° C in excess, unlabeled EGF to allow trafficking and minimize re-uptake of any recycled 125I-EGF. The reactions are stopped on ice, the media collected, and the surface bound 125I-EGF stripped (fraction A). The media is TCA precipitated, and the supernatant (containing the free 125I from degraded 125I-EGF (fraction B)), the pellet (containing intact, recycled 125I-EGF (fraction C)) and the remaining cells (containing intracellular 125I-EGF (fraction D)) solubilized in basic buffer, and fractions counted. The relative amount of recycled (A+C) or free (B) 125I-EGF, which closely mirrors the fate of EGFR (33,35,36), is calculated as a fraction of the total (A+B+C+D).
(33,35,36). If $G\alpha\delta$ depletion promotes recycling, the sum of fractions A and C would be greater than in controls. Since $G\alpha\delta$ depletion delays EGFR degradation (3), we would expect the fraction of free $^{125}\text{I}$ in (B) to be smaller in $G\alpha\delta$ depleted cells vs. controls.

**B) What are the effects of $G\alpha\delta$ on the distribution of EGFR over time?**

This will be investigated as follows: serum-starved control or $G\alpha\delta$-depleted HeLa cells will be stimulated with EGF or Alexa-647-EGF (0, 2, 5, 10, 15, 30, or 60 min), fixed, and stained for tEGFR or pEGFR (using site specific antibodies) and EEA1 (specific early endosome marker (37)), Hrs, Rab7 (late endosome marker (38)), or Lamp2 (lysosome marker (39)). Overlap is quantified using NIH Image software (40). If $G\alpha\delta$ depletion delays EGFR internalization, the time required for tEGFR, pEGFR, and 647-EGF to reach early endosomes and colocalize with EEA1 and/or Hrs would be expected to be longer (>15 min) compared to controls (<10 min). If $G\alpha\delta$ depletion increases EGFR recycling and/or inhibits efficient sorting for degradation, less receptor would be expected to reach lysosomes and colocalize with Lamp2 after 30 min of EGF stimulation compared to controls.

**C) Does $G\alpha\delta$ Promote Sorting of EGFR for Degradation?** Control or $G\alpha\delta$ depleted HeLa cells will be stimulated with EGF (0, 5, 15, 30 min), fixed, and processed for immunogold labeling (3,41) for EGFR (42). I will quantify the amount of EGFR found on the limiting membrane of the endosome (yet to be sorted) vs. the amount of EGFR that has been sorted and is located on the membrane of vesicles within the endosome lumen by counting the gold particles
in the two locations. If $G_\alpha$s depleted cells have a defect in sorting as I predict, there should be less EGFR on the membrane of vesicles within the endosome lumen compared to controls. I can further investigate whether transient expression of the internally-tagged, sr$G_\alpha$sGFP (3) WT and GA (inactive) mutant but not the active mutant or GFP alone might reverse any defects (Fig 4-1, 2) by co-immunolabeling with GFP (to monitor sorting in cells expressing GFP proteins) and EGFR antibodies. Overall, the immunogold, radio-ligand, and immunocytochemical experiments outlined above will help pinpoint the time and steps in EGFR trafficking that are regulated by $G_\alpha$s.

III. Does $G_\alpha$s affect downstream signaling and SH2 adaptor and Hrs recruitment to EGFR?

We observed that changes in EGFR autophosphorylation at Y992, Y1045, and Y1068 correlate with changes in SH2 adaptor recruitment (e.g., PLC$\gamma$, c-Cbl, and Grb2, respectively) (Fig. 2-2). I will investigate if $G_\alpha$s depletion affects adaptor recruitment compared to controls by co-immunoprecipitation assays (Fig. 2-2). Since $G_\alpha$s depletion enhances EGFR autophosphorylation at the above sites (Fig. 4-1, data not shown), I would expect an increase in adaptor recruitment. If $G_\alpha$s depletion impairs EGFR sorting (section II-C) and c-Cbl recruitment (above), I can investigate if depletion impairs c-Cbl mediated ubiquitination of (43) and ubiquitin-dependent Hrs recruitment to the receptor (3) by analyzing the co-immunoprecipitations above for Hrs and ubiquitin. I would expect both ubiquitin bands at and above the predicted m.w. for EGFR (180 kDa)
(laddering due to mono- and poly- ubiquitinated at multiple residues (44)) and Hrs bands only following ligand stimulation.

I recently used similar approaches as in Fig. 2-1 and Fig 4-1 to investigate any state-dependent effects of $G_{i3}$ on EGFR-induced downstream signaling to ERK 1/2 and Akt. My preliminary data indicate that inactive $G_{i3}$ is required to efficiently downregulate EGF-induced ERK 1/2 signaling (Fig. 4-2) which is consistent with the effect of inactive $G_{i3}$ on downregulation of autophosphorylation at Y1068 (Fig. 4-1), which mediates ERK 1/2 activation via Grb2 (45). In contrast, active $G_{i3}$ is required to downregulate Akt signaling (Fig. 4-2), which is consistent with a role for $G_{i3}$-based, cAMP/PKA signaling in attenuating Akt signaling (46,47). Future studies will test the effects of $G_{i3}$ on PLCγ and c-Src/STAT5b signaling. Overall, the studies outlined above should help define the effects of the $G_{i3}$ on EGFR trafficking and signaling.

IV. Is binding of GIV to EGFR direct and/or regulated by receptor autophosphorylation?

Our work in Chapter 2 indicates that GIV forms a ligand-independent complex with EGFR, yet EGF stimulation induces complex formation between EGFR, $G_{i3}$, and actin in cells expressing GIV-wt but not GIV-FA (Fig. 2-4). These data suggest that GIV’s GEF motif forms a critical, ligand-regulated link between EGFR, $G_{i3}$, and actin. To better understand how GIV interacts with EGFR and promotes formation of a complex between EGFR, actin, and $G_{i3}$, we need to understand 1) which domains of GIV are responsible for interaction with...
the receptor, 2) if the interaction is regulated by phosphorylation, and 3) whether the interaction is direct.

I recently tested whether the amino- or carboxy-terminus of GIV might interact with EGFR using immunoprecipitation assays. I found that GIV (1-1385) (GIV-NT) co-immunoprecipitates with FLAG-EGFR both before and after EGF stimulation (Fig. 4-4). I then carried out a similar experiment as above with immunoisolated FLAG-EGFR and purified, His-tagged, GIV-CT (a.a. 1623-1870) and found that it bound to FLAG-EGFR only after EGF stimulation (Fig. 4-5). These results indicate that binding of GIV-NT to EGFR is ligand-independent whereas binding of GIV-CT, which harbors the GEF motif and the actin and Akt binding sites, is ligand-dependent.

I will investigate which domain of GIV directly interacts with EGFR by pull downs assays using in vitro translated full length GIV, GIV-NT, or GIV-CT and GST-Gαi3 or GST-EGFR-CT (a.a. 650-1210, the cytoplasmic domain of the receptor) (12). Since GIV-CT binds to EGFR following ligand binding (Fig. 4-5), I predict that EGFR must be autophosphorylated for GIV-CT to bind the receptor. This can be tested by first in vitro phosphorylating GST-EGFR-CT as in (45) before it is used for pull downs.

V. What steps in EGFR trafficking are affected by the Gαi-GIV switch?

Our results indicate that an intact Gαi-GIV switch prolongs EGFR signaling from the actin bed at the PM, which could be due delayed internalization and/or enhanced receptor recycling (Fig. 2-3). Meanwhile, disruption of the switch prolongs receptor signaling from endosomes, which could
be due to enhanced receptor internalization, inhibited recycling, or delayed degradation (Fig. 2-3). I will pinpoint the steps in EGFR trafficking (see Chapter 1 and Fig. 1-3 for review) that are affected by the $G\alpha_i$-GIV switch by following receptor trafficking in control, GIV-wt, and GIV-FA cells using radioligand, immunocytochemistry, and immunogold labeling as in sections II-A-C above.

VI. Does the $G\alpha_i$-GIV Switch dictate pro-migration or proliferative signaling induced by the LPA receptor?

Our data shows that GIV-dependent activation of $G\alpha_i$ or expression of active $G\alpha_i$ mutant preferentially promotes cell migration, an intriguing finding since many potent mitogens (e.g., LPA) signal via $G\alpha_i$-coupled GPCRs (48,49) and transactivate EGFR (50,51). Our group previously determined that an intact $G\alpha_i$-GIV switch is required not only for EGF-induced but also LPA-induced activation of Akt (12). I will therefore assess the effects of the $G\alpha_i$-GIV switch on LPA-induced ERK 1/2 and Akt signaling by serum-starving control, GIV-wt, or GIV-FA cells, stimulating with 10 $\mu$M LPA as in (12), and assaying for cell migration, cell proliferation, and ERK 1/2 and Akt signaling as in Fig. 2-1. We can also analyze autophosphorylation of EGFR as in Fig. 2-2 + pre-incubation with an EGFR specific inhibitor (100 nM AG1478) prior to LPA stimulation to determine the EGFR-dependent signaling responses.

What are the similarities and differences in the effects of $G\alpha_s$ vs. $G\alpha_i$ on EGFR signaling?
G\(_i\) and G\(_s\) signaling are classically “ying-yang” in regards to cAMP signaling (52), yet I helped to find that these G-proteins have many similar effects on EGFR dynamics. Although our understanding of the mechanisms by which G\(_s\) affects EGFR signaling is in its infancy, it is still useful to compare the effects of both G-proteins. For example, activation of G\(_s\) and G\(_i\) might enhance ligand-induced EGFR autophosphorylation at multiple sites (Y992, Y1045, Y1068) (Fig. 2-2, 4-1, data not shown), and I predict that the changes in receptor autophosphorylation following G\(_s\) depletion with correlate with changes in adaptor recruitment (e.g., c-Cbl, PLC\(\gamma\), and Grb2) to these sites as in Fig. 2-2.

We also know that both G-proteins facilitate ligand-induced receptor degradation (Fig. 2-3, 3-3) by potentially affecting receptor sorting for degradation, albeit via different mechanisms. For example, G\(_s\) and Hrs form a complex that might be required for sorting (Fig 3-5, 4-2), whereas activation of G\(_i\) by GIV promotes recruitment of c-Cbl to EGFR (Fig. 2-2) which I expect facilitates ubiquitination and Hrs-dependent sorting of the receptor. Thus, both G-proteins regulate receptor trafficking and fine-tune the degree and overall timing of receptor signaling.

A critical difference in how these different G-proteins regulate EGFR signaling might lie in the effects on downstream signaling as GIV-dependent activation of G\(_i\) promotes EGF-induced Akt (pro-migration) but inhibits ERK 1/2 (proliferative) signaling (Fig. 2-2), whereas my preliminary data suggests that activation of G\(_s\) has the opposite effects (Fig. 4-3). Consistent with these
current findings is that $\Gamma_i$ but not $\Gamma_s$ is required for cell migration (19). The effects of $\Gamma_s$ affects on cell proliferation in our experimental systems remains to be determined, but it currently seems that differential regulation of EGFR downstream signaling by G-proteins might in part determine the overall cellular response. Additionally, it may turn out that signaling by additional classes of G-proteins (e.g., $\Gamma_q$ (53)) and GPCRs promote EGFR autophosphorylation yet achieve specificity in the overall cellular response by differential regulation of the spatial distribution of the EGFR, the timing of its sorting for degradation, and the downstream receptor-initiated signaling pathways.

Although many critical and complex questions continue to arise based on our work to date, it is of great satisfaction to see that my work together with the work from others in our own and other groups has revealed the very unique mechanisms that cells have evolved which employ G-proteins in fine-tuning the degree of EGFR signaling. Overall, the studies outlined above should help continue to unravel the mechanisms by which G-proteins affect EGFR induced signaling, cell migration, and proliferation in normal and abnormal processes.
Figure 4-1. Gαs Mediates Down-regulation of EGFR Auto-phosphorylation. (A) Gαs depletion leads to increased auto-phosphorylation of EGFR at Y1045 and Y1068 at 5 and 15 min after EGF stimulation. (B) Transfection of inactive but not active Gαs mutant can reverse this effect. HeLa cells were treated with Silencer negative control siRNA #1 (Ambion) or Gαs-specific siRNA and then transfected with either pCDNA3.1 (Vector) alone, siRNA-resistant (sr) Gαs-WT (n=4), constitutively active srGαs-Q227L (n=3), or inactive srGαs-G226A (n=3) mutant as indicated. Site-directed mutagenesis (primers available upon request) was used to make point mutations in human Gαs cDNA constructs within the region of homology to the siRNA oligo (3). 48 h after siRNA transfection, cells were serum starved overnight in 0.2% FBS DMEM, then stimulated with 50 nM EGF (0, 5, or 15 min) (Invitrogen), and whole cell lysates were analyzed by immunoblotting for phosphorylated (p) Y1068 pAb (Cell Signaling), pY1045 pAb (Cell Signaling), tEGFR pAb (Santa Cruz), Gαs pAb (Calbiochem), and actin mAb (Sigma). Bands were revealed and imaged using infrared-labeled secondary antibodies (goat anti-rabbit 680 nM and goat anti-mouse 800 nM) (Invitrogen) and the Odyssey Infrared Imaging system (LICOR).
Figure 4-1 (continued). (C-F) Bands were quantified using Odyssey imaging software, and the pY1068/actin (C, D) and pY1045/actin (E, F) ratios were calculated at each time point. Each ratio calculated was then divided by the ratio calculated for control siRNA cells transfected with vector alone at the same time point. The results are plotted as the fold increase in phosphorylation compared to control cells ± S.E.M.
Figure 4-2. Working Model for the Effects of Gαs on EGFR Trafficking and Signaling. When Gαs is inactive, efficient downregulation of EGF-induced receptor signaling ensues. 1) When Gαs is activated (or depleted), a GDI might be released that 2) can enhance ligand-induced EGFR signaling. 3) After EGFR is internalized and traffics to endosomes, 4) Gαs might be inactivated by RGS-PX1 and 5) inactive Gαs may preferentially form a complex with Hrs that is required to promote efficient sorting of the receptor for degradation.
Figure 4-3. \( \gamma \text{G} \) Promotes Down-regulation of Akt and ERK 1/2 Signaling. (A) \( \gamma \text{G} \) depletion leads to enhanced EGF-induced Akt and ERK 1/2 signaling. HeLa cells were treated with siRNA and transfected with vector or sr-\( \gamma \text{G} \) WT (n=4), active Q227L (n=3), or inactive G226A (n=3) mutant, starved, stimulated with EGF, analyzed by immunoblotting for total (t) and phosphorylated (p) ERK 1/2 and Akt, \( \gamma \text{G} \), and actin, and the bands quantified as described in Fig 4-1. The pAkt/actin (B) ratio at t=5 and the pERK/actin (C, D) ratios were calculated at t=5 and 15 min. Each ratio was then divided by the ratio at the same time point for control siRNA treated cells expressing vector alone. The results were then plotted as the fold increase in phosphorylation compared to control.
Figure 4-4. The Amino-terminus of GIV Interacts with EGFR Before and After EGF Stimulation. Cos7 cells were co-transfected with GIV-NT (a.a. 1-1385) and FLAG-EGFR (a gift from Howard Rockman, University North Carolina, Chapel Hill) using TransIT-LT1 transfection reagent (Mirus). 36 hr after transfection, cells were serum starved overnight in 0.2% FBS, and stimulated with 50 nM EGF for 0 or 5 min. Cell were washed on ice in cold PBS and lysates prepared by collecting cells in buffer (20 mM HEPES, pH 7.2, 5 mM Mg-acetate, 125 mM K-acetate, 0.4% Triton X-100, 1 mM DTT, supplemented with sodium orthovanadate (100 µM), phosphatase (Sigma) and protease (Roche) inhibitor cocktails). Lysates were then passed through a 28G needle at 4 °C, and cleared (10,000 x g for 5 min). FLAG M2 mAb (Sigma) was added to cleared lysates overnight before incubation with Protein G agarose (Zymed) for 1 hr and extensive washing. Bound proteins were boiled in sample buffer and analyzed by immunoblotting using FLAG mAb and affinity purified GIV pAb (18) as indicated.
Figure 4-5. The Carboxyl-terminus of GIV Interacts with EGFR After Ligand Stimulation. Cos7 cells were transfected with FLAG-EGFR, starved, and then stimulated with 50 nM EGF for 0 or 10 min as described in Fig 4-4. FLAG-EGFR was then immunoprecipitated, immobilized on beads, and the beads washed as described above. After washing, purified His-GIV-CT (a.a. 1623-1870, (12)) was incubated with immobilized FLAG-EGFR for an additional 4 h, washed, solubilized in sample buffer, and bound proteins were analyzed by immunoblotting for Gβ pAb (Santa Cruz), His mAb (Cell Signaling), and other IgG as indicated. C-terminally tagged Gαi3-FLAG was similarly expressed, immunoisolated, and used as a positive control for His-GIV CT binding (12).
REFERENCES


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