EPIDERMAL GROWTH FACTOR STIMULATES INTESTINAL EPITHELIAL CELL MIGRATION THROUGH ACTIVATION OF THE SMALL GTPASE RAC

By

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LIST OF ABBREVIATIONS

AR	Amphiregulin
BIM-1	Bisindolylmaleimide 1
BTC	Betacellulin
C	Carboxyl or Celsius
DAG	Diacylglycerol
DH	Dbl homology
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EPR	Epiregulin
ERK 1/2	Extracellular signal-regulated kinase 1/2
F	Phenylalanine or Filamentous
FACS	Fluorescence activated cell sorter
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Gab1	Grb2-associated binder-1
GAP	GTPase activating protein
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
Grb2	Growth factor receptor bound protein 2

GST	Glutathione S transferase
GTP	Guanosine triphosphate
HB-EGF	Heparin binding epidermal growth factor
HRP	Horseradish peroxidase
IFN	Interferon
IP_3	Inositol 1, 4, 5-trisphosphate
kDa	Kilodalton
MAPK	Mitogen activated protein kinase
MCE	Mouse colon epithelial
MEK 1/2	MAPK/ERK kinase 1/2
N	Amino
NRG	Neuregulin
PAK	p21 activated kinase
PBD	p21 binding domain
PBS	Phosphate buffered saline
PE	Phycoerythrin
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC-γ	Phospholipase C gamma
PMA	Phorbol 12-myristate 13-acetate
PTB	Phosphotyrosine binding
PtdIns	Phosphatidylinositol

pY	Phosphotyrosine
RPMI	Roswell Park Memorial Institute
SH2	Src homology 2
SH3	Src homology 3
Shc	Src homology collagen
siRNA	Small interfering ribonucleic acid
Sos	Son of sevenless
SV40	Simian virus 40
TGF-α	Transforming growth factor alpha
TM	Transmembrane
Y	Tyrosine
YAMC	Young adult mouse colon

CHAPTER I

INTRODUCTION

Biology of the intestinal epithelium

In humans, the first line of defense against harmful pathogens is the innate immune response. The most fundamental function of innate immunity is to provide a physical barrier that protects internal organs from being exposed to toxic substances. For instance, the epithelial lining of the gastrointestinal tract is an important impenetrable barrier in animals that is constantly exposed to pathogenic bacteria and other potentially harmful substances. Maintenance of this barrier is critical for normal growth, development, and disease prevention. Unlike most tissues, the gastrointestinal tract has the ability to rapidly regenerate and repair damage. The epithelial lining of the gastrointestinal tract consists of a single layer of columnar epithelial cells that are replaced every 4-5 days in humans. This epithelial sheet forms a dynamic barrier that processes and absorbs nutrients from digested food while preventing ingestion of noxious substances present in the lumen of the gut. The small and large intestine, which differ in their surface morphology, make up the largest part of the gastrointestinal tract. The surface of the small intestine is covered with luminal protrusions, termed villi, and invaginations, termed crypts of Lieberkühn (Figure 1) (Reya and Clevers, 2005). The villi function to increase the absorptive surface area of the small intestine, as most nutrient absorption occurs in this region (Sancho et al., 2004). The large intestine, or

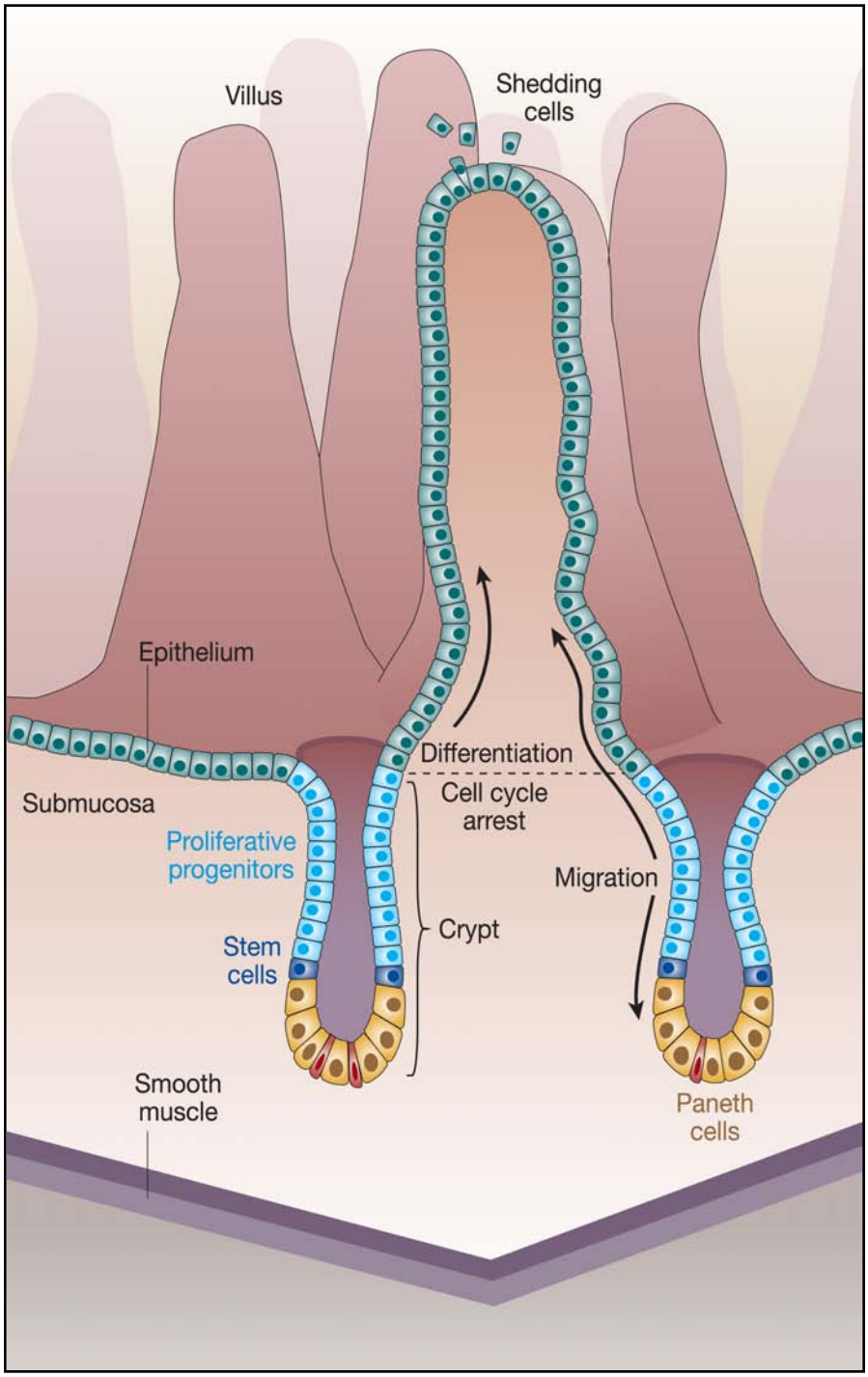

Figure 1. Tissue anatomy of the small intestine. Putative stem cells (dark blue) reside immediately above the Paneth cells (yellow) near the crypt bottom. Proliferating progenitor cells occupy the remainder of the crypt. Differentiated cells (green) populate the villus, and include goblet cells, enterocytes and enteroendocrine cells. From: Reya, T and Clevers, H. Nature. 2005. 434: 843-850.

colon, also contains crypts of Lieberkühn but has a flat surface epithelium devoid of villi (Figure 2A and 2B) (Reya and Clevers, 2005).

A delicate balance of proliferation, migration, and apoptosis is required for maintenance of a healthy gastrointestinal tract. Homeostasis is maintained as stem cells at the base of the crypts divide to give rise to proliferative progenitor cells (Figures 1 and Figure 2A) (Sancho et al., 2003). These cells rapidly divide and begin to migrate out of the crypts towards the tip of the villus where they will take the place of old cells that are sloughed off into the lumen. As these progenitor cells migrate up the crypts, they cease proliferation and commit to differentiate into one of three cell types: enteroendocrine, mucosecreting (Goblet), or absorptive (enterocyte) cells (Figures 1 and 2A). Enterocytes, the main absorptive cell in the intestine, are the most abundant cell type, accounting for approximately 80% of the intestinal epithelial cells (de Santa Barbara et al., 2003). Goblet cells are found scattered from the mid-crypt to the tip of the villus. These cells secrete mucus, which functions as an additional protective layer between the epithelium and the contents of the lumen (de Santa Barbara et al., 2003). In the small intestine, some cells migrate into the base of the crypt and differentiate into a fourth cell type, Paneth cells (Figure 1) (Sancho et al., 2003). After reaching the tip of the villus, the differentiated cells eventually undergo apoptosis and are shed into the lumen.

Our understanding of the processes that maintain homeostasis of the gastrointestinal tract is continually evolving. Defects in the regulation of proliferation, migration, or apoptosis result in a dysfunctional epithelium, which has been linked to the development of gastrointestinal disease and malignant transformation. One category of gastrointestinal disease under intense investigation is inflammatory bowel disease, which

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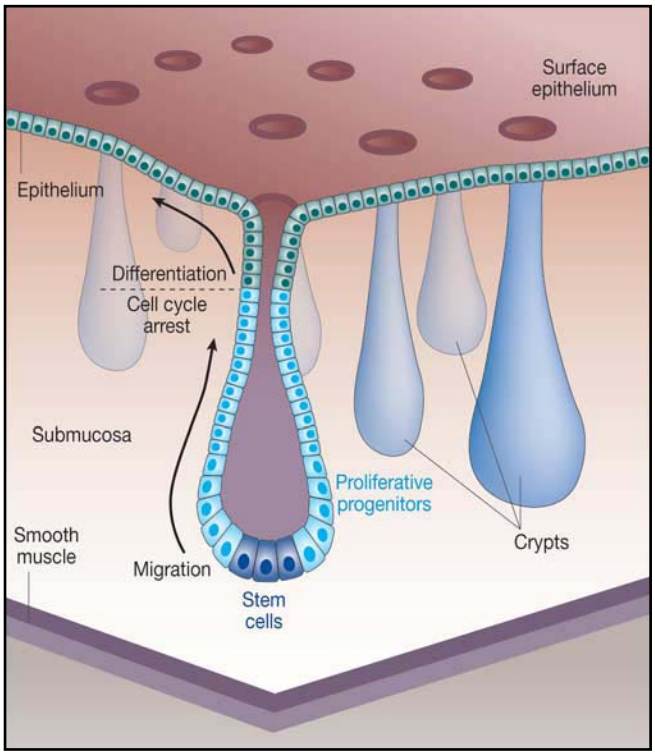

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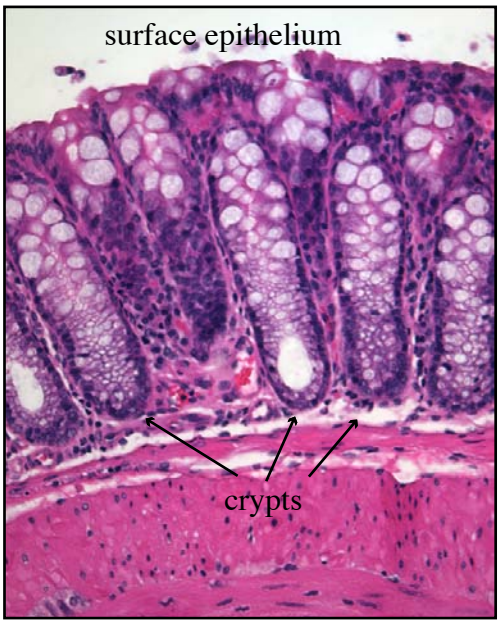


Figure 2. Tissue anatomy of the colonic epithelium. A. Putative stem cells (dark blue) reside at the crypt bottom. Proliferating progenitor cells occupy two-thirds of the crypt. Differentiated cells (green) populate the remainder of the crypt and the flat surface epithelium. From: Reya, T and Clevers, H. Nature. 2005. 434:843-850. B. Hematoxylin and eosin stain of colonic epithelium from an 8 week old SV129 mouse. Courtesy of Jeremy Goettel, 2006.

currently affects approximately 1 million people in North America (Bamias et al., 2005). Inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, are characterized by inflammation and epithelial cell damage in the intestine (Okamoto and Watanabe, 2005). The pathogenesis of these diseases is not completely understood, although a number of genetic and environmental factors have been suggested to contribute to disease progression. Inflammatory bowel diseases are thought to occur when the body mounts an inappropriate immune response to bacteria normally present in the lumen of the gut (Bamias et al., 2005). Alternatively, defects in the barrier function of intestinal epithelial cells have also been implicated in the pathology of these diseases by allowing bacteria to penetrate the gastrointestinal tract, resulting in an inflammatory immune response (Podolsky, 2002). Because the factors responsible for these diseases are not known, current therapeutic treatments include non-specific anti-inflammatory and immunosuppressive drugs to alleviate the inflammation. Characterization of the molecular mechanisms underlying inflammatory bowel diseases is critical for the development of more specific therapeutic treatments. As we learn more about the mechanisms that control the normal biological functions of these tissues, we also gain insight into how these pathways may be deregulated in diseases.

Wound healing in the intestine

Harmful substances present in the lumen of the gut continually challenge the integrity of the intestinal epithelium, sometimes resulting in cell damage (Figure 3A and 3B). In response to injury, intestinal epithelial cells initiate a wound repair process that can be divided into two distinct phases. Immediately following injury, epithelial cells

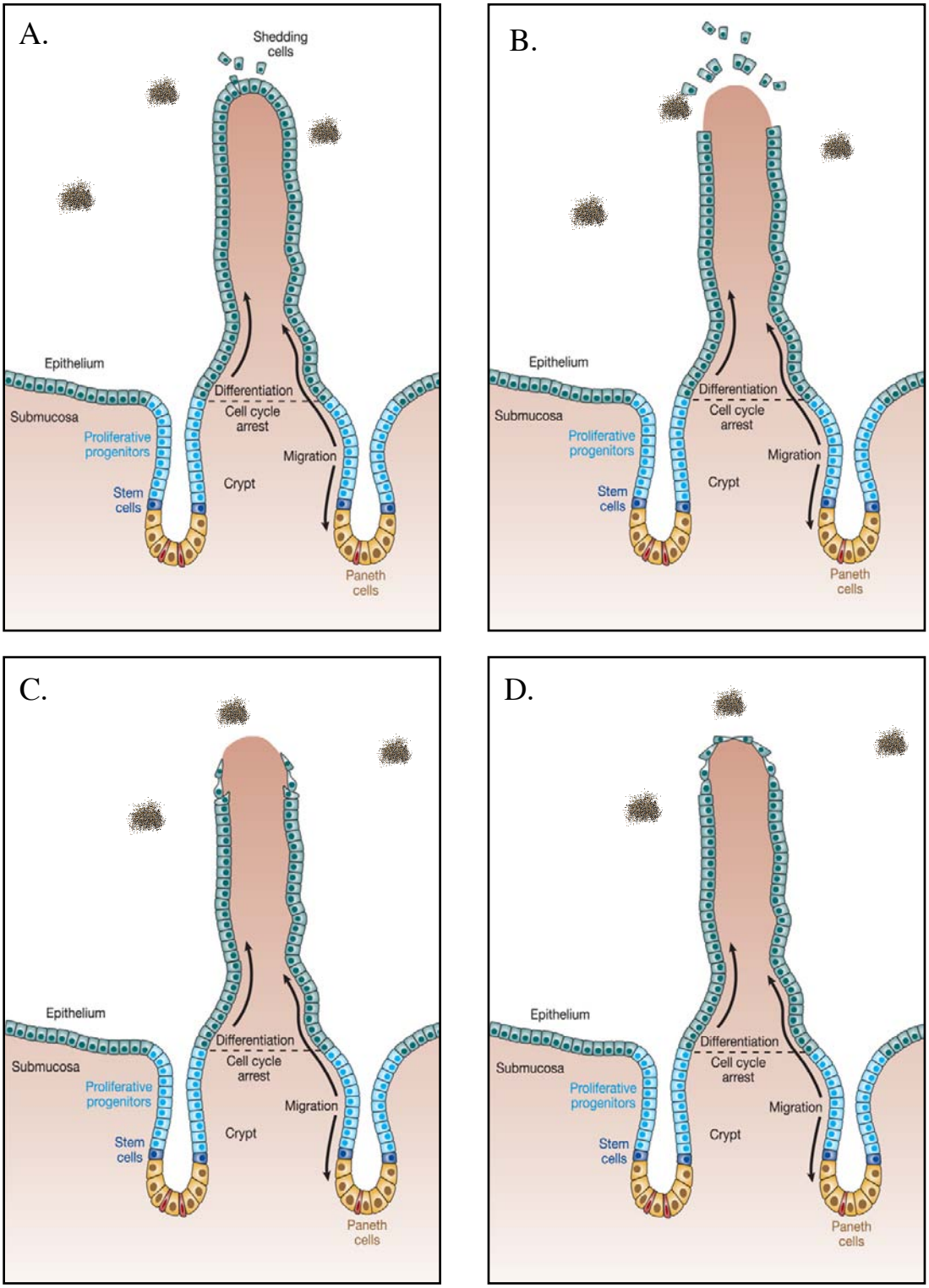


Figure 3. Restitution in the small intestine. A. Normal small intestinal epithelium. B. Damaged epithelial cells detach from the the tip of villus exposing the underlying basal lamina to the luminal contents. C. Epithelial cells surrounding the wound begin to migrate to cover the denuded area. D. Restitution is completed as epithelial cells close the wound, restoring the epithelium. Adapted from: Reya and Clevers, 2000.

surrounding the wound lose their polarity, taking on a flattened morphology, and rapidly migrate into the denuded area to restore the epithelial barrier (Figure 3C and 3D) (Feil et al., 1989) (Argenzio et al., 1988). This process, termed "restitution", has been shown to occur rapidly in *in vivo* models of injury, with complete re-epithelialization occurring as quickly as a few minutes after wounding, depending on the size of the injured area (Lacy, 1988). *In vivo* and *in vitro* models of intestinal injury have demonstrated that the first visible sign of restitution is the extension of lamellipodia over the denuded basal lamina (Feil et al., 1987) (Feil et al., 1989). In both the small and the large intestine, enterocytes were the only cell type observed migrating over the basal lamina, suggesting that only these cells contribute to the wound healing response (Lacy, 1988). After flattened epithelial cells completely cover the wound, these cells begin to regain their columnar shape and eventually return to polarized epithelial cells (Argenzio et al., 1988).

The second phase of wound healing, termed "regeneration", completes the process of wound repair (Okamoto and Watanabe, 2005). During this phase, proliferation is stimulated in crypts near the damaged mucosa to re-populate the epithelium. This process occurs over a much longer period of time as cells must undergo mitosis in the base of the crypts, followed by differentiation and migration up the crypt-villus axis (Okamoto and Watanabe, 2005). Alterations in either phase of the wound healing response are thought to contribute to chronic gastrointestinal diseases, such as inflammatory bowel disease (Bamias et al., 2005).

Rapid restitution is a critical part of maintaining a protective barrier in the gastrointestinal tract because slow or impaired restitution exposes the organism to harmful substances in the lumen. Much work has focused on trying to understand how

soluble factors present in the lumen contribute to restitution. A number of growth factors, including transforming growth factor α (TGF-α) and epidermal growth factor (EGF) (Riegler et al., 1996) (Wilson and Gibson, 1999), as well as trefoil factor peptides (Dignass et al., 1994) and transforming growth factor β (Dignass and Podolsky, 1993) accelerate restitution in *in vitro* models of intestinal injury. However, the molecular mechanisms by which these factors activate intestinal epithelial cell migration are poorly defined. Several of these factors are currently being tested in clinical trials as therapeutic treatments for inflammatory bowel diseases, of which the most promising so far is EGF. Administration of EGF-containing enemas to patients with ulcerative colitis in combination with the anti-inflammatory drug mesalamine induced remission in 10 out of 12 patients compared to only 1 out of 12 in patients receiving mesalamine only (Sinha et al., 2003). While larger studies are necessary to confirm the efficacy of EGF treatment for ulcerative colitis, this study demonstrates that data obtained from *in vitro* models of intestinal injury can be successfully applied to therapeutic treatments.

Cell migration and Rho GTPases

The process of cell migration is critical throughout the human life cycle (reviewed in (Horwitz and Webb, 2003)). In the adult, cell migration is required for homeostasis of the gastrointestinal tract as cells continually migrate up the crypt-villus axis. In humans, deregulated cell motility can cause pathologic conditions both when migratory cells become non-motile and when non-migratory cells aberrantly start to move. For example, when cancerous cells become migratory, they stray from the primary tumor, and can eventually move throughout the body to create secondary tumors. In contrast, defects in

intestinal epithelial cell migration during restitution contribute to an inability to heal ulcerations, perpetuating chronic intestinal diseases described above. Because it is a fundamental process throughout life, much work has focused on identifying the mechanisms that control cell migration.

The actin cytoskeleton consists of bundles and networks of actin filaments that provide the physical structure for cell shape and allow the capacity for cell migration through dynamic remodeling of actin filaments. Successful cell migration requires several phases of coordinated actin rearrangement. In response to an external migratory stimulus, a cell must first establish a direction of movement and then begin to polarize creating a front, termed the leading edge, and a rear, termed the trailing edge (Figure 4A) (reviewed in (Raftopoulou and Hall, 2004)). Actin polymerization and filament elongation at the leading edge create broad actin-rich membrane protrusions, called lamellipodia, which extend in the direction of movement. New sites of adhesion are then established to anchor the leading edge to the substratum (Figure 4B) (Ridley et al., 2003). Actin:myosin filament contractions at the rear of the cell move the cell body forward and cell adhesions at the rear are released, resulting in a net forward movement (Figure 4C).

The process of cell migration requires tight regulation of the actin cytoskeleton, which is controlled by members of the Rho family of small GTPases (reviewed in (Raftopoulou and Hall, 2004)). Rho GTPases are a distinct subfamily of the Ras family of small GTPases, named for their relatively small protein size of approximately 21 kDa. Currently 22 mammalian Rho GTPases have been identified and shown to regulate actin-dependent processes in all eukaryotic cells (reviewed in (Rossman et al., 2005) and (Jaffe and Hall, 2005)). Rho GTPases cycle between an active GTP-bound state and an inactive

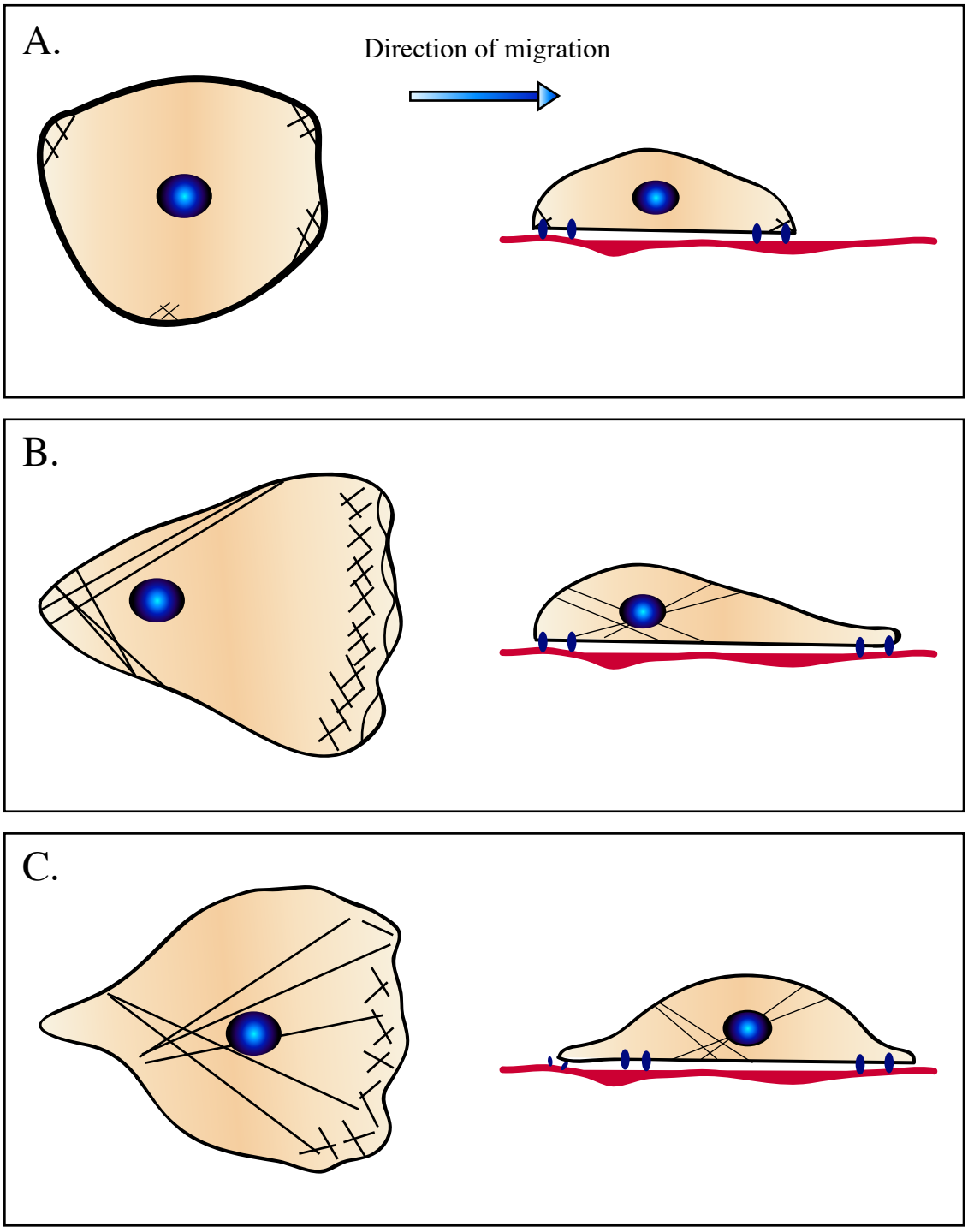


Figure 4. Cell migration. A. Upon sensing a migratory stimulus, the cell establishes a direction of migration. B. The cell extends lamellipodial protrusions in the direction of migration, forms new adhesions with the substratum, and stress fibers begin to form. C. Actin:myosin contraction pulls the cell body forward, adhesions at the rear release and the trailing edge begins to retract.

GDP-bound state (Figure 5). Three classes of regulatory proteins regulate this cycling both spatially and temporally. Guanine nucleotide dissociation inhibitors (GDIs) bind to and sequester inactive Rho GTPases in the cytoplasm. When stimulated, Rho-GTPases are released from the GDI, exposing a C-terminal prenylation, which translocates the small GTPases to the plasma membrane (Figure 5) (reviewed in (Seabra, 1998) and (Rossman et al., 2005)). Guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP to activate Rho GTPases at the plasma membrane. Active Rho GTPases initiate signaling through downstream effector proteins that specifically recognize and bind to the GTP-bound form of the GTPase. GTPase activating proteins (GAPs) stimulate the intrinsic GTPase activity to inactivate the GTPase, completing the cycle.

In an effort to identify a biological function for the Rho GTPases, work from the laboratory of Dr. Alan Hall in the 1990s revealed that three family members, Rac, Rho, and Cdc42, control individual elements of actin cytoskeletal rearrangement. Microinjection of constitutively active Rho into serum starved confluent Swiss 3T3 cells rapidly induced a dramatic reorganization of the actin cytoskeleton, activating the formation of dense microfilament networks, or stress fibers (Paterson et al., 1990). This study provided the first direct evidence that Rho is involved in regulating the organization of polymerized actin. Further experiments demonstrated that serum stimulation of quiescent cells induced the rapid formation of actin stress fibers, a phenotype similar to the injection of constitutively active Rho (Ridley and Hall, 1992). Treatment of cells with individual components of serum confirmed that growth factors alone produced similar effects. Further studies of the Rho family of GTPases revealed

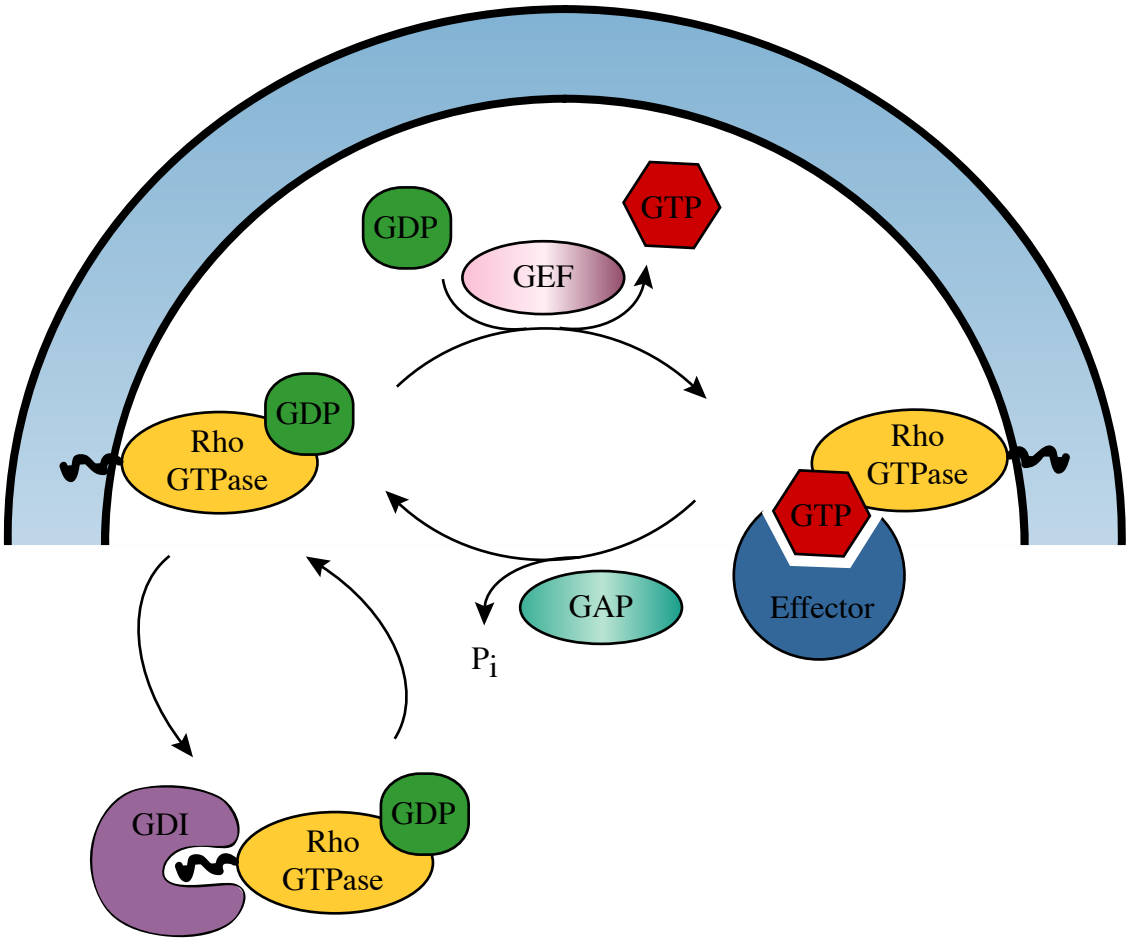


Figure 5. Regulation of Rho GTPases. Inactive GDP-bound Rho GTPases are sequestered in the cytoplasm by GDIs, which mask the c-terminal prenylation. Release of the GDI allows the GTPase to translocate to the plasma membrane. GEF activation catalyzes the exchange of GDP for GTP, activating the GTPase. The active GTPase binds to downstream effectors, which specifically recognize the GTP-bound form. GAPs stimulate intrinsic GTPase activity, inactivating the GTPase.

that microinjection of fibroblasts with wild type Rac induced an accumulation of actin-rich membrane ruffles at the cell periphery. This effect was enhanced by microinjection of constitutively active Rac and reversed by a Rac mutant in which a conserved threonine essential for activity was mutated to alanine (Ridley et al., 1992). Similar microinjection experiments with Cdc42 a few years later demonstrated that activation of Cdc42 results in the formation of spike-like filopodial extensions at the cell periphery (Nobes and Hall, 1995). An analysis of Rac, Rho, and Cdc42 activity in scrape-wound assays demonstrated that coordinated activation of these GTPases regulates processes required for cell migration. Cdc42 activity controls the extension of filopodia, which determines the direction of cell migration and is involved in the establishment of polarity. The extension of lamellipodia in the direction of movement is regulated by Rac activation, followed by contraction of the cell body mediated by Rho activation (Nobes and Hall, 1999). These studies established a basic paradigm for the control of cell migration by the Rho GTPases.

As rapid cell migration is critical for proper restitution in the gastrointestinal tract, the Rho GTPases are most likely required for proper intestinal epithelial wound healing. However, little is known about the requirement for Rho GTPases in restitution *in vivo*. Work in cultured intestinal epithelial cells has demonstrated that inhibition of Rho significantly reduces basal migration and completely blocks EGF induced migration in a scratch wound assay, suggesting that Rho may be involved in maintenance of the intestinal epithelium and restitution *in vivo* (Santos et al., 1997). While work in cultured fibroblasts has demonstrated that Rac is involved in wound closure, no work has been done in cultured intestinal epithelial cells to study the potential role of Rac in the process

of restitution. The Rac family consists of three different genes, Rac1, Rac2, and Rac3. While Rac1 is ubiquitously expressed, Rac2 is hematopoietic specific and Rac3 is expressed only in the nervous system. Rac1 deficient mice die at embryonic day 9.5 as a result of severe developmental defects (Sugihara et al., 1998). In contrast, mice with a targeted deletion of Rac2 or Rac3 are viable with no obvious phenotype. Because Rac1 null mice are not viable, a chimeric mouse expressing dominant negative Rac1 in the small intestine was generated to investigate the *in vivo* function of Rac1 in the intestinal epithelium. In these mice enterocyte migration along the crypt-villus axis is significantly reduced and epithelial cell differentiation is inhibited, demonstrating that Rac1 GTPase activity is required for intestinal epithelial homeostasis (Stappenbeck and Gordon, 2000). However, the signaling mechanisms that regulate the activation of Rac in the intestine have not been defined and more work is necessary to understand the role of this GTPase in intestinal epithelial cell migration.

ErbB family of receptor tyrosine kinases

Growth factor stimulation of cultured fibroblasts induces a dramatic rearrangement of the actin cytoskeleton (Ridley and Hall, 1992) and induces cell proliferation (Carpenter and Cohen, 1976). Soluble growth factors bind to receptor tyrosine kinases, which transmit signals from the extracellular milieu to the inside of the cells. Receptor tyrosine kinases are characterized by an extracellular ligand binding domain, a single pass transmembrane domain, and a cytoplasmic portion containing a conserved protein tyrosine kinase domain as well as multiple potential sites of tyrosine autophosphorylation (Schlessinger, 2000). In response to ligand binding, the receptors

dimerize and trans-autophosphorylate on tyrosine residues, which initiates downstream signaling cascades. The ErbB family of receptor tyrosine kinases consists of four closely related members: ErbB1, ErbB2, ErbB3, and ErbB4. The founding member of this family, ErbB1, better known as the epidermal growth factor receptor (EGFR), was identified by Dr. Stanley Cohen and co-workers in their search to identify the receptor that bound epidermal growth factor (EGF) (Cohen et al., 1982) (Buhrow et al., 1982). Soon after Cohen's discovery of the first receptor protein tyrosine kinase, EGFR was identified as the cellular homolog of the v-erbB oncogene and was subsequently designated ErbB1 (Downward et al., 1984b) (Ullrich et al., 1984).

ErbB receptor tyrosine kinases are activated by the EGF family of ligands, which consists of over 10 structurally related polypeptides, including several alternatively spliced variants. In response to ligand binding, ErbB receptors form homo- or heterodimers, depending on the ligand specificity and availability of other family members for dimerization (Figure 6A). EGF family ligands are characterized by an EGF-like domain that contains six spatially conserved cysteine residues (Groenen et al., 1994). These conserved cysteines form three disulfide-bonded intramolecular loops required for biological activity of these polypeptides (Kumar and Vadlamudi, 2000). EGF ligands are synthesized as active, transmembrane precursors, which can signal through a juxtacrine pathway (Massague and Pandiella, 1993). Cell-surface proteases cleave the extracellular domain to release a soluble form of the active ligand (Harris et al., 2003). Although the EGF family of ligands share considerable sequence identity in their EGF-like domains, ErbB receptor binding affinity is highly variable (Jones et al., 1999). EGF, transforming growth factor α (TGF-α), and amphiregulin (AR) bind specifically to ErbB1 (EGFR),

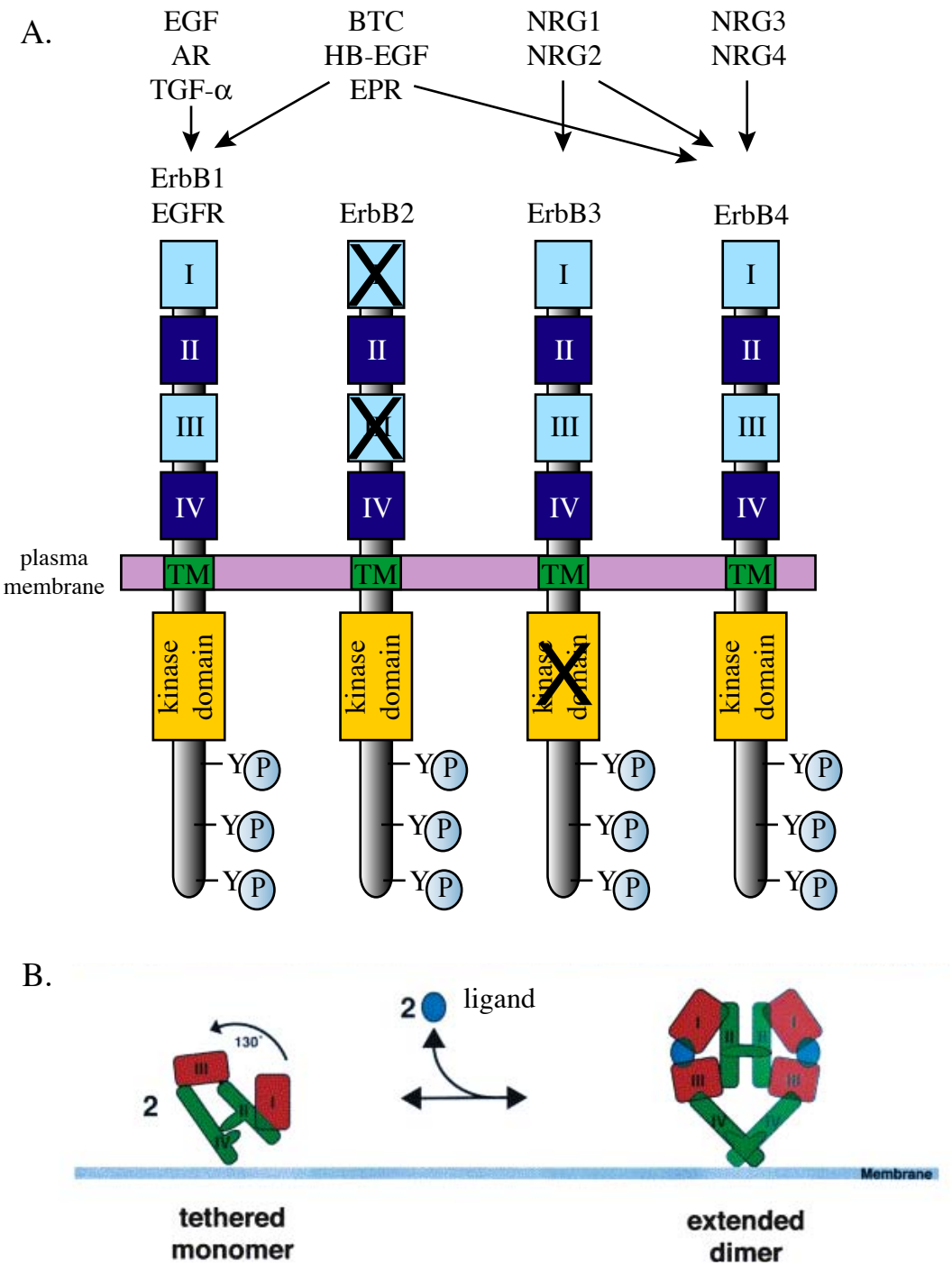


Figure 6. Structure of ErbB receptors and model of activation. A. Structure of ErbB receptors. Extracellular domains I-IV are indicated, followed by a short transmembrane domain (TM), the intracellular tyrosine kinase domain, and the c-terminal tail containing multiple phosphorylation sites. Binding specificity of the EGF family ligands is shown at the top. The inactive ligand binding domains of ErbB2 and inactive kinase domain of ErbB3 are marked with an X. B. Proposed model of ErbB activation. A 130^{o} rotation of the tethered monomer allows ligand binding and dimerization to occur. From: Burgess, A.W., *et. al.*, Molecular Cell. 2003. 12: 541-552.

while betacellulin (BTC), heparin-binding EGF (HB-EGF), and epiregulin (ER) bind to both ErbB1 and ErbB4 (Figure 6A) (Holbro and Hynes, 2004). The neuregulins (NRG) can be divided into two subclasses based on their binding affinity: NRG-1 and NRG-2 bind to ErbB3 and ErbB4, while NRG-3 and NRG-4 only bind ErbB4 (Figure 6A) (Holbro and Hynes, 2004). Investigation of EGF ligands has greatly expanded this field in the years since the identification of EGF, the founding member of this family (Cohen, 1962) (Cohen, 1964); however, no ligand has been identified for ErbB2, making this the only orphan receptor in the ErbB family.

Recent reports of the crystal structure of the ErbB receptors has shed light onto the mechanism of receptor activation, and the culmination of this work has led to a comprehensive model for ligand binding and dimerization (Figure 6B). Crystallographic studies have revealed that in the absence of ligand ErbB receptors exist in an inactive, monomeric state (reviewed in (Burgess et al., 2003). Intramolecular interactions between extracellular domains II and IV prevent the formation of a ligand binding pocket between domains I and III. In the presence of ligand, domains I and II rotate 130º, creating a high affinity binding pocket between domains I and III (Figure 6B). In the extended monomer, each receptor molecule binds to a single ligand molecule, stabilizing the active conformation. This conformational change exposes a dimerization arm, previously hidden by the domain II/IV tether that allows the receptor to participate in intermolecular interactions with other ErbB receptors (Burgess et al., 2003). While this model agrees with all of the available data for EGFR, ErbB3, and ErbB4, this paradigm does not apply to ErbB2. The crystal structure for ErbB2 has revealed that this receptor exists in an active conformation with an extended dimerization arm in the absence of ligand,

explaining why this receptor is the preferred dimerization partner for the other ErbB family members (Garrett et al., 2003).

While the four ErbB receptors share considerable sequence homology, their biological functions are not redundant. In transgenic mice, individual null mutations for any of the ErbB genes results in embryonic lethality, demonstrating that each of these receptors is required for mammalian development (reviewed in (Olayioye et al., 2000)). A closer look at the amino acid sequences of the ErbB receptors reveals several other differences that result in divergent biological functions. Variable residues in the extracellular domain of ErbB2 alter the 3-dimensional structure of this domain, making it unable to bind ligand. Three of the seven conserved residues involved in stabilizing the intramolecular tether in the other ErbB family members are different in ErbB2 (Garrett et al., 2003). While these amino acids substitutions allow the extracellular domain of ErbB2 to constitutively form an extended conformation, they also create a domain I/III pocket that mimics the presence of ligand, making it difficult for ligand to bind (Garrett et al., 2003). Furthermore, several amino acids required for ligand binding in the other ErbBs are not conserved in ErbB2 (Met 10, Arg13, and Pro15), further distinguishing the extracellular domain of ErbB2 from the conserved ectodomains of ErbB1, ErbB3, and ErbB4 (Roskoski, 2004a).

Although the kinase domain of ErbB3 is highly homologous to the other family members, kinase activity is impaired in this receptor (Guy et al., 1994). In ErbB3 the conserved protein tyrosine kinase catalytic loop amino acids HRDLAARN found in EGFR, ErbB2, and ErbB4 are altered to HR**N**LAARN (Plowman et al., 1990). This conserved aspartic acid forms the catalytic base required for phosphotransfer, and

substitution of this base with asparagine yields a catalytically inactive kinase. As a result, ErbB3 homodimers are unable to trans-autophosphorylate, forming an inactive dimer. Activation of ErbB3 occurs through heterodimerization with other ErbB family members.

The ErbB family of receptors and their ligands constitutes a highly complex signaling network with multiple input layers that regulates diverse biological outcomes from embryogenesis through adulthood (reviewed in (Yarden and Sliwkowski, 2001)). The availability and specificity of the numerous ligands provides the first layer of complexity, followed by the capacity of these receptors to form different combinations of homo- and heterodimers. Activation of downstream signaling pathways results from trans-autophosphorylation of the ErbB receptors on C-terminal tyrosine residues, which create docking sites for signaling proteins with Src-homology 2 (SH2) and phosphotyrosine-binding (PTB) domains. The amino acid sequences surrounding the C-terminal phosphorylated tyrosines create specificity for different signaling molecules (see below) (Songyang et al., 1993) (Uhlik et al., 2005). Some of these recruited proteins require phosphorylation by ErbB kinases for their activation, such as Shc (Pronk et al., 1994). The ErbB family contains both redundant and unique phosphotyrosine sequences in their C-terminus. For example, all of the ErbB receptors couple to the mitogen activated protein kinase (MAPK) cascade through Shc binding, while only ErbB3 and ErbB4 possess direct binding sites for the p85 subunit of phosphoinositide 3-kinase (PI3K) (reviewed in (Olayioye et al., 2000)).

Signaling through epidermal growth factor receptor

Initial studies by Dr. Stanley Cohen provided the first glimpse of EGFR biological activity by demonstrating that EGF stimulated increased levels of phosphotyrosine in A431 cells (Ushiro and Cohen, 1980). In the years since, much work has focused on identifying the signaling pathways that are activated by EGFR. Specificity for downstream signaling is determined by the tyrosine residues trans-autophosphorylated in the C-terminal tail, and while a clear requirement has emerged for several consensus tyrosines, the importance of other residues is still under investigation. Unlike platelet derived growth factor receptor, in which specific tyrosine residues are required for different downstream signaling pathways, the autophosphorylation sites of EGFR have redundant binding partners and can become compensatory in the absence of other sites, making mutational analyses difficult to interpret (Soler et al., 1994). This work is also complicated by the ability of this receptor to heterodimerize with the other ErbBs, making it difficult to establish which pathways are specific to activation of EGFR. A further complicating factor is that the Src family of tyrosine kinases has been shown to phosphorylate EGFR on conflicting tyrosine residues and the importance of these phosphorylation events is not yet known. However, treatment of cells with ligands that preferentially activate EGFR (EGF, amphiregulin, and TGF-α) has revealed a number of signaling molecules that are direct targets of EGFR activation. While a clear model for EGFR activation has recently emerged, the complex signaling pathways and functional consequences downstream of the receptor are still being elucidated.

Autophosphorylation sites

The identification of phosphorylated tyrosine residues and their corresponding binding partners has provided important insight into the mechanism by which EGFR elicits downstream biological effects. Initial phosphopeptide mapping studies of EGFR identified tyrosines 1068, 1148, and 1173 as *in vitro* sites of autophosphorylation, all of which are in the C-terminal tail (Downward et al., 1984a). *In vivo* labeling of A431 cells confirmed that these tyrosines were phosphorylated in response to EGF treatment, with tyrosine 1173 being the major site of tyrosine phosphorylation *in vivo* (Downward et al., 1984a). Further phosphopeptide mapping studies identified additional sites of phosphorylation at tyrosine 1086 and tyrosine 992 both *in vitro* and in intact cells (Margolis et al., 1989b) (Walton et al., 1990). Since the discovery of tyrosines 1068, 1148, and 1173 as the major sites of EGFR autophosphorylation and tyrosines 992 and 1086 as minor autophosphorylation sites, much work has focused on the identification of binding partners and signaling pathways downstream of receptor activation and this is still an ongoing area of investigation (Figure 7).

Activation of the mitogen activated protein kinase cascade

All ErbB receptors activate the mitogen activated protein kinase (MAPK) pathway either directly through Grb2 or indirectly through the adaptor protein Shc. In unstimulated cells, Grb2 constitutively associates with proline rich sequences in Sos, the guanine nucleotide exchange factor for Ras, through both of its SH3 domains (Li et al., 1993) (Egan et al., 1993). Upon EGF stimulation, the Grb2/Sos complex is recruited to EGFR through direct binding of the Grb2 SH2 domain to phosphorylated tyrosine

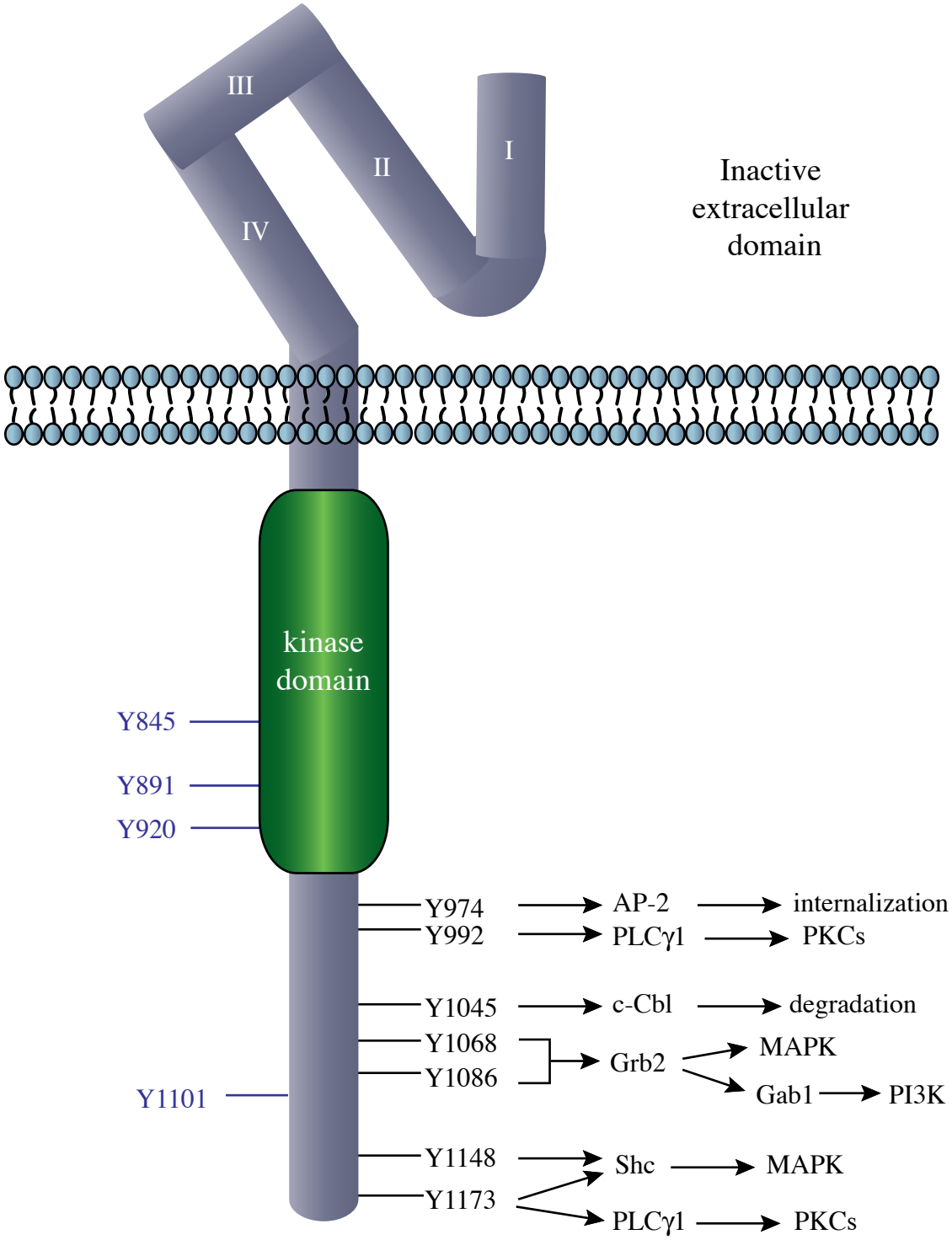


Figure 7. Structure of EGFR. The extracellular ligand binding domain is in an inactive conformation. The intracellular kinase domain (green) does not contain any auto-phosphorylation sites. Autophosphorylation sites (black), their binding partners, and downstream targets are shown. Src phosphorylation sites are shown to the left (blue).

residues on the receptor (Rozakis-Adcock et al., 1993). When recruited to the membrane via Grb2, Sos stimulates the exchange of GDP for GTP on Ras. Activated GTP-bound Ras initiates the MAPK cascade through binding and activating Raf kinase, which phosphorylates MEK1/2 on serine residues. MEK1/2, a dual specificity kinase, phosphorylates ERK1/2 on tyrosine and threonine residues, targeting it to the nucleus where it phosphorylates and activates a number of transcription factors involved in proliferation.

EGFR has been shown to activate the MAPK cascade through each of its major autophosphorylation sites. Tyrosine to phenylalanine (Y-F) mutational analysis of EGFR revealed that phosphorylation of tyrosines 1068 and 1086 creates high affinity binding sites for the SH2 domain of Grb2 (Okutani et al., 1994). Alternatively, EGFR can recruit Grb2 indirectly through the adaptor protein Shc. In response to growth factor stimulation, Shc binds to EGFR and is tyrosine phosphorylated (Pelicci et al., 1992). Grb2 binds to phosphorylated Shc proteins through its SH2 domain and this recruitment of Grb2/Sos activates the Ras/MAPK pathway (Rozakis-Adcock et al., 1992). EGFR Y-F mutational analysis demonstrated that Shc binds with high affinity to phosphotyrosine 1148, and to a lesser extent with phosphotyrosine 1173 (Okabayashi et al., 1994). Mutation of both of these tyrosines abolished most of the Shc binding, however, tyrosine 992 was found to exhibit some compensatory Shc binding in the absence of the other two tyrosines, suggesting that this is not a major site of Shc recruitment. Further analysis revealed that the PTB domain of Shc is required for its interaction with tyrosine 1148, and its SH2 domain mediates interaction with tyrosine 1773, which may explain the differences in binding affinity (Sakaguchi et al., 1998).

Activation of phosphoinositide 3-kinase

Another signaling pathway activated by all members of the ErbB family is phosphoinositide 3-kinase (PI3K). The PI3K superfamily is made up of a large family of enzymes that all phosphorylate the 3' hydroxyl group of phosphatidylinositol (PtdIns). However, they differ in their substrate specificity, mechanism of regulation, and subunit composition (reviewed in (Foster et al., 2003)). Class IA PI3Ks are heterodimeric enzymes that consist of a p85 regulatory subunit and a p110 catalytic subunit. The p85 subunit contains two SH2 domains, which have been shown to bind with high affinity to the sequence pYXXM (Songyang et al., 1993). The p110 subunit contains a stretch of 100 amino acids at its N-terminus that constitutively binds to the p85 subunit. *In vivo*, the class I PI3Ks selectively phosphorylate PtdIns 4,5-bisphosphate (PtdIns-4,5-P_2), but they are capable of phosphorylating PtdIns, PtdIns 4-phosphate (PtdIns-4-P), and PtdIns-4,5-P_2 *in vitro*.

Class IA PI3Ks are activated in response to growth factor stimulation through binding of p85 SH2 domains to phosphorylated receptors. Binding of the p85 subunit to phosphorylated tyrosines increases the catalytic activity of the p110 subunit 2-3 fold (Carpenter et al., 1993) and also positions PI3K in close proximity with its substrates in the plasma membrane. In resting cells, PtdIns-4-P and PtdIns-4,5-P_2 account for 90% of the phosphoinositide in the plasma membrane. Activation of PI3K generates a rapid and transient increase in the levels of PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 in the plasma membrane at the sites of PI3K recruitment. The generation of these lipid products recruits proteins with lipid binding domains, such as pleckstrin homology (PH) or FYVE domains, to the plasma membrane (Rameh and Cantley, 1999). One class of proteins that

contain a phospholipid binding domain are the guanine nucleotide exchange factors (GEFs) for the Rho family of GTPases. Over 60 mammalian GEFs have been identified, most of which contain a characteristic tandem PH domain and Dbl homology (DH) domain (Rossman et al., 2005). While the DH domain is critical for nucleotide exchange, the PH domain is thought to contribute to GEF activation by recruiting it to the plasma membrane in response to PI3K activity. However, other factors are required for full GEF activation as PH domain membrane recruitment is not sufficient for activation (Welch et al., 2003).

The best-characterized target downstream of PI3K is the serine/threonine kinase Akt. In response to PI3K activation, the N-terminal PH domain of Akt binds with high affinity to PtdIns-3,4-P_2, recruiting it to the plasma membrane (Franke et al., 1997). Another serine/threonine kinase, phosphoinositide-dependent kinase-1 (PDK-1), is also recruited to the membrane through binding of its PH domain to PtdIns-3,4,5-P_3. PDK1 is a constitutively active kinase that has been shown to phosphorylate Akt on threonine 308 in response to PI3K activation (Stephens et al., 1998). Full activation of Akt requires phosphorylation on threonine 308 by PDK1 and on serine 473 by an unknown kinase (Alessi et al., 1996). When activated, Akt promotes cell survival through a number of different pathways, including phosphorylation and inactivation of a number of pro-apoptotic proteins, such as BAD (reviewed in (Song et al., 2005)). Because a large number of proteins contain phospholipid binding domains, PI3K activation contributes to activation of many different signaling pathways in addition to cell survival.

Interestingly, activation of PI3K in response to EGF treatment has been reported despite the lack of p85 consensus binding sites in EGFR (Bjorge et al., 1990). Mutational

analysis of EGFR revealed that the SH2 domain of the p85 subunit of PI3K does not bind with high affinity to any of the autophosphorylation sites (Soler et al., 1994). An adaptor protein, called Grb2-associated binder-1 (Gab1), provided the missing link for the mechanism of PI3K activation by EGFR. Gab1 binds to the SH3 domain of Grb2, becomes tyrosine phosphorylated in response to EGF treatment, and contains three binding sites for the SH2 domains of p85 (YXXM motifs) (Holgado-Madruga et al., 1996). Gab1 association with EGFR requires tyrosines 1068 and 1086 (Rodrigues et al., 2000), as well as the SH3 domain of Grb2 (Lock et al., 2000), suggesting that Gab1 binds to EGFR indirectly through association with Grb2. A recent study demonstrates that EGF treatment does not stimulate Akt activation in Gab1 null fibroblasts, but expression of Gab1 in the same null cells restores EGF stimulated Akt activation (Mattoon et al., 2004). These studies suggest that both Grb2 and Gab1 are essential for activation of PI3K by EGFR; however, this paradigm is still under investigation.

Activation of phospholipase Cγ

Initial studies investigating the physiological substrates of EGFR discovered that phosphotyrosine proteins isolated from EGF treated A431 cells exhibited phospholipase C activity (Wahl et al., 1988). Phospholipase Cγ (PLCγ) was soon identified as a substrate for EGFR tyrosine kinase (Meisenhelder et al., 1989) (Margolis et al., 1989a). In response to EGFR activation, PLCγ1 binds to phosphorylated tyrosines 992 and 1173, with highest affinity to tyrosine 1173, through its SH2 domains (Rotin et al., 1992) (Chattopadhyay et al., 1999). Recruitment of PLCγ1 to the activated receptor results in its tyrosine phosphorylation, a requirement for its activation (reviewed in (Carpenter and

Ji, 1999)). Activated PLCγ1 hydrolyzes PtdIns-4,5-P_2 to generate the second messengers diacylglycerol and inositol trisphosphate (IP_3). Binding of IP_3 to receptors on the endoplasmic reticulum stimulates the release of calcium pools, resulting in increased intracellular calcium concentrations. Diacylglycerol, in combination with increased calcium levels, activates the protein kinase C family of serine threonine kinases, which in turn phosphorylate and activate numerous signaling proteins. The exact biological consequences of PLCγ activation in response to EGF have been difficult to identify because numerous calcium-dependent and PKC dependent pathways are turned on in response to diacylglycerol and IP_3 production. Previous work from our lab, as well as others, has demonstrated that phospholipase C (PLC) activity is required for EGF stimulated cell migration (Chen et al., 1994b) (Polk, 1998). However, the mechanism by which PLC contributes to EGF stimulated migration is still not clear. We have demonstrated that activation of PKCs downstream of PLC is required for EGF stimulated migration (Polk, 1998), while studies by a different group suggested that PLC activates the small GTPase Cdc42 to promote directed cell migration in response to EGF (Chou et al., 2003).

Receptor internalization and degradation

Increased activation or expression of EGFR has been detected in many different cancers and correlates with poor prognosis, suggesting that constitutive signaling through EGFR is deleterious to cells (reviewed in (Pal and Pegram, 2005). In untransformed cells, EGFR signaling is attenuated by the rapid endocytosis and subsequent degradation of this receptor in response to ligand binding (reviewed in (Wiley, 2003)). Unlike the

other ErbB receptors, which remain at the cell surface after ligand binding, EGFR is internalized through clathrin-coated pits within minutes of ligand stimulation and binds to the clathrin associated protein complex AP-2 (Baulida et al., 1996). Two internalization motifs, FYRAL at 973-977 and QQGFF at 996-100, were discovered in the C-terminal tail of EGFR that are not found in the other ErbBs (Chang et al., 1993). Mutation of tyrosine 974 in the FYRAL sequence to phenylalanine blocks AP-2 binding and slows ligand-stimulated internalization (Sorkin et al., 1996); however, phosphorylation of this tyrosine residue has not been demonstrated and is not required for AP-2 binding.

Activation of EGFR in response to ligand binding, but not the other ErbB family members, stimulates phosphorylation of the E3 ubiquitin ligase c-Cbl (Levkowitz et al., 1996). Cbl binds to phosphotyrosine 1045 of EGFR, a previously unidentified autophosphorylation site, stimulating EGFR ubiquitination and subsequent degradation (Levkowitz et al., 1999). The exact requirement for c-Cbl activity in EGFR trafficking is still under intense investigation as this study conflicts with a previous report in which a C-terminal truncation mutant (1-1022) lacking tyrosine 1045 did not exhibit impaired degradation in response to ligand (Wiley et al., 1991). Interestingly, a recent study demonstrated that EGFR mutations found in non-small-cell-lung cancers exhibited a specific increase in phosphorylation of tyrosine 1045 in response to EGF (Chen et al., 2005) suggesting a potential role for this autophosphorylation site and subsequent c-Cbl activation in carcinogenesis.

Activation of Src family kinases

EGFR activation stimulates increased tyrosine phosphorylation of a number of proteins, some of which are direct targets of EGFR kinase activity, and others which are substrates for the Src family kinases. These nonreceptor tyrosine kinases share a highly conserved structure consisting of an SH3 domain, followed by an SH2 domain, a kinase domain, and a C-terminal inhibitory tyrosine (Y527) (reviewed in (Roskoski, 2004b)). In the inactive conformation, the SH2 domain of Src binds to phosphorylated tyrosine 527, creating an intramolecular inhibition of its kinase activity. Dephosphorylation of tyrosine 527 relieves this inhibition and exposes another highly conserved residue in the activation loop of the kinase, tyrosine 416. Subsequent autophosphorylation of tyrosine 416 greatly enhances Src kinase activity (reviewed in (Roskoski, 2005)). Alternatively, Src can be activated when phosphorylated tyrosine residues on other proteins bind to its SH2 domain, displacing the intramolecular inhibition and opening the conformation of the kinase. Unlike the receptor tyrosine kinases, which are anchored in the plasma membrane, the Src family kinases are targeted to the membrane by an N-terminal myristoylation sequence, positioning these kinases in close proximity with transmembrane receptors. Src family kinases are involved in signal transduction downstream of a broad range of cell surface receptors, including integrins, G-protein coupled receptors, and growth factor receptors. The Src family consists of 11 members in humans, three of which, Src, Yes and Fyn, are ubiquitously expressed. The remaining family members exhibit a more restricted pattern of expression, many of which are specific to hematopoietic cells. The characteristic functional domains and regulatory

properties of the Src family kinases are conserved in many other tyrosine kinases, making Src a prototype for the study of other tyrosine kinases.

Overexpression of both Src and EGFR has been reported in several different human tumors, suggesting that these kinases synergize to promote tumor formation (reviewed in (Biscardi et al., 1999b)). Despite intense investigation, the exact nature of the relationship between these two kinases is unclear. In cells engineered to overexpress both EGFR and Src at very high levels, two novel EGFR phosphopeptides were observed, which were predicted to be sites of Src phosphorylation (Maa et al., 1995). Phosphopeptide mapping identified tyrosines 845 and 1101 as the residues phosphorylated *in vitro* and *in vivo* in response to EGF in these cells, but these sites could not be detected in cells expressing endogenous levels of these kinases (Biscardi et al., 1999a). Tyrosines 891 and 920 were also identified as sites of Src phosphorylation (Stover et al., 1995). Phosphorylation of tyrosine 920 creates a high affinity binding site for the SH2 domain of p85, and both tyrosine 891 and 920 are predicted to bind to the SH2 domain of Src with high affinity (Stover et al., 1995). However, several other labs have not detected any of these sites in their phosphopeptide mapping studies. While the reason for this discrepancy is unclear, one possibility is that cell type specificity determines the requirement for Src phosphorylation. *In vivo* phosphopeptide mapping studies identifying the autophosphorylation sites were generated either from A431 cells, a human epidermoid carcinoma cell line that overexpresses EGFR to very high levels (Downward et al., 1984a) (Hsuan et al., 1989), or cells transfected with human EGFR (Margolis et al., 1989b) (Walton et al., 1990). In these cell lines, autophosphorylation may be favored due to the low availability of endogenous Src molecules per receptor.

The studies of Stover, *et. al.,* which identified tyrosines 891 and 920 as *in vivo* Src phosphorylation sites were performed in breast and colorectal cancer cell lines, which may overexpress both Src and EGFR family members as a result of transformation, favoring Src phosphorylation sites on EGFR. Phosphorylation of tyrosines 845 and 1101 were also identified only in cells overexpressing both Src and EGFR, further suggesting that these phosphorylation events do not occur in untransformed cells. Further studies are necessary to determine the importance of these sites.

Initial sequencing of EGFR revealed that the predicted intracellular domain exhibited a high degree of conservation with the Src family of tyrosine kinases, including the highly conserved tyrosine residue required for Src kinase activation (tyrosine 845 of human EGFR) (Ullrich et al., 1984). Based on the importance of this residue in the activation of other kinases, phosphorylation of EGFR by Src at tyrosine 845 in the activation loop was proposed to be an important regulatory event for EGFR kinase activity (Biscardi et al., 1999a). In the absence of ligand, most receptor tyrosine kinases adopt an inactive conformation in which the activation loop of the kinase domain inhibits substrate binding (Huse and Kuriyan, 2002). Ligand-induced receptor dimerization results in autophosphorylation of a highly conserved tyrosine residue in the activation loop, relieving the inhibition and creating an optimal conformation for substrate binding and ATP transfer (Hubbard and Till, 2000). However, unlike all other receptor tyrosine kinases studied, the crystal structure of the inactive EGFR kinase domain reveals that the activation loop exists in an active conformation (Stamos et al., 2002). In fact, the conformation of the unphosphorylated EGFR kinase domain is identical to the structure of the insulin growth factor receptor with its activation loop tyrosine phosphorylated.

These studies suggest that EGFR exists as a constitutively active kinase regulated by ligand binding and dimerization. Furthermore, these data are consistent with the observation that tyrosine 845 phosphorylation is not required for EGFR activation (Gotoh et al., 1992) and the inability to detect endogenous phosphorylation at this residue in response to EGF.

While the importance of EGFR phosphorylation by Src remains an open question, activation of Src downstream of EGFR has been documented. Src family kinases are activated rapidly in response to EGF treatment and the level of Src family kinase activation is dependent on the expression level of EGFR, suggesting a direct activation by the receptor (Osherov and Levitzki, 1994). Overexpression of Src stimulates a 2-5 fold increase in EGF induced DNA synthesis (Luttrell et al., 1988) and inhibition of Src activity by microinjection of antibodies blocks EGF stimulated DNA synthesis (Roche et al., 1995), demonstrating that Src is involved in mitogenic pathways downstream of EGFR. However, as both Src and EGFR are tyrosine kinases, it has been difficult to identify specific substrates of Src phosphorylation in response EGF. While it is generally accepted that Src family kinases are activated downstream of EGFR, the mechanism of their activation and their role in downstream signaling is not understood. Further analysis of the role of Src activation in normal signaling through EGFR is required to understand more about the signaling pathways that may be deregulated by these kinases in cancer.

Summary

The ErbB family of receptors and their ligands constitutes a highly complex signaling network that regulates diverse biological outcomes. In particular, the ErbB

family member EGFR has been implicated in the control of intestinal epithelial cell migration required for proper restitution. Our lab, as well as others, has demonstrated that EGFR ligands stimulate increased intestinal cell migration *in vitro* and clinical trials have confirmed that EGF may play a role in intestinal wound healing *in vivo*. The goal of these studies was to investigate the molecular mechanism by which EGF treatment of intestinal epithelial cells accelerates cell migration. I have found that EGF stimulates a rapid and robust activation of the small GTPase Rac1, which requires the kinase activity of EGFR (Chapter III). Further studies demonstrate that synergistic activation of PI3K and Src downstream of EGFR are required for EGF stimulated Rac activation (Chapter IV). These data provide important insights into the mechanisms that regulate intestinal epithelial cell restitution with implications for potential therapeutic intervention.

CHAPTER II

MATERIALS AND METHODS

Cell Culture

The conditionally immortalized young adult mouse colon (YAMC) epithelial cell line isolated from the colonic epithelium of an H-2K^{b}-tsA58 (Immorto) mouse has been previously described (Whitehead et al., 1993). YAMC cells express a temperature-sensitive mutation of SV40 large T antigen under the control of an interferon γ inducible promoter. EGFR-null mouse colon epithelial (EGFR$^{-/-}$MCE) cells were isolated from the colonic epithelium of a homozygous EGFR-null Immorto mouse (Threadgill et al., 1995) (Whitehead et al., 1993). Cells were maintained on rat tail collagen (Mediatech, Herndon, VA) coated plates (5μg/cm^{2}) in complete media (RPMI 1640 supplemented with 5% FBS, 100 U/ml penicillin and streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenous acid (BD Biosciences, San Jose, CA)) with the fresh addition of 5 U/ml mouse interferon gamma (IFN-γ) (Intergen, Norcross, GA) at 33°C (permissive conditions). Prior to all experiments cells were washed twice in warm PBS and then incubated for 16-18 hours in starve media (RPMI 1640 containing 0.5% FBS and 100U/ml penicillin and streptomycin) at 37°C (nonpermissive conditions).

Antibodies, growth factors, and pharmacological inhibitors

FITC-conjugated goat anti-mouse secondary antibody was from Jackson ImmunoResearch Labs (West Grove, PA). Alexa Fluor 594 Phalloidin was purchased

from Molecular Probes (Eugene, OR). Mouse monoclonal Rac, Src, and rabbit polyclonal EGFR antibodies were from Upstate (Charlottesville, VA). Mouse monoclonal EGFR antibody conjugated to phycoerythrin, EGFR (528) PE, (Santa Cruz Biotechnology, Santa Cruz, CA) was used for FACS analysis. Mouse monoclonal HA-Tag (262K), rabbit monoclonal phospho-EGFR (Tyr1173), rabbit polyclonal phospho-Akt (Ser 473), phospho-FAK (Tyr925), ERK 1/2, Akt antibodies, and horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody were from Cell Signaling Technology (Beverly, MA). Anti-active ERK 1/2 polyclonal antibody was from Promega Corp. (Madison, WI). Mouse monoclonal actin antibody was from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal Cdc42, Fyn, and HRP-conjugated PY20 antibodies, and HRP-conjugated goat anti-mouse IgG were from BD Transduction Labs (San Diego, CA). Rabbit polyclonal ErbB2, PKCα, PKCδ, and PKCε antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human EGF was a gift from Stanley Cohen (Vanderbilt University, Nashville, TN). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma (St. Louis, MO). Rac1 inhibitor NSC23766, MEK 1/2 inhibitor U0126, PI3K inhibitor wortmannin, and PKC inhibitors Bisindolylmaleimide 1 (BIM-1) and Go6976 were from Calbiochem (San Diego, CA). MEK 1/2 inhibitor PD98059 and phosphoinositide 3-kinase inhibitor LY294002 were from Cell Signaling Technology (Beverly, MA). Src inhibitors PP1 and PP2, and phospholipase C inhibitors U73122, D609, and L108, were from Biomol (Plymouth Meeting, PA). Src inhibitor CGP77675 was a gift from Anna Suter (Novartis, Basel, Switzerland).

Transient transfections

Prior to transfection, cells were trypsinized, resuspended in complete media without penicillin and streptomycin, and then counted using a hemacytometer. Cells were seeded in the appropriate plates as follows: 2.5 x 10^5 cells per 35mm plate, 5 x 10^5 cells per 60mm plate, 1.5 x10^6 cells per 100mm plate. The cells were grown overnight at 33ºC in the presence of IFN-γ. The next morning, the cells were transfected with vector control, dominant negative Akt1, or dominant negative Akt2 by Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, using exactly one half of the recommended concentrations of DNA and Lipofectamine 2000. 32 hours after transfection the cells were washed twice with warm PBS, and then starve media was placed on the cells. The cells were serum starved for 16-18 hours at 37ºC. Dominant negative Akt constructs were a gift from Dr. Joseph Testa.

Generation of stable cells

Tyrosine to phenylalanine (Y-F) EGFR mutations were generated from pcDNA3.1/Zeo(-)/EGFR (human) using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). $EGFR^{-/-}$MCE cells were stably transfected with pcDNA3.1/Zeo(-) empty vector (Invitrogen, Carlsbad, CA), pcDNA3.1/Zeo(-)/wild type EGFR, pcDNA3.1/Zeo(-)/kinase dead (K721R) EGFR, or pcDNA3.1/Zeo(-)/Y-F EGFR mutants using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described above for transient transfections. 48 hours after transfection 200μg/ml Zeocin (Invitrogen, Carlsbad, CA) was added to the cells to begin the stable cell selection. The cells were grown in complete media with 200μg/ml Zeocin until the cells reached confluence, and

then the cells were expanded. Zeocin selected pools of cells were trypsinized, suspended in 5% FBS/RPMI 1640, and then spun at 1500 rpm for 5 minutes. The cells were washed twice in 5% FBS/PBS and then resuspended in 5% FBS/PBS at 5 x 10^6 cells/ml. 50μl of anti-EGFR 528-PE antibody was added per ml of cell suspension. The cells were incubated on ice for 30 minutes, washed in PBS, and then resuspended in PBS at 5 x 10^6 cells/ml. EGFR positive cells were sorted at the Vanderbilt VAMC Flow Cytometry Special Resource Center using the Beckton Dickinson FACSAria. Rounds of cell sorting were repeated until a pure population of cells was obtained. Stable pools of cells expressing EGFR were maintained in 100μg/ml Zeocin.

siRNA experiments

Smart pools of small interfering RNA (siRNA) directed against murine Rac1, Src, and Fyn were purchased from Dharmacon (Lafayette, CO). Non-targeting smart pool siRNA was purchased from Upstate (Charlottesville, VA). One day prior to transfection, cells were plated in 35mm or 60mm plates as described above for transient transfections. The media was changed to Opti-MEM (Invitrogen, Carlsbad, CA) immediately before transfection and the cells were transfected with Lipofectamine 2000 according to the manufacturer's protocol for siRNA transfection. The cells were incubated overnight (16-18 hours) at 33ºC in Opti-MEM containing the DNA/lipid complexes. The next morning, the transfection media was removed and fresh growth media was added to the cells. For Rac activation assays, the cells were grown for 48 hours after transfection, serum starved overnight at 37ºC, and lysed the following morning. For the migration assays, the cells

were grown for 24 hours after transfection, the cells were serum starved overnight at 37ºC, and then wounded the following morning.

Cell lysis, SDS-PAGE, and Western blot analysis

Serum starved cells were treated with 10ng/ml EGF at 37ºC for the times indicated. For inhibitor studies, cells were treated with inhibitor or an equal volume of vehicle for 45 minutes at 37ºC prior to EGF stimulation. Immediately after treatment, cells were placed on ice, washed twice with cold PBS, and then lysed in Rac lysis buffer (50mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 200mM NaCl, 1% (v/v) Nonidet P-40, 5% (v/v) glycerol) with freshly added protease and phosphatase 1 and 2 inhibitor cocktails (Sigma, St. Louis, MO). The lysates were cleared by centrifugation and the protein concentration was determined with the DC protein assay (Bio-Rad Laboratories, Hercules, CA). An equal volume of 2X sample buffer was added to each cell lysate and the lysates were heated at 95ºC for 5 minutes. Equal protein amounts of each sample were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked in 5% (w/v) nonfat dry milk in Tris buffered saline (50mM Tris, 138mM NaCl, 2.7mM KCl, pH 8.0) with 0.05% Tween 20 (TBST) for 1 hour. The membranes were incubated in primary antibody at the appropriate dilution in TBST at 4ºC overnight. The blots were washed in TBST 6 x 5 minutes and then incubated in HRP conjugated secondary antibody diluted in TBST (1:5000 for anti-rabbit secondary or 1:10,000 for anti-mouse secondary antibody) for 1 hour at room temperature. The blots were washed in TBST 6 x 5 minutes. Bound HRP

was detected using Western Lightning enhanced chemiluminescence kit (Perkin-Elmer, Boston, MA) and the blots were exposed to film.

Rac activation assays

Rac activation was assayed as described (Benard and Bokoch, 2002). Briefly, the p21-binding domain (PBD) of PAK fused to GST was expressed from pGEX-PBD, a gift from Josephine Adams (University College of London, London). Recombinant GST-PBD was purified with glutathione-Sepharose 4B beads (Pharmacia, Piscataway, NJ) and stored at –80ºC in a solution of 10% glycerol. Immediately after treatment, cells were lysed in Rac lysis buffer and the protein concentration determined, as described above. Equal amounts of each total lysate (500μg-1000μg) were nutated with GST-PBD beads at 4ºC for 1 hour. The beads were collected by centrifugation, washed 3 times with washing buffer (25mM Tris-HCl (pH7.6), 1mM DTT, 30mM $MgCl_2$, 40mM NaCl, 1% (v/v) Nonidet P-40), and twice with washing buffer without Nonidet P-40. The beads were suspended in 2X SDS gel loading buffer, heated at 95ºC for 5 minutes, separated on a 15% SDS-polyacrylamide gel and blotted for Rac.

Migration assays

Cells were trypsinized, resuspended in complete media, and counted using a hemacytometer. 5×10^5 cells were seeded onto each 35mm plate coated with 2.5μg of human fibronectin (BD Bioscience, Bedford, MA) and the cells were incubated at 33ºC overnight. The next day the cells were washed twice with PBS, and then starve media was placed on the cells. The cells were incubated in starve media at 37ºC for 16-18

hours. Eight circular wounds were made in each confluent plate of cells using a rotating silicon tip. The cells were washed twice with PBS to remove the cell debris, and then fresh starve media with or without 10ng/ml EGF was placed on the cells. For migration assays using inhibitors, the cells were pre-treated with the inhibitor for 45 minutes prior to wounding, and then starve media containing fresh inhibitor was added with or without EGF after wounding. The wounds were photographed at 0 and 8 hours after wounding and the size of each wound at 0 and hours was determined in arbitrary units using ImageJ software (NIH, Bethesda, MD). The data for each wound was entered into an Excel spreadsheet and the percent wound closure is calculated using the following equation:

(0 hour wound size – 8 hour wound size) / 0 hour wound size x 100 = % closed

The average wound closure rates and the standard deviation for all eight wounds were calculated and displayed graphically. Statistical differences in wound closure rates between control and EGF treated wound closure rates were determined by a paired t-test.

Immunofluorescence

YAMC cells were trypsinized, resuspended in complete media, and counted using a hemacytometer. 4 x 10^4 cells were seeded into each well of a fibronectin coated glass chamber slide and the cells were incubated at permissive conditions for 24 hours. Cells were serum starved at nonpermissive conditions for 16-18 hours and then circular wounds were made as described above. After wounding, the cells were washed twice with PBS, and then treated with or without 10ng/ml EGF in fresh starve media for 5 or 10 minutes. Cells were fixed in 4% paraformaldehyde/PBS and permeabilized with 0.4% Triton-X-100 in PBS. The cells were then stained with a monoclonal Rac antibody (1:50)

followed by anti-mouse FITC secondary and then Alexa-594-phalloidin (3.3 nM). Coverslips were mounted using the Prolong Antifade Kit (Molecular Probes), and imaged on a Zeiss Axiovert 200 microscope.

Immunoprecipitation

YAMC, EGFR$^{-/-}$ MCE vector control, wild type EGFR, and kinase dead EGFR stable cells were grown to confluence in 100mm plates, serum starved overnight at 37ºC, and treated with or without 10 ng/ml EGF for 5 minutes. The cells were lysed in Rac lysis buffer and the protein concentration was determined for each sample. 2µg of ErbB2 polyclonal antibody was added to 600µg of protein for each sample. 5µl of polyclonal EGFR (986) rabbit sera was added to 600µg of protein for each sample. The samples were nutated for 2 hours at 4ºC and then 35µl of a 50:50 slurry of Protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA) was added to each sample. The samples were nutated for 1 hour with the beads and then the beads were washed three times with 500µl of lysis buffer. 100µl of 2X sample was added to the beads and the samples were heated at 95ºC for 5 minutes. The immunoprecipitations were subjected to Western blot analysis for EGFR, ErbB2, or phosphotyrosine.

Statistical analysis

Statistical significance between control and EGF treated migration rates were determined in Excel using a paired two-sample student's t-test with a confidence level of 0.05. All data presented are representative of at least three repeat experiments.

CHAPTER III

EPIDERMAL GROWTH FACTOR ACTIVATES THE SMALL GTPASE RAC1 TO STIMULATE INTESTINAL EPITHELIAL CELL MIGRATION

Introduction

Regulated intestinal epithelial cell migration is critical for maintenance of a healthy gastrointestinal tract. In response to damage, intestinal epithelial cells must rapidly migrate to close the wounded area and restore the integrity of the epithelium. Previous work from our lab and others has demonstrated that epidermal growth factor (EGF) treatment stimulates a migratory response from cells at the wound margin. However, the mechanism by which EGF elicits this effect is not understood. Recently, a role for EGF in wound healing *in vivo* was demonstrated in a clinical trial in which administration of EGF-containing enemas to patients with ulcerative colitis induced remission in 10 out of 12 patients (Sinha et al., 2003). In an effort to elucidate the molecular mechanisms involved in growth factor stimulated wound closure, I have investigated the role of the small GTPase Rac in EGF mediated cell migration. Using a biochemical approach as well as a cell culture model of wound healing, I demonstrate that EGFR mediates activation of Rac in EGF stimulated intestinal epithelial cells. These studies provide insight into the role of EGFR in normal intestinal epithelial cell migration and expand our knowledge of the pathways that may be deregulated in chronic gastrointestinal disorders.

Results

Rac is required for EGF stimulated migration

We have previously reported that young adult mouse colon (YAMC) epithelial cells exhibit increased rates of wound closure in response to EGF treatment in a wound closure assay that mimics ulceration *in vivo* (Corredor et al., 2003) (Frey et al., 2004). In this assay, circular wounds are made in a confluent monolayer of serum starved cells with a rotating silicon tip and the cells are treated with or without 10 ng/ml EGF. Phase contrast images of the wounds are taken at 0 and 8 hours after wounding (Figure 8A). The cell-free area of the wound is measured at these time points and the average percent wound closure for 8 wounds in each treatment condition is calculated and represented graphically (Figure 8B). This assay mimics the process of restitution *in vivo* by creating a wound in the epithelial monolayer similar to ulceration *in vivo*. In response to the damage, cells at the wound margin migrate over the denuded area in an attempt to reduce the size of the wound and restore the integrity of the epithelial layer (Figure 8A, compare 0 hour wounds to 8 hours wounds), similar to the restitution phase *in vivo*. We have previously demonstrated that proliferation does not contribute to the wound healing process observed in our assay (Corredor et al., 2003). In fact, the rate of cell proliferation around the wounded area decreases in response to EGF treatment, supporting the idea that, similar to the phases of restitution and regeneration observed *in vivo*, proliferation is not a part of the immediate response to damage. Therefore, we can conclude that our calculation of the percent wound closure is a direct measure of the rate of cell migration. Unlike the commonly used scrape wound assay of cell migration, which produces linear wounds of variable size, this assay provides a very consistent measure of cell migration

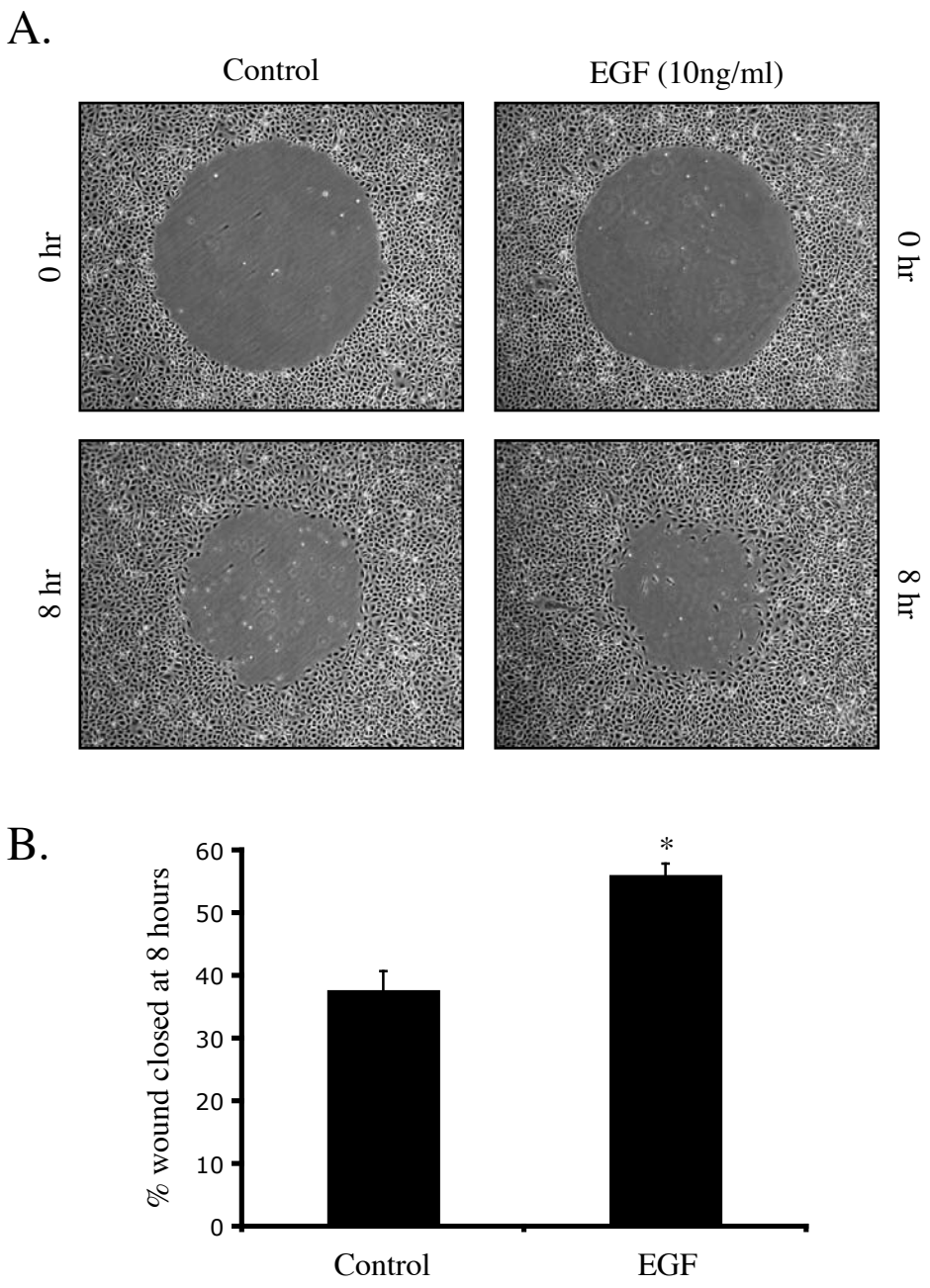


Figure 8. Wound closure assay. Circular wounds were made in confluent monolayers of serum starved YAMC cells, cells were washed twice with PBS, and then treated in the presence or absence of 10 ng/ml EGF. The wounds were photographed at 0 and 8 hours. Representative wounds are shown for control and EGF treated cells at 0 and 8 hours (A). The cell-free area was measured for each wound at 0 and 8 hours and the percent wound closure was calculated. The average wound closure for 8 control wounds (37.78% +/- 2.97) and 8 EGF treated wounds (56.13% +/- 1.77) from the experiment shown in A was calculated and graphed +/- the standard deviation (B). $*$ p value < 0.001

because the initial size of the wounds is similar within the 8 wounds of each treatment condition and between the treated and untreated cells (Figure 8A). Because EGF consistently stimulates a 1.5 fold increase in wound closure (56% closed with EGF vs. 38% closed in control) (Figure 8B), this assay is amenable to the use of pharmacological inhibitors and other perturbations of cell signaling to study the pathways involved in EGF stimulated migration.

As lamellipodia formation is the first visible sign of wound closure in both *in vitro* and *in vivo* models of wounding (Lacy, 1988), I hypothesized that EGF stimulates increased lamellipodia formation at the wound margin to promote cell migration. While the requirement for activation of the small GTPase Rac in lamellipodia formation has been well documented (Ridley et al., 1992), the signals that activate this GTPase are still under investigation. When activated, GTP-bound Rac translocates to the plasma membrane through its C-terminal prenylation, distinguishing it from the inactive, cytoplasmic Rac in the cell. To determine if EGF stimulates Rac membrane translocation, Rac was immunolocalized in wounded YAMC cells treated in the presence or absence of 10 ng/ml EGF for 10 minutes to visualize the cellular distribution of Rac. The cells were co-stained with fluorescent-conjugated phalloidin, a toxin that binds to F-actin, to visualize the actin cytoskeleton. While both control and EGF treated cells contain cortical actin at the cell periphery, only YAMC cells treated with EGF exhibited prominent Rac staining in membrane ruffles at the wound margin (Figure 9A, see arrows). To quantify this, circular wounds were made in confluent monolayers of YAMC cells treated with or without EGF for 10 minutes and the cells were visualized by phase contrast microscopy. To determine the percentage of cells with lamellipodia at the

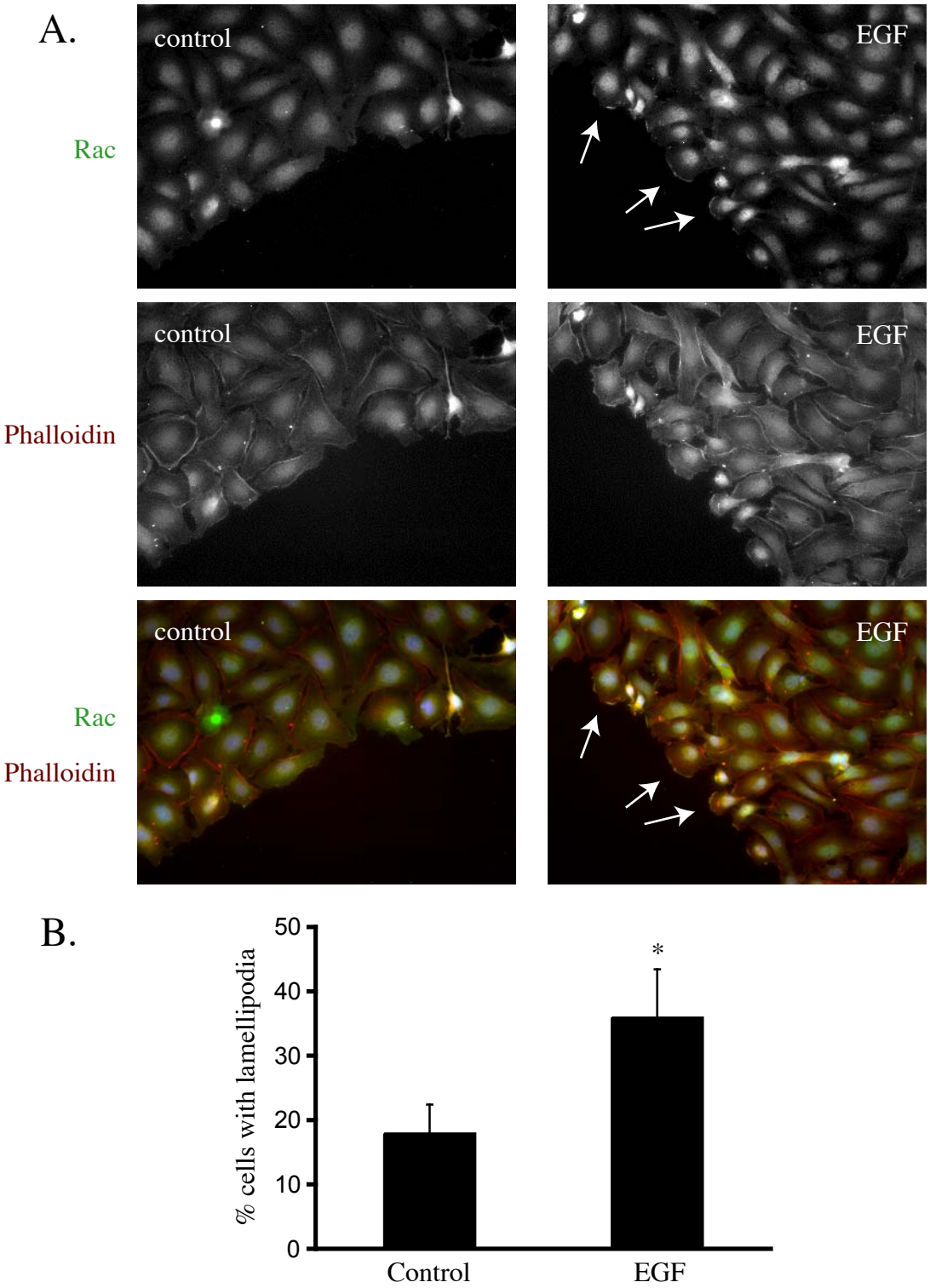


Figure 9. EGF stimulates lamellipodia formation at the wound margin. YAMC cells were wounded, treated with or without 10 ng/ml EGF for 10 minutes, fixed, and stained with Rac and Phalloidin to visualize lamellipodia formation at the wound margin (A). The percent of cells at the wound margin with visible lamellipodia was quantified with or without EGF (B). $*$ p value < 0.001

wound margin, the number of cells exhibiting visible lamellipodia at the wound margin were counted and divided by the total number of cells at the wound margin. As shown in Figure 9B, EGF treatment stimulates a twofold increase in the percentage of cells exhibiting prominent lamellipodia at the wound margin (35.97 ± 7.53 vs. 17.98 ± 4.48). These data confirm that lamellipodia formation and Rac membrane localization are increased at the wound margin in response to EGF.

Because Rac membrane translocation was increased in the presence of EGF, I next wanted to determine if Rac is required for EGF stimulated cell migration. To test this, two different approaches were used to inhibit Rac activity. The first approach uses a pharmacological Rac1 inhibitor, NSC23766, which has been shown to specifically block Rac-GTP loading without interfering with either Cdc42 or Rho activation (Gao et al., 2004). In the second approach, control non-targeting siRNA or Rac1 siRNA was transfected into YAMC cells to specifically reduce Rac1 protein levels. YAMC cells pre-treated with vehicle only or 50μM Rac1 inhibitor were wounded, treated with or without 10 ng/ml EGF in the continued presence of vehicle or Rac1 inhibitor, and the percent wound closure was calculated. In YAMC cells pre-treated with vehicle only, EGF stimulates a statistically significant increase in wound closure after 8 hours when compared to untreated cells (Figure 10A). However, EGF stimulated wound closure is completely blocked in YAMC cells treated with the Rac1 inhibitor. Consistent with these data, wound closure in response to EGF is completely blocked in cells transfected with 50nM Rac1 siRNA, while cells transfected with 50nM non-targeting siRNA show the expected rate of increased wound closure in response to EGF (Figure 10B). To determine the efficacy of the siRNA in the transfected cells, the cells were lysed after the

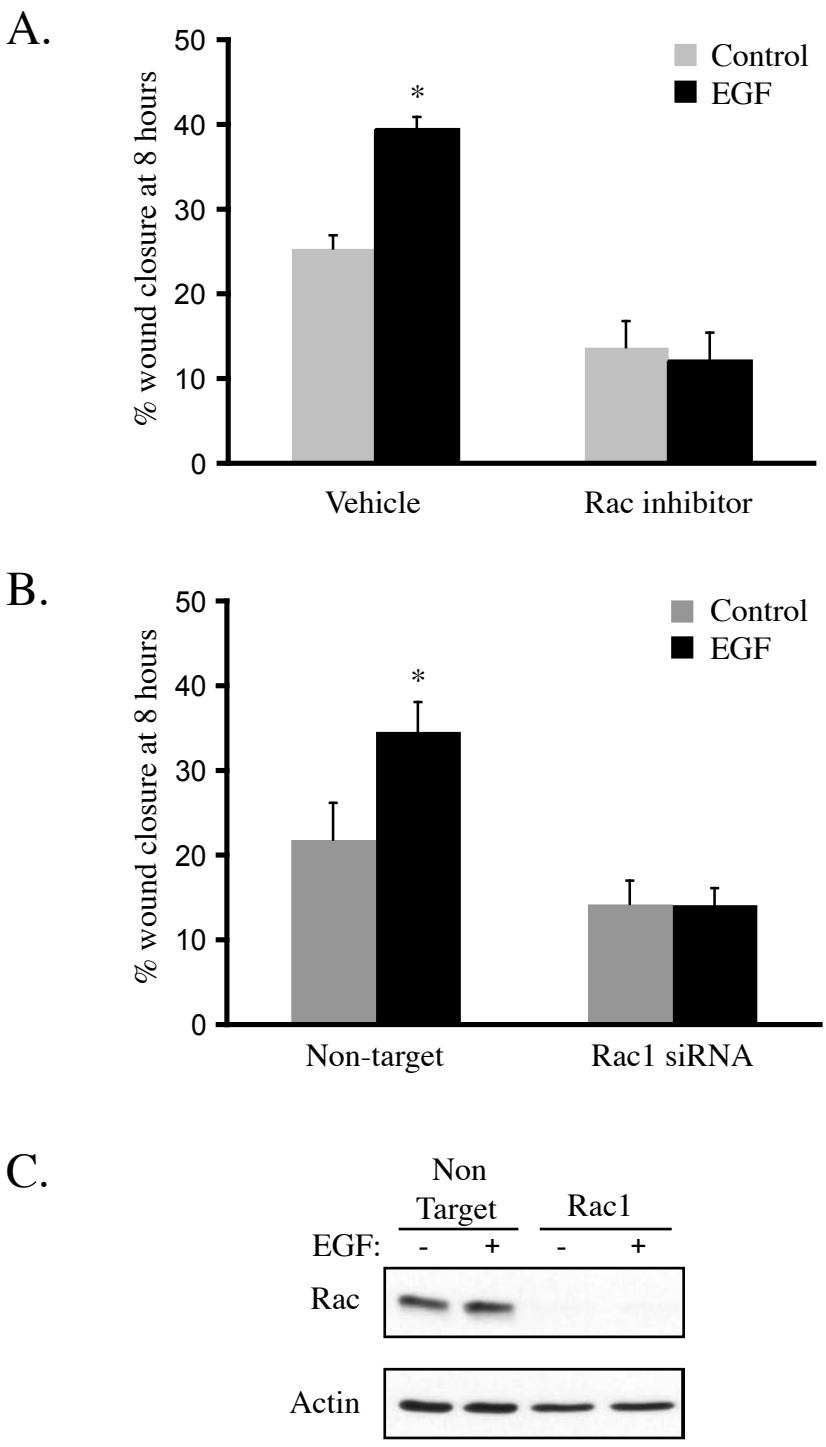


Figure 10. Rac is required for EGF stimulated migration. Wound healing assays were performed with serum-starved YAMC treated with or without 10 ng/ml EGF either in the presence of 50μM Rac inhibitor (A) or after transfection with 50nM non-targeting or 50nM Rac1 siRNA (B). The cells used for the migration assay in B were lysed and blotted for Rac or actin as a loading control (C). ∗ p value < 0.001

migration assay and Rac protein levels were detected by Western blot analysis (Figure 10C). Transfection with Rac1 siRNA significantly reduced the amount of Rac expressed in YAMC cells compared to the non-targeting siRNA, while the total amount of protein in each lane did not change as assessed by immunoblot analysis for actin (Figure 10C). The specificity of the siRNA confirms that Rac1, and not other isoforms that may be expressed, is required for EGF stimulated wound closure and cell migration in YAMC cells.

EGF rapidly activates Rac in YAMC cells

To biochemically determine if EGF treatment stimulates increased lamellipodia formation and wound closure through activation of Rac, a GST pulldown assay was used to isolate GTP-bound Rac from cell lysates (Benard and Bokoch, 2002). In this assay, a GST fusion protein encoding the p21 binding domain (PBD) of p21 activated kinase (PAK), which is activated downstream of Rac and Cdc42, is bacterially produced and bound to glutathione sepharose beads. The PBD domain of PAK binds with high specificity to GTP-bound Rac and Cdc42, but does not recognize the GDP-bound form of these proteins. Using the GST-PBD pull-down assay, confluent monolayers of YAMC cells treated with EGF for 0 to 60 minutes were assayed for Rac and Cdc42 activation, as shown in Figure 11A. While the total cellular lysate from each time point contained equal levels of Rac, GTP-bound Rac was detected only in cells treated with EGF for 3 and 5 minutes. Active Rac was not detected at any of the later time points examined, suggesting that EGF rapidly and transiently activates Rac (Figure 11A). Densitometry was used to determine the fold increase in Rac activation. Compared to control cells,

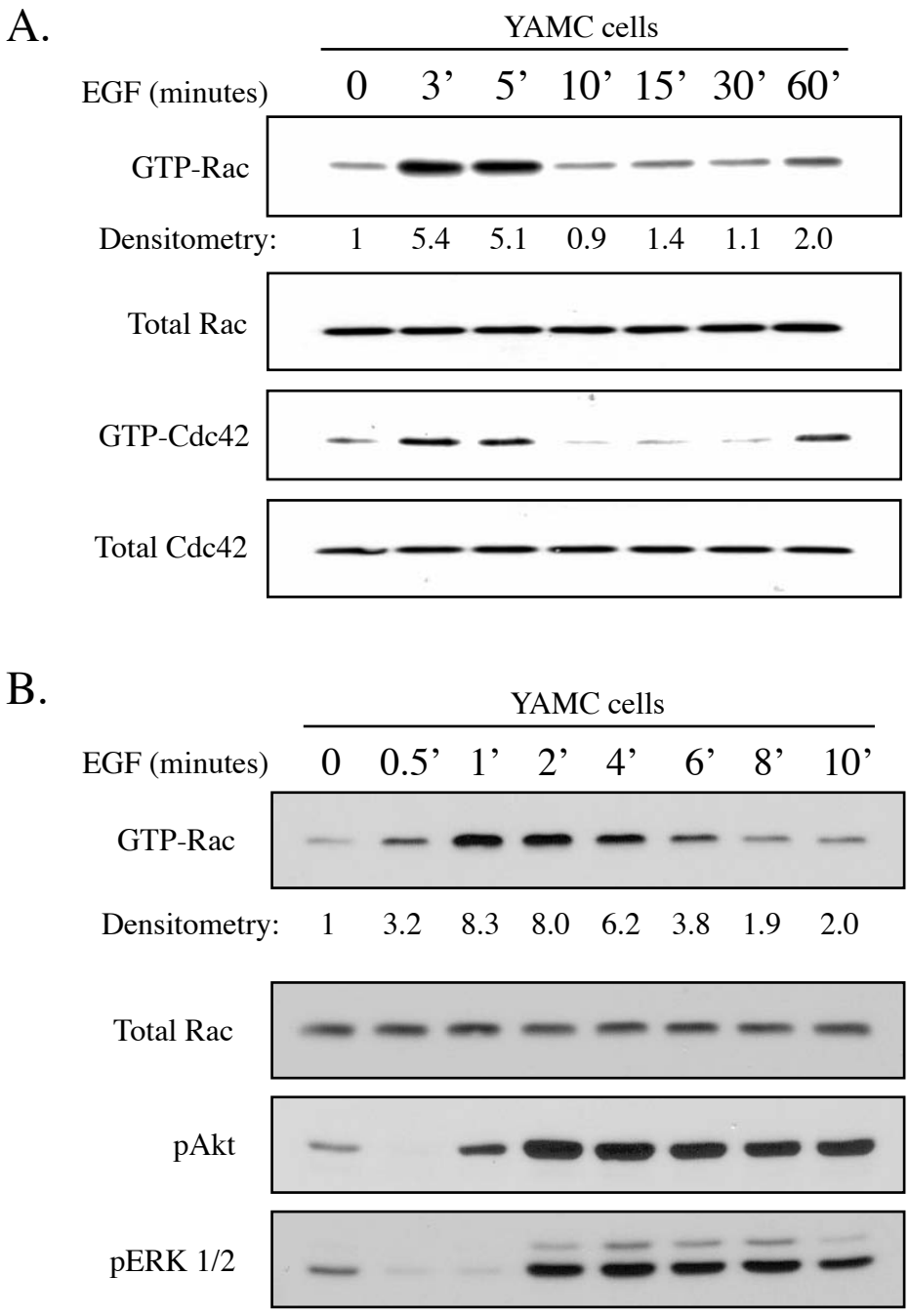


Figure 11. EGF treatment of YAMC cells rapidly activates Rac. YAMC cells were treated with 10 ng/ml EGF for a long (A) or a short (B) time course for the indicated times. Rac and Cdc42 activation was assessed by GST-PBD pull-down followed by Western blot analysis for Rac (A and B) or Cdc42 (A). The total lysates from the short time course were immunoblotted with phospho-Akt and phospho-ERK 1/2 antibodies to detect activation of these proteins (B). Densitometry was used to determine the fold increase in Rac activation compared to untreated cells.

EGF treatment for 3 and 5 minutes induced a 5.4-fold and 5.1-fold increase in GTP-Rac levels, respectively. At 10, 15, and 30 minutes of EGF exposure GTP-Rac was similar to control levels (approximately 1 by densitometry), followed by a two-fold increase at 60 minutes, which may represent a second wave of Rac activation. Although a small increase in Cdc42 activation was detected at 3, 5, and 60 minutes post-EGF treatment in this experiment (Figure 11A), in repeated experiments performed under identical assay conditions, Cdc42 was not activated above basal levels in response to EGF, despite very consistent Rac activation at 3 and 5 minutes. Because the activation of Cdc42 was inconsistent and not as robust as Rac activation, I decided to focus the remainder of my studies on the activation of Rac.

To further investigate the timing of Rac activation, YAMC cells were treated with EGF for shorter time points between 0 and 10 minutes, as indicated, and Rac activation was determined by GST-PBD pull-down as above. Densitometry was used to quantify the fold increase in Rac activation at each time point (Figure 11B). Again, EGF stimulates rapid activation of Rac that begins with a 3-fold increase at 0.5 minutes after treatment, followed by a robust, 8-fold increase at 1 and 2 minutes after treatment. Rac activation persists at a significant increase over control up to 6 minutes (3.8-fold), followed by a weaker, two-fold increase at 8 and 10 minutes (Figure 11B). EGF treatment of YAMC cells also rapidly activates Akt and ERK 1/2 (Figure 11B), as detected by phospho-specific antibodies that recognize only the active, phosphorylated forms of these proteins. Interestingly, maximal Rac activation is detected 1 minute after EGF treatment, prior to ERK 1/2 and concomitant with minimal Akt activation, further

supporting that the signaling pathways that activate Rac are independent or upstream of the cell proliferative (ERK 1/2) and cell survival (Akt) pathways.

In our wound healing assay, lamellipodia formation at the wound margin is stimulated following EGF treatment, but lamellipodia are also visible in untreated cells, suggesting that Rac is activated in these cells as well (Figure 9B). Consistent with this, basal cell migration is observed in untreated cells in response to wounding, albeit at a much slower rate than EGF treated cells (Figure 8B). Because the biochemical experiments assessing Rac activation were performed in confluent monolayers of cells that were not actively migrating, it is plausible that the increase in GTP-Rac levels in response to EGF may not be maximal under these conditions. To examine this possibility, confluent plates of serum starved YAMC cells were wounded using a gel comb to make multiple scratch wounds across the plate. After wounding, cells were incubated either in starve media only for 3 or 60 minutes or in starve media containing 10 ng/ml EGF for 3, 10, 15, 30, or 60 minutes. The cells were lysed, Rac activation was determined by GST-PBD pull-down, and the fold increase in GTP-Rac was quantified by densitometry. As shown in Figure 12, wounding alone stimulated a 6-fold increase in GTP-Rac after 3 minutes, representing a fold increase in Rac activation similar to EGF treatment alone in confluent cells (Figure 11A). The combination of wounding and EGF treatment increased GTP-Rac levels 11-fold, demonstrating that GTP-Rac levels activated by wounding alone are enhanced by EGF treatment. Interestingly, Rac activation was sustained 5- to 6-fold in wounded cells treated with EGF over control cells at all of the longer time points examined (Figure 12). This increase in Rac activation at later time points was not observed in previous experiments in which confluent YAMC

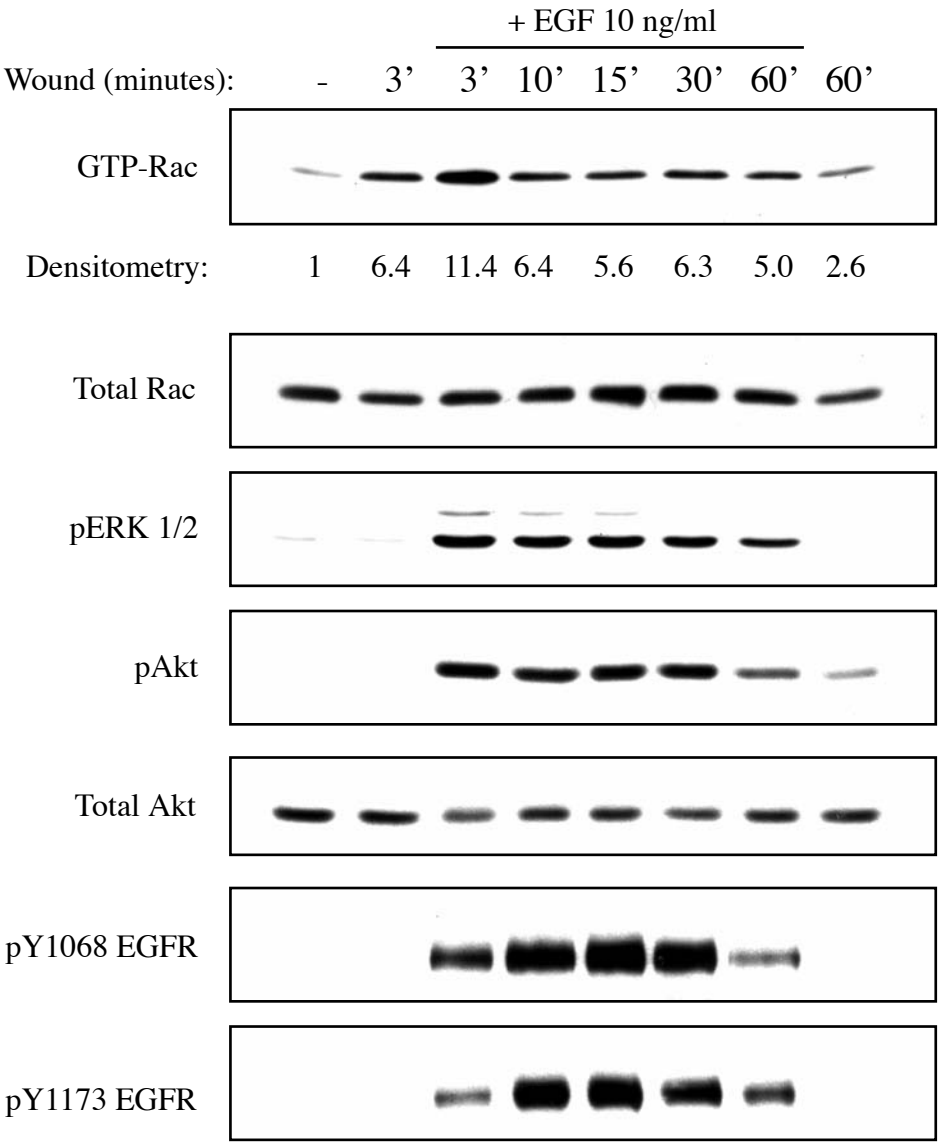


Figure 12. Wounding activates Rac and sustains EGF stimulated Rac activation. Multiple scrape wounds were made in confluent, serum-starved YAMC cells and the cells were incubated for 3 or 60 minutes after wounding only or treated with 10 ng/ml EGF immediately after wounding for 3, 10, 15, 30, or 60 minutes. The cells were lysed and Rac activation was determined by GST-PBD pull-down. Western blot analysis of the total lysate was used to detect activation of ERK 1/2, Akt, or EGFR using phospho-specific antibodies. Immunoblot analysis of total Rac and Akt confirmed equal loading of the gels. Densitometry was performed to determine the fold increase in Rac activation compared to untreated cells.

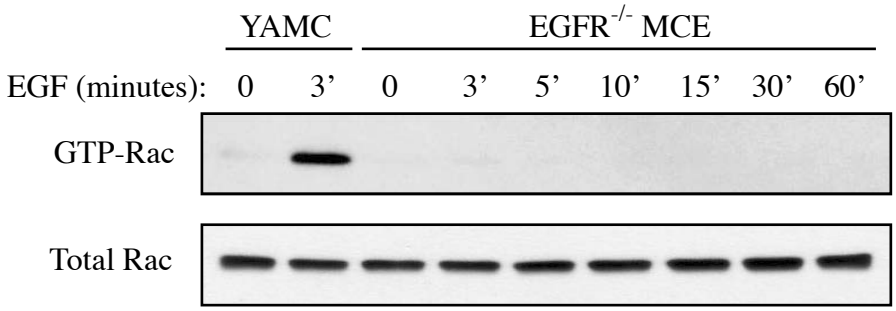
cells were treated with EGF only (see Figure 11A). Rac activation was slightly elevated (two-fold) after 60 minutes of wounding only (Figure 12), similar to the elevation observed following EGF treatment of confluent monolayers for 60 minutes (Figure 11A). These data demonstrate that Rac is activated by the mechanical stimulus of wounding and that this activation is both increased and potentiated by the presence of EGF.

To determine if wounding activated any proteins known to be downstream of EGF, Western blot analysis was used to assess activation of ERK 1/2, Akt, and EGFR with phospho-specific antibodies. Interestingly, these proteins were only activated by EGF treatment, including EGFR as determined by antibodies that recognize two of its major autophosphorylation sites, pY1068 and pY1173. These results demonstrate that basal Rac activation in response to wounding may not require activation of EGFR, and further suggest that enhanced Rac activation in response to EGF likely occurs through a different pathway.

EGFR kinase activity is required for EGF stimulated Rac activation

Because EGF binds preferentially to EGFR among the ErbB family receptors (Jones et al., 1999), EGFR is most likely the receptor required for EGF stimulated Rac activation. To test the contribution of the other ErbB family members to EGF stimulated Rac activation, I utilized $EGFR^{-/-}$ mouse colon epithelial (MCE) cells derived from an immortomouse homozygous for targeted disruption of EGFR (Threadgill et al., 1995). These cells express endogenous levels of ErbB2, ErbB3, and ErbB4, but lack any detectable EGFR expression. $EGFR^{-/-}$ MCE or YAMC cells were treated with 10 ng/ml EGF for the indicated times (Figure 13A), and Rac activation was assessed by GST-PBD

A.



B.

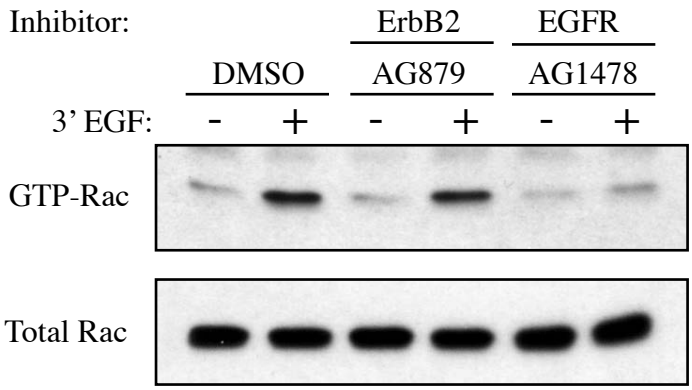


Figure 13. EGFR, but not ErbB2 is required for EGF stimulated Rac activation. Serum starved EGFR$^{-/-}$ MCE cells were treated with 10 ng/ml EGF from 0 to 60 minutes as indicated. Rac activation was assessed by GST-PBD pulldown assay followed by Western blot analysis for Rac (A). YAMC cells were used as a positive control for Rac activation (A). Serum starved YAMC cells were pre-treated with either DMSO, 10μM AG879 (ErbB2 inhibitor), or 150nM AG1478 (EGFR inhibitor), stimulated with 10 ng/ml EGF for 3 minutes, and Rac activation was assessed as in A (B).

pull-down. Whereas EGF exposure activated Rac in YAMC cells after 3 minutes as expected, $EGFR^{-/-}$ MCE cells failed to activate Rac at any of the time points tested (Figure 13A). These data confirm that EGF stimulates Rac activation only in the presence of EGFR.

Exposure of cells to EGF preferentially activates EGFR homodimerization, but EGF can also stimulate EGFR heterodimerization with ErbB2, ErbB3, or ErbB4. ErbB2 is the most likely candidate for EGFR heterodimerization because it is the preferred heterodimerization partner for all of the ErbB family members (Graus-Porta et al., 1997). To determine if ErbB2 is required for EGF stimulated Rac activation, pharmacological inhibitors of ErbB2 (AG879) and EGFR (AG1478) were used to block activation of each of these receptors individually. YAMC cells were pre-treated with vehicle only (DMSO), 10μM AG879, or 150nM AG1478 prior to treatment with 10 ng/ml EGF for 3 minutes and Rac activation was assessed as described above. As shown in Figure 13B, pre-treatment with an EGFR specific inhibitor completely blocks EGF stimulated Rac activation. Pre-treatment of these cells with vehicle only (DMSO) or an ErbB2 inhibitor fails to attenuate activation of Rac, suggesting that ErbB2-EGFR heterodimers are not the active dimers upstream of Rac activation (Figure 13B).

EGF binding to EGFR not only induces receptor dimerization, but also is associated with increased kinase activity and trans-autophosphorylation of tyrosine residues in the C-terminus of EGFR. Inhibition of Rac activation by AG1478 suggests that activation of EGFR kinase activity is required for Rac activation (Figure 13B). To test this more thoroughly, $EGFR^{-/-}$ MCE cells were transfected with either empty vector, wild type human EGFR, or kinase dead (K721R) human EGFR. Stable pools of cells

expressing these transgenes were generated by FACS analysis using an antibody that recognizes the extracellular domain of EGFR, thus allowing the requirement for EGFR kinase activity in downstream signaling pathways to be assessed. A time course of EGF treatment was performed to determine if Rac is activated in the wild type EGFR add-back cells. $EGFR^{-/-}$MCE wild type EGFR cells were treated with 10 ng/ml EGF for 0, 1, 3, 5, 7, or 9 minutes and Rac activation was assessed. As shown in Figure 14A, GTP-Rac is detected in the wild type add-back cells at 1 and 3 minutes after EGF treatment. YAMC cells were used as a positive control. Interestingly, Rac activation in the wild type add-back cells is very transient, with robust GTP-Rac detected only at 1 minute, compared to the YAMC cells in which Rac activation is sustained up to 6 minutes post EGF treatment (Figure 11B). The differences in Rac activation between YAMC and $EGFR^{-/-}$ MCE wild type cells are not the result of a generalized defect in signaling as activation of ERK 1/2 is identical between these cell lines (compare Figure 14A with Figure 11B), but instead may reflect alterations in specific signaling pathways that result from the introduction of exogenous EGFR in these cells. A similar time course was performed in the kinase dead EGFR stable cells to assess the effect of this mutation (K721R) on Rac activation. In contrast to the wild type add-back cells, kinase dead stable cells do not activate Rac or ERK 1/2 at any of the time points tested, demonstrating that the kinase activity of EGFR is required for activation of both of these downstream targets in response to EGF (Figure 14B). Western blot analysis confirmed EGFR expression in the stable cells, but the relative levels of expression can not be compared to the YAMC cells because the antibody used for immunoblot analysis may not recognize mouse (YAMC) and human (stable cells) EGFR with the same affinity (Figure 14 A and B).

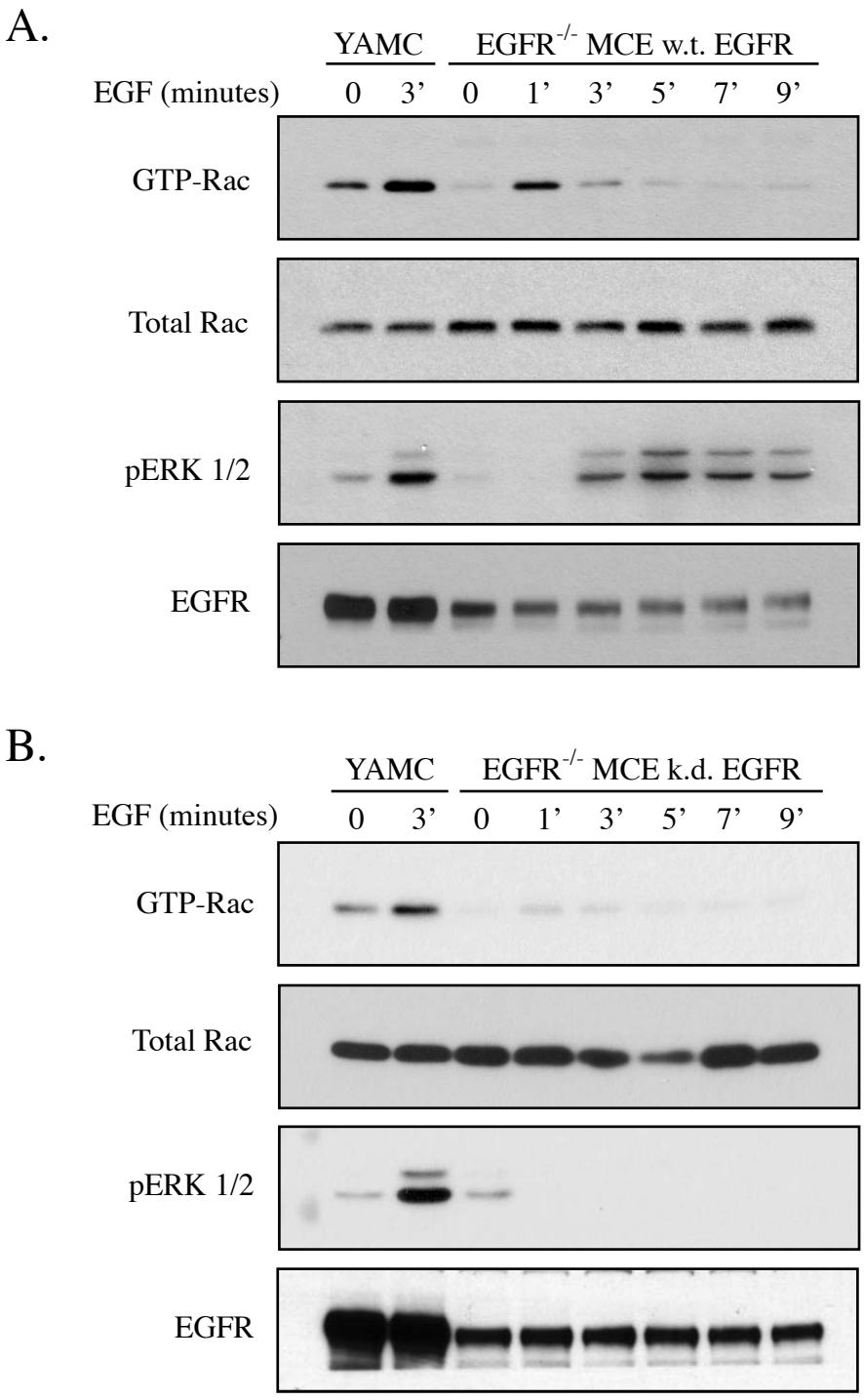


Figure 14. EGFR kinase activity is required for Rac activation. $EGFR^{-/-}$ mouse colon epithelial (MCE) cells stably transfected with either wild type (w.t.) EGFR (A) or kinase dead (k.d.) EGFR (B) were treated with 10 ng/ml EGF for the times indicated. Rac activation was assessed by GST-PBD pulldown. The total lysates were blotted for phospho-ERK 1/2 and total EGFR to confirm expression in the stable cells.

To directly compare EGFR levels and Rac activation in the wild type and kinase dead add-back cells, YAMC, EGFR$^{-/-}$ MCE vector control, wild type EGFR, and kinase dead EGFR stable cells were treated with 10 ng/ml EGF for 3 minutes and Rac activation was analyzed by GST-PBD pull-down. Although Rac activation is not maximal at 3 minutes in the wild type EGFR stable cells, this time point was selected because ERK 1/2 is activated at this time, allowing comparison of downstream signaling pathways between the different cell lines. As shown in Figure 15A, GTP-Rac is detected in both YAMC and wild type EGFR cells, but not in vector control or kinase dead EGFR cells treated with EGF. Western blot analysis for EGFR confirms that the receptor is expressed in both of the stable pools. Interestingly, the kinase dead stable pools express more EGFR than the wild type stable pools; however, Rac, Akt, and ERK 1/2 are not activated in these cells, confirming that activation of each of these pathways requires EGFR kinase activity. In agreement with this, expression of wild type EGFR restores EGF stimulated Akt and ERK 1/2 activation in the null cells to levels comparable to those observed in YAMC cells.

The ability of wild type EGFR stable cells to activate Rac in response to EGF treatment (Figure 15A) suggests that EGF stimulated migration should similarly be restored in these cells. To confirm that loss of EGFR kinase activity and downstream signaling in response to EGF translates to reduced cell migration, YAMC, EGFR$^{-/-}$ MCE vector control, wild type, or kinase dead stable cells were subjected to our wound healing assay in the presence or absence of 10 ng/ml EGF. As predicted, EGFR$^{-/-}$ MCE vector control cells did not exhibit increased migration in the presence of EGF (Figure 15B), consistent with the lack of signaling in response to EGF in these cells (Figure 15A).

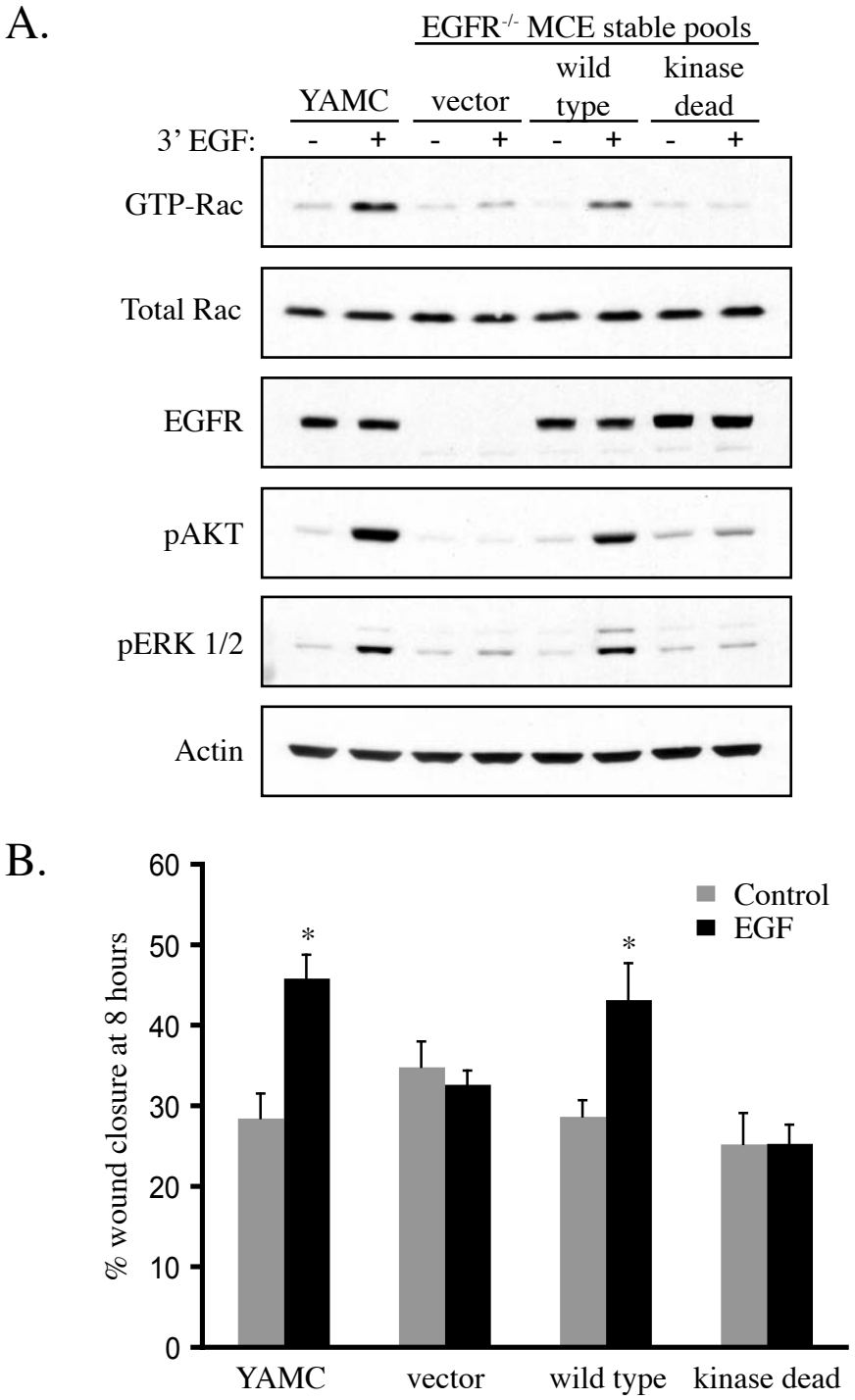


Figure 15. Wild type, but not kinase dead, EGFR stimulates Rac activation and cell migration in response to EGF. YAMC or EGFR$^{-/-}$ stable cellls expressing empty vector, wild type, or kinase dead EGFR were treated with or without 10 ng/ml EGF and Rac activation was assessed by GST-PBD pulldown. The total cell lysates were blotted for EGFR, phospho-Akt and ERK 1/2, or actin as a loading control (A). A wound healing assay was performed with the cells used in A in the presence or absence of 10 ng/ml EGF. $*$ p value < 0.001

EGFR$^{-/-}$ MCE cells transfected with wild type EGFR respond to EGF treatment with an increased wound closure rate similar to YAMC cells (Figure 15B). EGFR$^{-/-}$ MCE kinase dead stable cells showed no increase in cell migration at 8 hours, similar to the vector control cells (Figure 15B). These data are consistent with the signaling data (Figure 15A) and confirm that wild type, but not kinase dead, EGFR can restore EGF stimulated Rac activation and cell migration in EGFR$^{-/-}$ MCE cells.

One interesting caveat to these experiments appeared in a total phosphotyrosine blot of the EGFR$^{-/-}$ stable cells. As expected, EGFR$^{-/-}$ vector control cells fail to respond to EGF treatment, while wild type add-back cells show a profile of phosphorylated proteins in response to EGF similar to the YAMC cells (data not shown). Unexpectedly, a high molecular weight band at approximately 180 kDa appeared in the EGF treated kinase dead cells that was not present in the untreated cells. To determine if phosphorylation of this band correlated with the levels of kinase dead EGFR expression, EGFR$^{-/-}$ stable cells were generated expressing either low or high levels of kinase dead EGFR. YAMC, EGFR$^{-/-}$ vector control, kinase dead low, or kinase dead high expressing stable cells were treated with EGF for 3 minutes and EGFR and phosphotyrosine levels were determined by Western blot analysis. Intriguingly, increased phosphorylation of this high molecular weight band directly correlated with increased expression of kinase dead EGFR, suggesting that kinase dead EGFR is required for this phosphorylation (Figure 16A). Because kinase dead EGFR contains a functional ligand binding domain, this band may be the result of heterodimerization of kinase dead EGFR with ErbB2 in response to ligand. Kinase dead EGFR-ErbB2 heterodimerization can result in trans-phosphorylation of EGFR by ErbB2, autophosphorylation of ErbB2, or phosphorylation

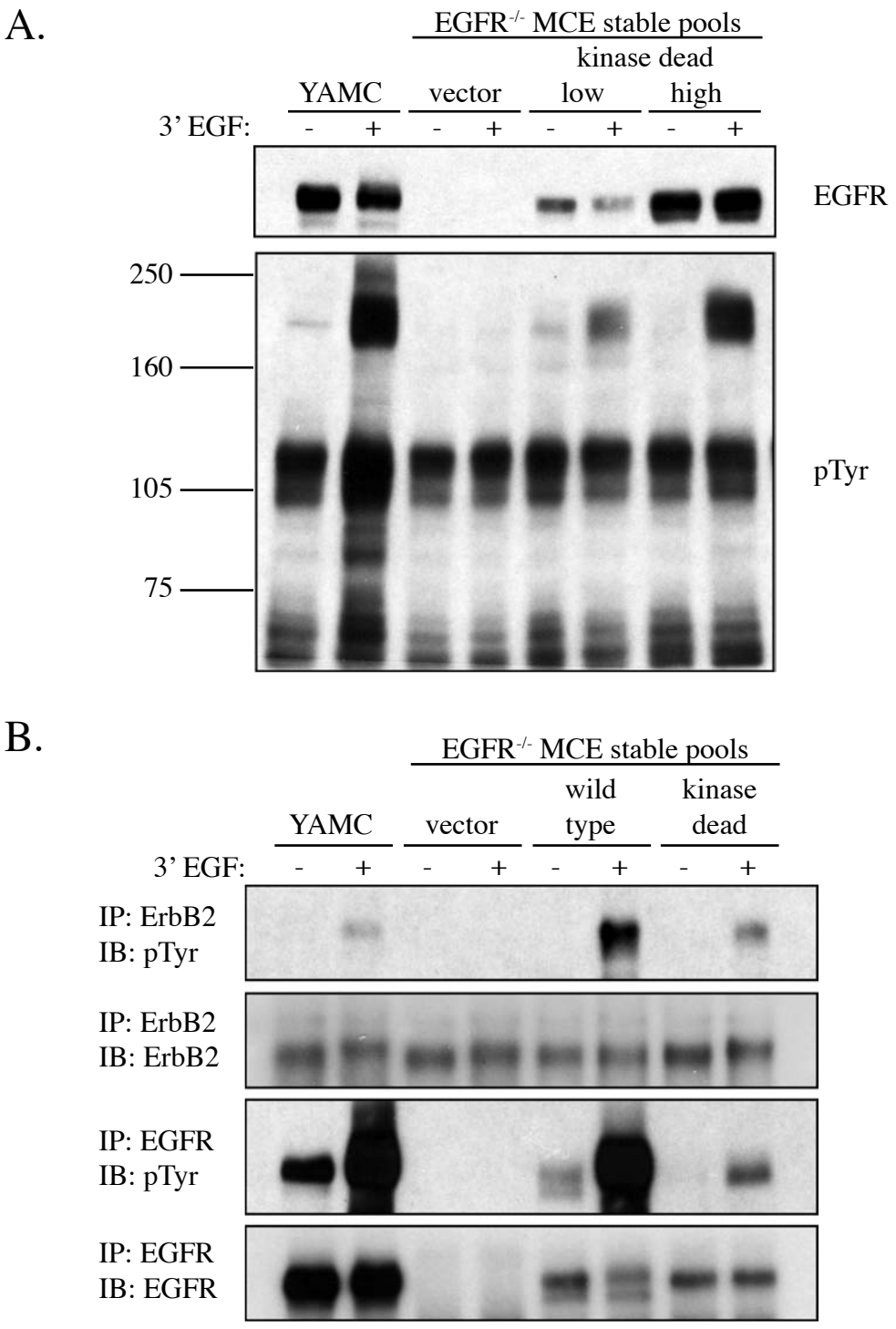


Figure 16. ErbB2 and EGFR are phosphorylated in EGFR$^{-/-}$ MCE kinase dead stable cells. YAMC, vector, or kinase dead EGFR$^{-/-}$ MCE stable cells expressing low and high levels of receptor were treated with 10 ng/ml EGF for 3 minutes, lysed and immunoblot analysis for EGFR and phosphotyrosine was performed (A). ErbB2 and EGFR were immunoprecipitated from YAMC, vector, wild type, kinase dead EGFR$^{-/-}$ MCE stable cells, and immunoblotted for ErbB2, EGFR, or phosphotyrosine (B).

of both receptors by ErbB2, but not phosphorylation of any direct substrates of EGFR. Consistent with this idea, the only phosphorylated proteins observed in the total phosphotyrosine blot of kinase dead cells treated with EGF are similar in size to EGFR and ErbB2, and no lower molecular weight proteins representative of EGFR substrates are increased even in cells expressing high levels of kinase dead EGFR (Figure 16A). To determine if kinase dead EGFR and/or ErbB2 are phosphorylated in the kinase dead cells, EGFR and ErbB2 were immunoprecipitated from YAMC, $EGFR^{-/-}$ vector control, wild type EGFR, or kinase dead EGFR cells treated with or without 10 ng/ml EGF for 3 minutes and phosphorylation of the receptors was detected by immunoblot analysis for total phosphotyrosine. In YAMC and $EGFR^{-/-}$ wild type EGFR cells, EGFR was heavily tyrosine phosphorylated in response to EGF (Figure 16B). While equal amounts of ErbB2 were immunoprecipitated from all of the cell lines, the levels of ErbB2 phosphorylation are increased in wild type cells compared to YAMC cells, suggesting that more EGFR-ErbB2 heterodimers are formed in these cells in response to EGF. However, the robust phosphorylation of EGFR in both YAMC and wild type add-back cells suggests that EGFR homodimers are predominant in both cell lines in response to EGF. Interestingly, in $EGFR^{-/-}$ kinase dead stable cells, both EGFR and ErbB2 are phosphorylated in response to EGF, although phosphorylation of both of these receptors is significantly reduced compared to wild type add-back cells (Figure 16B). Consistent with the phosphotyrosine blot in Figure 16A, EGFR and ErbB2 are not phosphorylated in vector control cells. These data suggest that kinase inactive EGFR heterodimerizes with ErbB2 in the $EGFR^{-/-}$ kinase dead stable cells; however, the biological consequences of these dimers, if any, is unclear. Together, these data demonstrate that increased Rac

activation is induced by signaling pathways downstream of functional EGFR homodimers and that enhanced activation of Rac contributes to increased cell motility in response to EGF.

Discussion

Previous work from our lab as well as others has demonstrated that EGF stimulates increased motility in a wound healing model of cell migration (Frey et al., 2004) (Wilson and Gibson, 1999). My studies have extended these previous observations to characterize the role of the small GTPase Rac1 in EGF stimulated migration. While many different signaling pathways contribute to the complex process of cell migration, I have demonstrated that activation of Rac1 is necessary for EGF stimulated migration in our wound closure assay. Targeted reduction of Rac1 protein expression inhibits YAMC cell migration in response to EGF. More specifically, the GTPase activity of Rac is required, as treatment of cells with a pharmacological inhibitor that blocks Rac GTP-binding also inhibits EGF stimulated migration. Biochemical experiments used to determine GTP-Rac levels confirm that Rac is rapidly and robustly activated in YAMC cells either in response to EGFR ligand binding or through wounding of the cell monolayer. In fact, Rac activation precedes the activation of other well-known signaling pathways downstream of EGFR, underscoring its importance in the immediate phase of the restitution process. These data provide important insight into the mechanism by which growth factors stimulate actin-based motility.

Whereas EGF stimulates increased migration in YAMC cells in our wound closure assay, lamellipodial extension and cell movement into the denuded area still

occur in the absence of EGF, albeit with reduced kinetics (Figure 8A and 8B, Figure 9B). As these processes are regulated by the small GTPase Rac, it is likely that Rac is activated in response to wounding and therefore, an effector of both growth factor stimulated and basal migration (Nobes and Hall, 1999). Consistent with this idea, scrape wounding of confluent YAMC cells in the absence of growth factor induces Rac activation (Figure 12) to a similar extent to which EGF activates Rac in confluent monolayers. I have demonstrated that EGF treatment in combination with wounding of the monolayer both increases and potentiates Rac activation. This suggests that EGF enhances the basal migratory response, potentially through sustained activation of signaling pathways that contribute to Rac activation. However, it is still unclear whether the upstream signals that contribute to Rac activation in response to wounding and in response to EGF are similar. One possibility is that loss of cell:cell contacts and the ensuing actin cytoskeletal rearrangement in response to the mechanical stimulus of wounding activates a Rac-specific GEF and that EGF treatment activates this GEF as well. Alternatively, EGF treatment might activate a completely different GEF from that activated by wounding, and the combination of this activation leads to increased GTP-Rac levels. Future experiments will be directed at understanding the signals that contribute to basal migration in response to wounding to determine if they are distinct from EGF stimulated signaling pathways.

Interestingly, one study has demonstrated that inhibitors of EGFR significantly block basal migration of colon cancer cells in a wound healing assay similar to our own (Wilson and Gibson, 1999). Additionally, blocking antibodies to TGFα, but not to EGF, also inhibit basal migration. This suggests that wounding stimulates the release of TGFα,

which in turn activates EGFR to stimulate migration in intestinal cells. However, the signaling pathways activated by EGFR that may contribute to basal migration have not been identified. Inconsistent with this idea, phospho-specific antibodies to two different EGFR phosphorylation sites failed to detect EGFR activation in response to wounding (Figure 12). It is possible that wounding may stimulate EGFR phosphorylation on sites different from those examined in my studies, or that EGFR activation may occur very transiently in response to wounding. A more detailed time course is necessary to determine if wounding alone activates EGFR. Furthermore, it is possible that EGFR is only activated in cells at the wound margin and this activation may not be detectable by our phospho-specific antibodies. Together, these data suggest a model in which wounded cells activate Rac at the wound margin and begin to migrate slowly as they sense the denuded area. I propose that in cells exposed to EGF, Rac activation occurs more rapidly as cells at the wound margin both sense the denuded area and bind to ligand, coordinately and potently activating the migratory pathways downstream of EGFR to enhance cell migration. It will be interesting to determine in future studies whether the different signaling cues of growth factors and the mechanical stimulus of wounding employ similar downstream effector molecules to elicit cell migration.

In confluent monolayers of cells, EGF stimulates a rapid and transient activation of Rac in the absence of wounding (Figure 11). Activation of Rac begins within 30 seconds of EGF exposure and reaches maximal activation within 1 minute, prior to maximal activation of Akt or ERK 1/2 (Figure 11B). The rapid time course of activation in relation to other well-characterized EGFR downstream pathways suggests that signals upstream of Rac must be activated almost immediately upon EGF exposure.

Characterization of the signaling pathways activated within the first 30 seconds of EGF treatment will provide candidate signaling molecules upstream of Rac. Interestingly, studies have demonstrated that filopodial extensions direct the formation of lamellipodia, suggesting that Cdc42 activation is upstream of Rac activation (Nobes and Hall, 1995). Although I was unable to consistently detect Cdc42 activation, these experiments are not conclusive enough to assess the requirement for Cdc42 in Rac activation. Cdc42 induces the formation of transient and labile filopodial extensions that may only require a low level of Cdc42 activity that may be below the threshold of detection by GST-PBD pulldown assays. Alternatively, Cdc42 activation might reach maximal levels prior to the time points examined in these experiments. Further studies are necessary to determine whether Cdc42 is activated by EGF and whether if functions upstream of Rac activation in intestinal epithelial cells.

Unlike activation of Akt and ERK 1/2, which are sustained for up to 10 minutes following EGF treatment, Rac activation occurs more transiently, returning to basal levels at 8 minutes after treatment. This transient activation of Rac is characteristic of the small GTPases, which rapidly hydrolyze GTP to GDP after activation through their intrinsic GTPase activity. Interestingly, increased Rac activation was observed 60 minutes after EGF treatment, although this activation was reduced compared to activation at 3 and 5 minutes (Figure 11A). As cells move forward, they must continually cycle through the processes of lamellipodial extension, cell body contraction, and rear release, requiring cyclical activation of the Rho GTPases as well (reviewed in (Raftopoulou and Hall, 2004)). This increase in GTP-Rac levels at 60 minutes may represent a second

cycle of Rac activation; however, a more detailed time course of EGF treatment from 30 to 60 minutes is necessary to confirm this observation.

Rac activation in response to EGF requires the kinase activity of EGFR, but not ErbB2 kinase activity, suggesting that signaling pathways downstream of EGFR homodimers induce GTP-Rac. EGFR null cells do not activate Rac or migrate in response to EGF, suggesting that EGF does not signal through other receptors in these cells. These cell provide a perfect model system for reconstitution of EGFR signaling by expressing wild type and kinase dead EGFR in the absence of endogenous receptor. Lysine 721 of EGFR is required for ATP binding and mutation of this residue abrogates all tyrosine kinase activity and cellular responses to EGF, but does not alter ligand binding affinity (Chen et al., 1987) (Honegger et al., 1987). Surprisingly, expression of kinase dead EGFR in the $EGFR^{-/-}$ cells results in phosphorylation of EGFR and ErbB2 in response to EGF treatment, suggesting that EGFR ligand binding activates ErbB2. In the low and high $EGFR^{-/-}$ kinase dead expressing cells, phosphorylation of both of these receptors directly correlates with increased levels of kinase dead EGFR, suggesting that increased availability of the active ligand binding domain of EGFR drives activation of ErbB2. It has yet to be determined if these heterodimers are functional, as no signaling proteins have been found to be activated in these cells. In contrast, co-expression of K721M kinase dead EGFR and ErbB2 in 32D myeloid cells, which do not express any endogenous ErbB receptors, resulted in phosphorylation and activation of ERK 1/2 and Akt in response to EGF treatment for 5 minutes (Deb et al., 2001). This demonstrates that signaling pathways can potentially be activated by kinase dead EGFR-ErbB2 heterodimers. These contradictory data may be explained by differences in ErbB2

expression levels, as our cells express endogenous levels of ErbB2. However, it is also possible that our kinase dead cells activate different signaling pathways in response to tyrosine phosphorylation. One possibility is that a unique subset of EGFR tyrosine residues are phosphorylated by ErbB2, limiting the recruitment of adaptor proteins to the receptor. These cells provide an excellent model to study the contributions of ErbB2 to EGFR phosphorylation. Determining the identity of the phosphorylated tyrosine residues on ErbB2 and kinase dead EGFR is necessary to learn more about how these receptors may signal. The inability of the kinase dead cells to migrate in response to EGF demonstrates that if signaling pathways are activated in these cells, they are not sufficient to induce cell motility. Furthermore, these studies need to be extended to determine if EGF activates ErbB3 and ErbB4 in these cells. In summary these studies provide a foundation for further investigation into the signaling pathways involved in both basal and EGF stimulated migration that contribute to Rac activation.

CHAPTER IV

RAC ACTIVATION REQUIRES PHOSPHOINOSITIDE 3-KINASE AND SRC FAMILY KINASE ACTIVITY

Introduction

In response to ligand binding, EGFR dimerizes, trans-autophosphorylates on tyrosine residues located in its C-terminal tail, and recruits a number of adaptor proteins with SH2 and PTB binding domains to activate downstream signaling events (see Figure 7). In addition to autophosphorylation of the receptor, a number of other proteins are tyrosine phosphorylated in response to EGF, either directly by EGFR or by kinases activated downstream of EGFR, such as the Src family kinases. Data presented in the previous chapter demonstrated that the small GTPase Rac1 is activated in response to EGF treatment and that this activation requires the kinase activity of EGFR. However, the pathways downstream of EGFR required for Rac activation have not been investigated. The purpose of these experiments was to investigate the molecular mechanism by which ligand engaged EGFR stimulates Rac-GTP levels. Because EGFR activation leads to many different cellular processes, I hypothesized that a subset of signaling pathways downstream of EGFR would be required for Rac activation while others would be dispensable for its activation. To identify those specific pathways, a panel of small molecule inhibitors and dominant negative constructs were used to disrupt signaling through EGFR. Included in these studies were pathways previously shown to be required for EGF stimulated migration, but whose role in Rac activation was

unknown. Collectively, I find that stimulation of Rac-GTP levels is complex and requires the synergistic effects of two different signaling pathways.

Results

PLC and PKCs are not required for Rac activation

Previous work has demonstrated that phospholipase C (PLC) activity is required for EGF stimulated cell migration (Chen et al., 1994b) (Polk, 1998), suggesting that activation of PLC by EGF may be involved in Rac activation. To test this, PLC activity was inhibited in YAMC cells with three different pharmacological inhibitors. YAMC cells were pre-treated with either vehicle only (DMSO) or the PLC inhibitors 2μM U73122, 100μM D609, and 10μM L108 prior to stimulation with 10 ng/ml EGF for 3 minutes and Rac activation was determined by GST-PBD pull-down. As shown in Figure 17A, Rac was activated in the presence of each of the PLC inhibitors similar to the vehicle treated cells. In each case, the PLC inhibitors had no effect on activation of ERK 1/2, suggesting that they did not non-specifically inhibit EGFR signaling (Figure 17A). In this experiment the efficacy of the PLC inhibitors was not confirmed; however, we have previously demonstrated that EGF stimulated IP_3 formation is inhibited by U73122, D609, and L108 at the concentrations used in this experiment (Polk, 1998). Therefore, these data demonstrate that Rac activation does not require PLC activity.

When activated by EGF, PLC generates diacylglycerol (DAG) and IP_3, which in turn activate protein kinase C (PKC) family members. As we have previously shown that activation of PKCs downstream of PLC is also required for EGF stimulated migration (Polk, 1998), the PLC inhibitor data suggest that PKCs are not involved in Rac

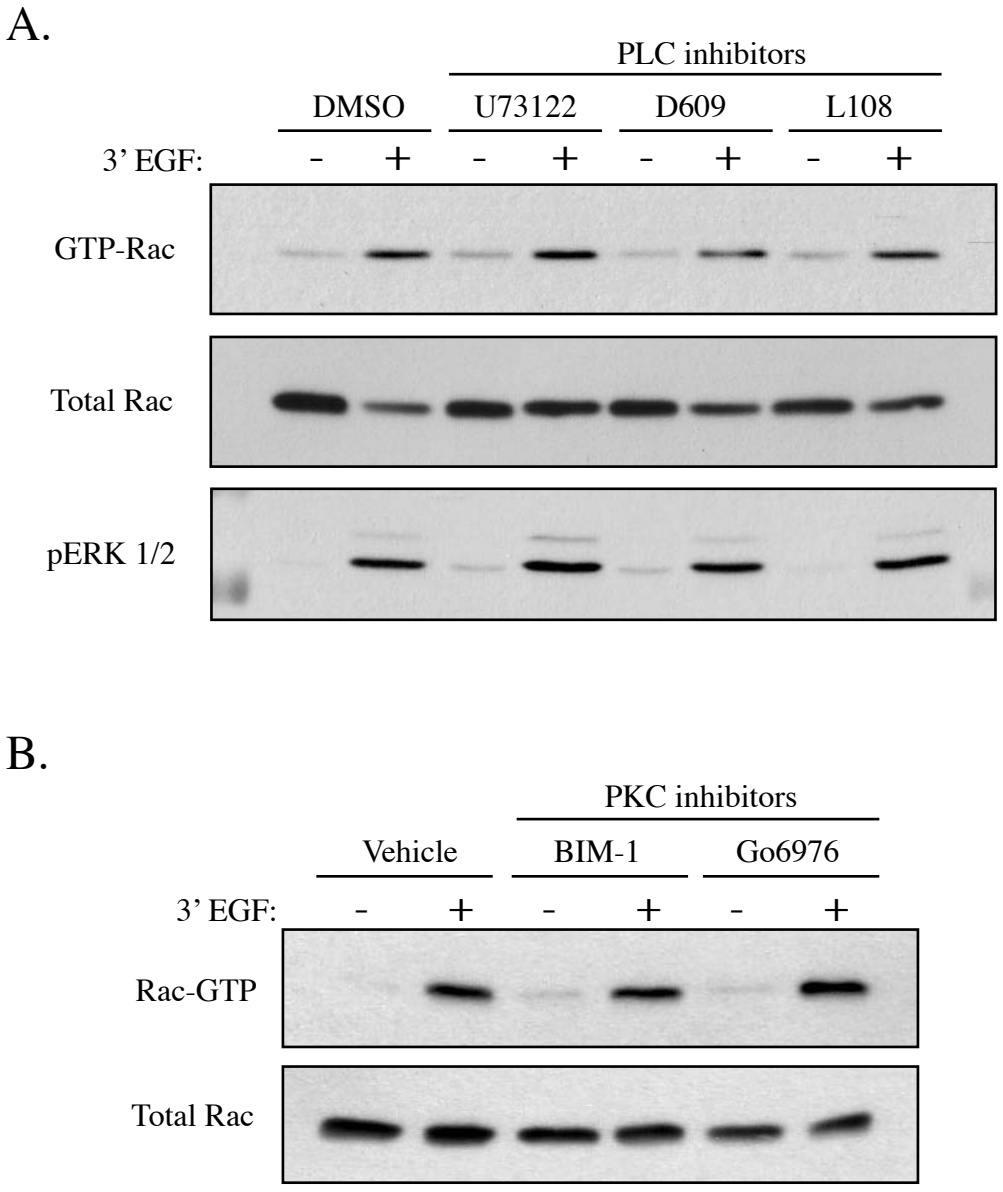


Figure 17. PLCγ and PKC activation are not required for EGF stimulated Rac activation. Serum-starved YAMC cells were pre-treated with either DMSO or PLCγ inhibitors 2μM U73122, 100μM D609, or 10μM L108 and then treated with 10ng/ml EGF for 3 minutes. Rac activation was assessed by GST-PBD pulldown and Western blot analysis for Rac. The total lysate was probed for total Rac and phospho-ERK 1/2 (A). Rac activation was assessed as in A in YAMC cells pre-treated with DMSO or PKC inhibitors 5μM BIM-1 or 1μM Go6976 (B).

activation. To test the requirement for PKCs, YAMC cells were pre-treated with either vehicle or pharmacological PKC inhibitors, 5μM Bisindolylmaleimide 1 (BIM-1) and 1μM Go6976, prior to treatment with 10ng/ml EGF and Rac activation was assessed. Pharmacological inhibition of PKCs did not affect GTP-Rac levels in response to EGF, demonstrating that these kinases are not involved in Rac activation (Figure 17B).

The PKC family consists of 11 different isoforms, some of which are not effectively blocked by these inhibitors. With this in mind, I utilized a more general approach to inhibit PKC activity. Phorbol 12-myristate 13-acetate (PMA) is a potent analog of DAG that directly binds to and activates PKCs, bypassing the requirement for PLC activity. In response to continuous activation, PKC activity is inhibited through its subsequent downregulation. Long-term treatment with PMA results in a potent reduction in PKC levels within the cell as a result of downregulation, while short-term PMA treatment potently activates PKCs. I have taken advantage of both of these mechanisms of PKC regulation to determine the requirement for PKCs in Rac activation. YAMC cells were treated with vehicle or 1μM PMA for 24 hours prior to stimulation with 10 ng/ml EGF for 3 minutes. The cells were lysed and Rac activation was determined using the GST-PBD pull-down assay. Western blot analysis for PKCα and PKCε, two predominant PKC isoforms found in YAMC cells, demonstrates a significant reduction in the expression levels of both of these proteins after 24 hour treatment with PMA (Figure 18A). PKCδ, a third isoform expressed in YAMC cells, is also downregulated in response to 24 hour PMA treatment (data not shown). Despite a significant reduction in the protein expression levels of all PKC isoforms known to be expressed in these cells, Rac was still activated in response to EGF (Figure 18A). Alternatively, YAMC cells

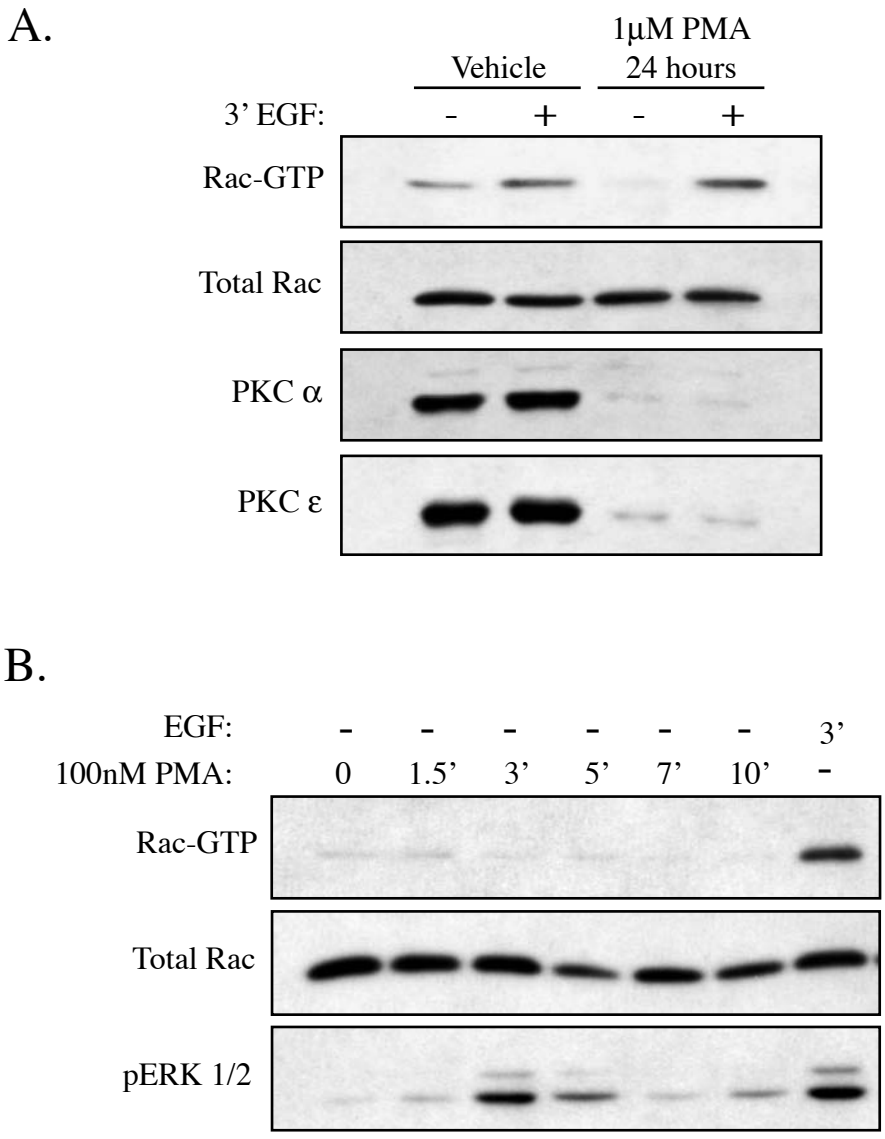


Figure 18. Rac activation does not require PKCs. YAMC cells pre-treated with 1μM PMA for 24 hours to downregulate PKCs were stimulated with 10 ng/ml EGF for 3 minutes and Rac activation was assessed by GST-PBD pulldown. Reduction of PKCα and ε was confirmed by Western blot analysis (A). YAMC cells were treated with 100nM PMA for the times indicated to stimulate PKC activation and Rac activation was assessed as in A. 10 ng/ml EGF treatment for 3 minutes was used as a positive control and phospho-ERK 1/2 Western blot analysis confirmed that the cells responded to the PMA treatment (B).

were treated with 100nM PMA for either 1, 1.5, 3, 5, 7, or 10 minutes to stimulate PKCs. While ERK 1/2 was activated in response to PMA treatment, PKC activation failed to stimulate an increase in GTP-Rac levels compared to 3-minute EGF treatment as a positive control at any of the time points tested (Figure 18B).

PI3K, but not Akt or MEK 1/2 activity, is required for Rac activation

As demonstrated in Figure 11B, both Akt and ERK 1/2, downstream targets of phosphoinositide 3-kinase (PI3K) and MAPK, respectively, are rapidly and robustly activated in YAMC cells in response to EGF as detected by phospho-specific antibodies. While EGFR does not possess any direct binding sites for the p85 subunit of PI3K within its C-terminus, PI3K activity is required for Akt activation. Therefore, it is assumed that PI3K is activated in response to EGF in YAMC cells, presumably through recruitment of the adaptor protein Gab1 to EGFR (Mattoon et al., 2004). In addition to Akt, many other signaling proteins that contain pleckstrin homology (PH) or FYVE domains are recruited to the membrane in response to PI3K activation (Rameh and Cantley, 1999). Tandem PH and Dbl homology (DH) domains characterize guanine nucleotide exchange factors (GEFs) for the Rho family of GTPases (Rossman et al., 2005). While the DH domain is required to catalyze the exchange of GDP for GTP, the PH domain is thought to localize the GEF to the membrane. Therefore, I hypothesized that EGF stimulated PI3K activity would be required for Rac activation through recruitment of a Rac-specific GEF. Recruitment of either Grb2 or Shc to EGFR phosphotyrosines initiates the MAPK cascade through activation of Sos, followed by Ras, Raf, MEK 1/2, and ERK 1/2 activation to stimulate cell proliferation. Because EGF stimulated proliferative signals

are likely to be independent from migratory signals, I hypothesized that activation of the MAPK cascade would not be required for Rac activation. To test these hypotheses, pharmacological inhibitors of PI3K and MEK 1/2 were employed to inhibit each of these signaling pathways. First, YAMC cells were pre-treated with vehicle only (DMSO), the PI3K inhibitors 50μM LY294002 or 200nM Wortmannin, or the MEK 1/2 inhibitor 10μM U0126. The cells were stimulated with 10 ng/ml EGF and Rac activation was assessed by GST-PBD pull-down. Treatment of YAMC cells with PI3K inhibitors, LY294002 or Wortmannin, inhibited EGF stimulated Rac activation, whereas MEK 1/2 inhibition had no effect (Figure 19A). The efficacy of these inhibitors was confirmed by immunoblot analysis of the total cell lysate for phospho-Akt and phospho-ERK 1/2. Both of the PI3K inhibitors blocked Akt activation, while the MEK 1/2 inhibitor blocked activation of ERK 1/2, as expected (Figure 19A). Because Akt is activated in response to PI3K, it was plausible that Akt activation and not PI3K activity is required for Rac activation. To test this, YAMC cells were transiently transfected with dominant negative Akt1 or Akt2, treated with or without 10ng/ml EGF for 3 minutes, and then subjected to Rac activation assays. Western blot analysis for phospho-Akt confirmed that expression of dominant negative Akt1 and Akt2 attenuated EGF stimulated Akt activation (Figure 19B). Despite the decrease in Akt activity, GTP-Rac levels were increased similarly in response to EGF in vector and dominant negative Akt transfected cells. Western blot analysis for HA confirmed that dominant negative Akt was expressed at equal levels in the transfected cells (Figure 19B). These data demonstrate that PI3K activity is required for EGF stimulated Rac activation independent of Akt activity. Because pharmacological inhibitors can exhibit non-specific effects, a different MEK 1/2 inhibitor, PD98059, was

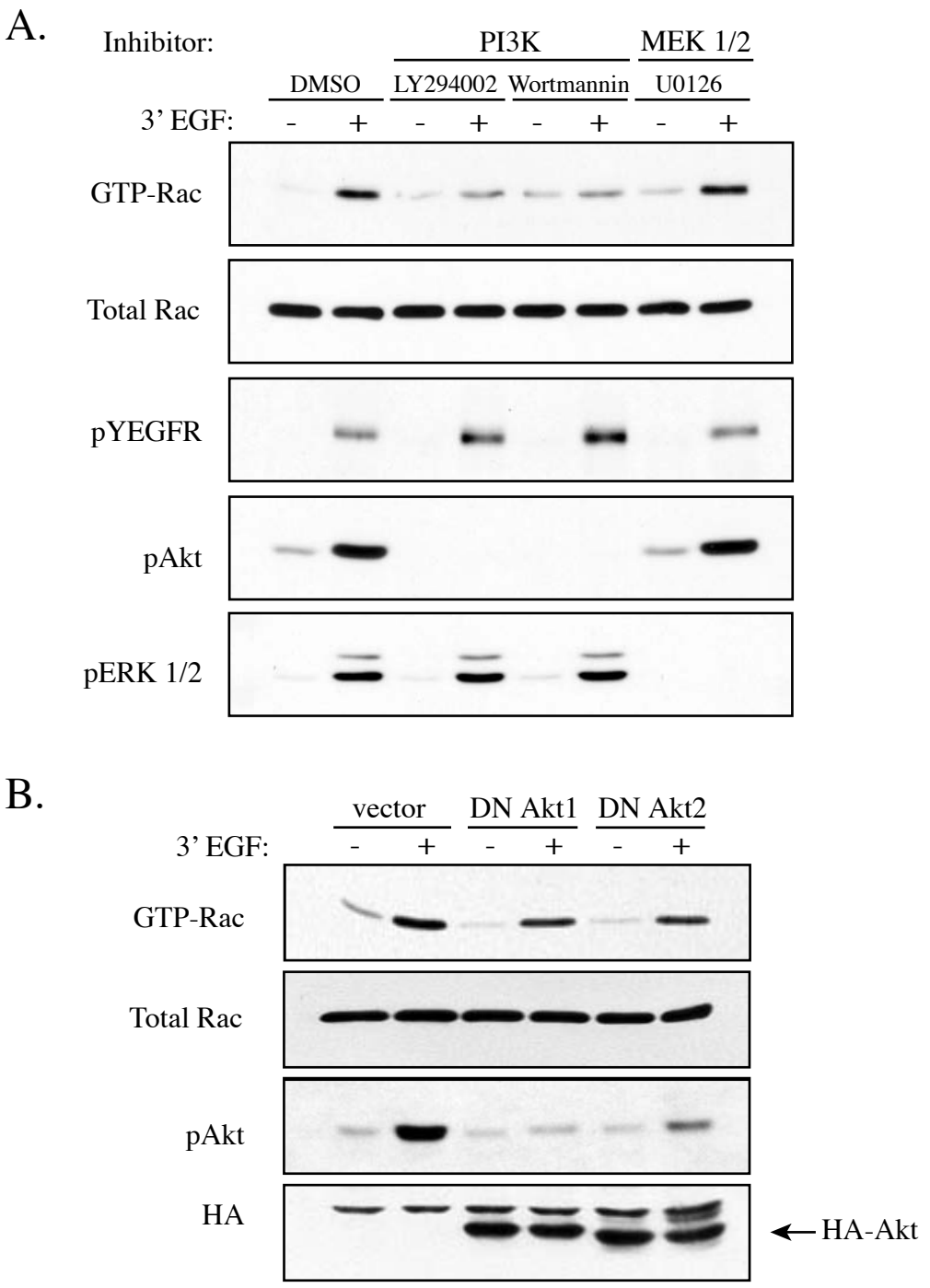


Figure 19. PI3K activity is required for Rac activation independent of Akt. YAMC cells were pre-treated with DMSO, 50µM LY294002, 200nM Wortmannin, or 10µM U0126, stimulated with 10 ng/ml EGF, and Rac activation was assessed by GST-PBD pulldown. The total lysates were probed with phospho- EGFR, Akt, ERK 1/2 and total Rac as a loading control (A). YAMC cells were transiently transfected with dominant negative (DN) Akt1 or Akt2 and Rac activation was assessed as in A. Transfection of DN Akt was assessed by Western blot analysis for phospho-Akt or HA (B).

used to confirm the data obtained with U0126. YAMC cells were pre-treated with DMSO, 50μM LY294002, or the MEK 1/2 inhibitors 10μM U0126 and 10μM PD98059. The cells were stimulated with 10 ng/ml EGF for 3 minutes, lysed, and Rac activation was assayed. Consistent with the previous experiment, Rac was activated in cells treated with the MEK 1/2 inhibitors, but not in cells treated with the PI3K inhibitor (Figure 20). Western blot analysis confirmed that both of the MEK 1/2 inhibitors blocked activation of ERK 1/2, and that Akt activation was blocked by the PI3K inhibitor (Figure 20). These data are also consistent with the time course presented in Figure 11B in which Rac is activated prior to Akt and ERK 1/2 activation, suggesting that these proteins do not act upstream of Rac.

Src family kinases are required for Rac activation

Src family kinases are activated in response to EGF treatment (Osherov and Levitzki, 1994), resulting in phosphorylation of Src targets and phosphorylation of EGFR on several tyrosine residues (reviewed in (Jorissen et al., 2003)). Because Src family kinases play a key role in activation of EGFR and its downstream signaling pathways, the involvement of Src family kinases in EGF stimulated Rac activation was investigated. Because available pharmacological inhibitors do not inactivate the Src family members equally, three different inhibitors, CGP77675, PP1, and PP2 were used in these experiments. All three of these inhibitors were tested in a concentration curve to determine the lowest concentration that inhibits phosphorylation of FAK on tyrosine 925 (a Src specific phosphorylation site) in response to EGF without inhibiting the phosphorylation of EGFR, as determined by phosphorylation of tyrosine 1173 (data not

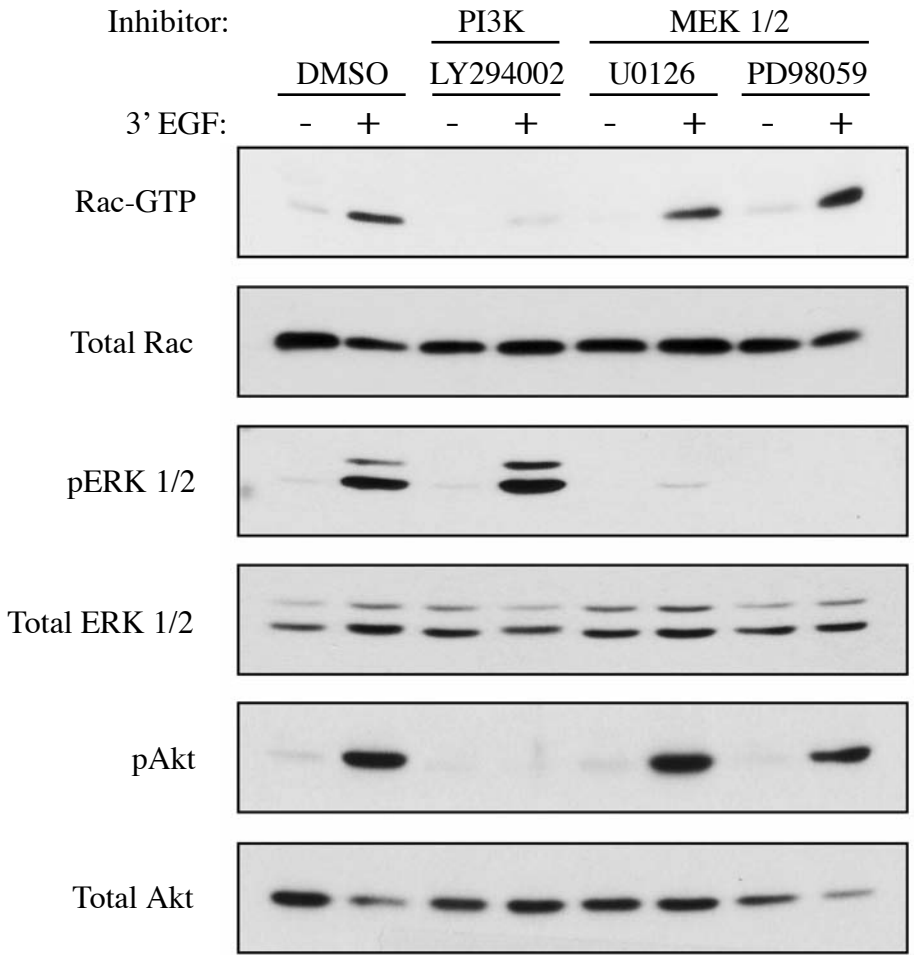


Figure 20. MEK 1/2 and ERK 1/2 activity are not required for Rac activation. Serum-starved YAMC cells were pre-treated with DMSO, 50μM LY294002, 10μM U0126, or 10μM PD98059 prior to 10 ng/ml EGF stimulation and Rac activation was assessed by GST-PBD pulldown. The total lysates were probed with phospho-Akt, phospho-ERK 1/2, or total Rac, ERK 1/2, and Akt as loading controls.

shown). YAMC cells were pre-treated with vehicle only (DMSO) or the Src inhibitors at 2μM CGP77675, 12.5μM PP1, and 7.5μM PP2 prior to stimulation with 10 ng/ml EGF for 3 minutes and Rac activation was assessed. As shown in Figure 21A, Rac activation was reduced in the presence of all three Src inhibitors compared to DMSO treated cells. Treatment of YAMC cells with Src inhibitors did not change EGF stimulated EGFR phosphorylation at tyrosine 1173, a major phosphorylation site, or activation of ERK 1/2. The efficacy of the inhibitors was confirmed by Western blot analysis for phosphorylation of FAK at tyrosine 925, which was inhibited by all of these inhibitors, but to varying degrees. Interestingly, the ability of these inhibitors to block FAK phosphorylation correlated with their ability to inhibit Rac activation. Because Src family kinase inhibitors effectively blocked Rac activation, these inhibitors were also tested in a wound healing assay to determine if Src family kinases are required for EGF stimulated wound closure. YAMC cells were treated with vehicle only (DMSO) or each of the Src inhibitors in the absence or presence of EGF and wound closure rates were determined. As shown in Figure 21B, each of the Src inhibitors blocked EGF stimulated wound closure. Interestingly, CGP77675 was the most effective inhibitor of Rac activation and FAK phosphorylation at tyrosine 925 and also inhibited EGF stimulated migration to the greatest extent (Figure 21 A and B).

Previous work from our laboratory demonstrated that Src family kinases Src, Fyn, and Yes are expressed in YAMC cells, but only Src and Fyn are activated in response to EGF treatment (Frey et al., 2004). To further investigate the role of each of these Src family members in Rac activation, small-interfering RNA (siRNA) was used to specifically reduce the expression of Src or Fyn in YAMC cells. Rac1 siRNA was used

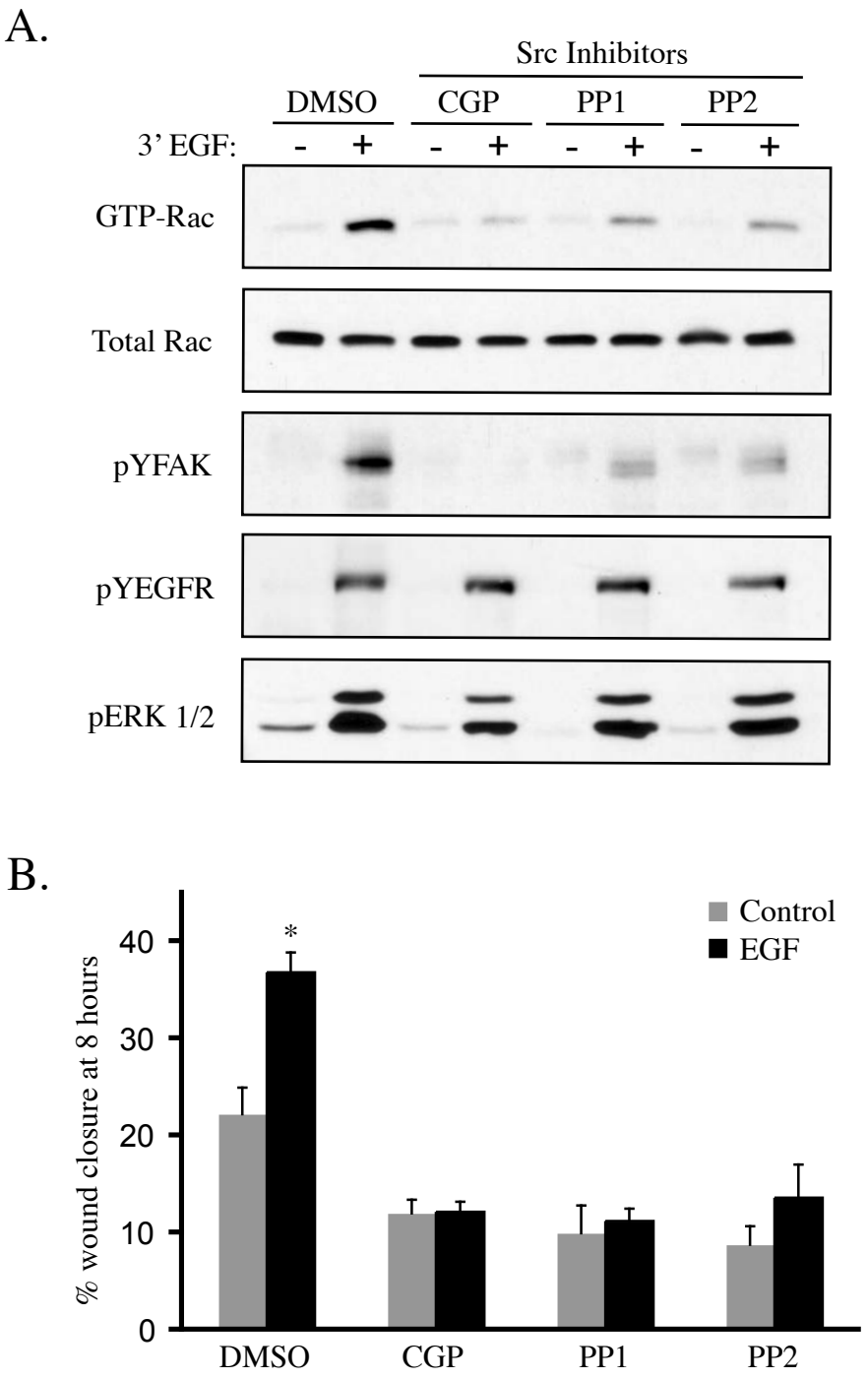


Figure 21. Src family kinases are required for EGF stimulated Rac activation and migration. Rac activation was assessed in YAMC cells pre-treated with DMSO, 2μM CGP77675, 12.5μM PP1, or 7.5μM PP2 in the presence or absence of 10 ng/ml EGF. Western blot analysis was performed for phospho- FAK, EGFR, ERK 1/2, and total Rac (A). Cell migration was assessed in YAMC cells in the presence of Src inhibitors treated with or without 10 ng/ml EGF. $*$ p value < 0.001

as a positive control for inhibition and non-targeting siRNA was used as a negative control. YAMC cells were transiently transfected with non-targeting, Rac1, Src, or Fyn murine siRNA smart pools. Each smart pool contains four different short interfering RNA duplexes specifically designed to knockdown mRNA levels of the target gene, resulting in decreased protein expression. Western blot analysis confirmed that Rac, Src, and Fyn protein levels were specifically reduced in cell transfected with the corresponding siRNA smart pools, although only partially (Figure 22). Reduction of Rac protein levels decreased Rac activation in response to EGF, as expected. Interestingly, decreased levels of Fyn, but not Src, reduced the activation of Rac in response to EGF. While the Src inhibitors used in the previous experiment inhibited phosphorylation of FAK at tyrosine 925, reduction of Src or Fyn expression only slightly reduced FAK phosphorylation, suggesting that the pharmacological inhibitors were more potent than the siRNA at inhibiting Src family kinase activity. Inconsistent with the previous experiment using Src pharmacological inhibitors, phosphorylation of EGFR at tyrosine 1173 was slightly reduced in both the Src and Fyn siRNA transfected cells in this experiment, but this result was not consistently observed. Decreased expression of Rac did not inhibit activation of FAK, Akt, or ERK 1/2, demonstrating that Rac is not required for signaling upstream of these pathways. These data suggest that activation of Fyn, but not Src, by EGF may be upstream of Rac activation.

Src and PI3K synergize to activate Rac

While I observed significantly reduced activation of Rac with either PI3K or Src inhibitors, Rac-GTP was still stimulated in response to EGF treatment. Therefore, it was

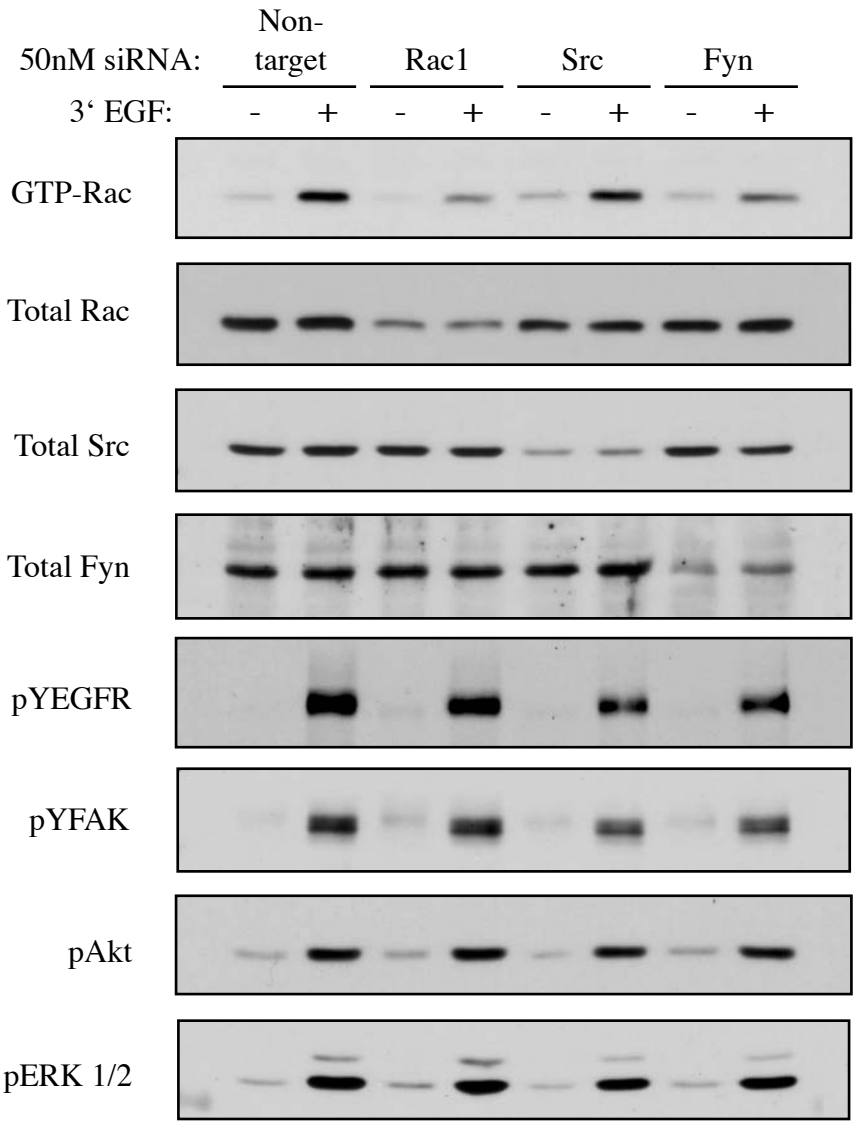


Figure 22. Reduced expression of Rac or Fyn inhibits Rac activation. YAMC cells were transfected with 50nM Non-targeting, Rac, Src, or Fyn siRNA, serum-starved, and then treated with or without 10 ng/ml EGF for 3 minutes. Rac activation was assessed by GST-PBD pull-down. Rac, Src, and Fyn Western blot analysis confirmed that siRNA transfections were effective. The total lysates were also blotted with phospho-EGFR (pY1173), FAK, Akt, and ERK 1/2 to detect activation of these proteins.

plausible that Src and PI3K act in parallel pathways to activate Rac. To test this, YAMC cells were again pre-treated with vehicle only (DMSO), 50μM LY294002, 2μM CGP77675, or concomitantly with 50μM LY294002 and 2μM CGP77675, and EGF stimulated Rac activation was assessed. While treatment of YAMC cells with LY294002 or CGP77675 incompletely inhibited Rac activation as shown above, the combination of both LY294002 and CGP77675 completely blocked Rac activation in response to EGF (Figure 23). These inhibitors did not affect EGF stimulated EGFR phosphorylation on tyrosine 1173, showing that the inhibitors did not have a non-specific effect on EGFR activation. CGP77675 alone or in combination with LY294002 blocked FAK phosphorylation at tyrosine 925, while LY294002 alone did not inhibit FAK Y925 phosphorylation. LY294002 alone or in combination with CGP77675 completely blocked activation of Akt, while CGP77675 alone did not. These data confirm that both Src and PI3K contribute to Rac activation in response to EGF, suggesting that these kinases act in parallel pathways downstream of EGFR activation and upstream of Rac activation.

Effect of tyrosine mutations on EGF stimulated signaling and migration

Phosphorylated tyrosine residues in the C-terminal tail of EGFR create binding sites for proteins containing SH2 and PTB domains. Mutational analyses of EGFR autophosphorylation sites have revealed a number of binding partners and their preferred tyrosine residues as shown in Figure 7. Tyrosines 1068 and 1086 have been shown to bind to Grb2, which couples to Gab1 to activate PI3K in response to EGF. Having demonstrated that PI3K is required for Rac activation, I hypothesized that mutation of tyrosines 1068 and 1086 would inhibit EGF stimulated cell migration. Because the

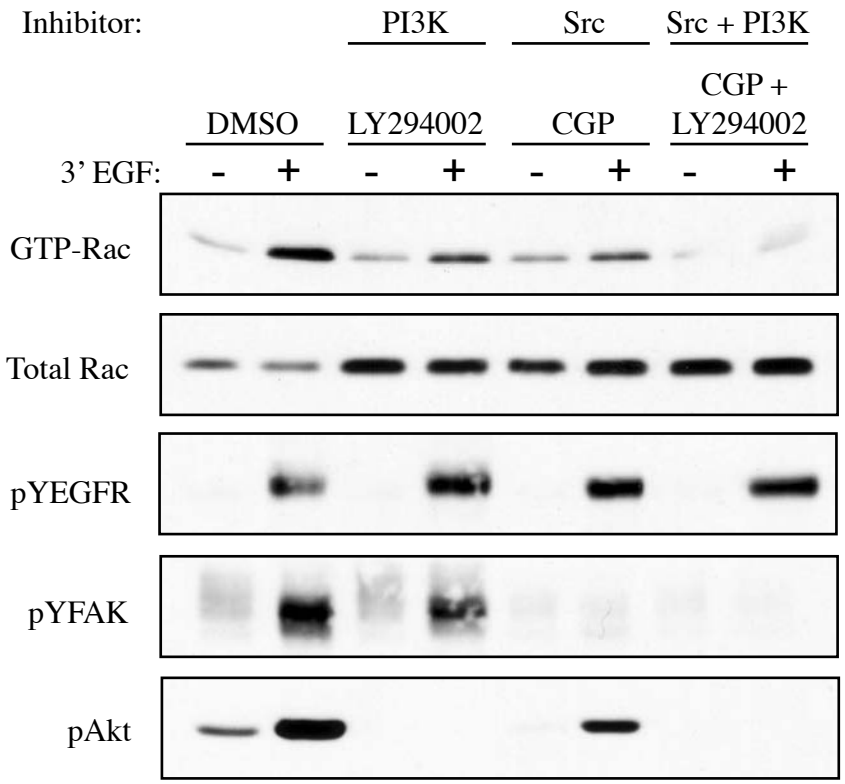


Figure 23. PI3K and Src family kinases synergize to activate Rac. YAMC cells were pre-treated with DMSO, 50µM LY294002, 2µM CGP77675, or 50µM LY294002 and 2µM CGP77675, stimulated with 10ng/ml EGF and Rac activation was assessed with GST-PBD pulldown. Activation of EGFR (pY1173), FAK, and Akt in the total lysate was assessed by Western blot analysis using phospho-specific antibodies. Equal loading was confirmed by Western blot analysis for Rac.

autophosphorylation sites required for Src activation are not known, I also wanted to test the requirement of other tyrosine residues for EGF stimulated cell migration. $EGFR^{-/-}$ MCE stable cell lines expressing single tyrosine to phenylalanine (F) mutations at residues 992, 1045, 1068, 1086 were generated in the same manner as the vector, wild type, and kinase dead stable cells described above. Tyrosines 992 and 1173 have both been shown to bind to PLCγ1 and Shc, suggesting that these tyrosines have redundant functions. Similarly, tyrosines 1068 and 1086 have both been identified as binding sites for Grb2, so double mutants of F992/F1173 and F1068/F1086 were also generated. After generating cells stably expressing these various mutants in collaboration with Toshimitsu Yamaoka, I determined their ability to respond to EGF treatment by treating serum starved cells with 10 ng/ml EGF for 3 minutes. The cells were lysed and immunoblot analysis for EGFR, phospho-Akt, and total phosphotyrosine was performed (Figure 24). Compared to wild type and kinase dead stable cells, the EGFR mutants were expressed to similar levels, except the double mutation of F992/F1173, which is expressed at very low levels (Figure 24). Interestingly, Akt was activated in response to EGF in all of the mutants, including the double F1068/F1086 mutant. Akt was not activated in kinase dead EGFR stable cells, confirming that EGFR kinase activity is required for its activation. These data suggest that none of the tyrosine mutations tested inhibit EGF stimulated PI3K activation. Total phosphotyrosine immunoblot analysis revealed that the double mutants F992/F1173 and F1068/F1086 show reduced phosphorylation of a high molecular weight band that most likely represents EGFR. The profile of EGF stimulated phosphotyrosine looked similar between wild type, F1045, F1068, and F1086 suggesting that these mutations do not have much effect on downstream signaling (Figure 24).

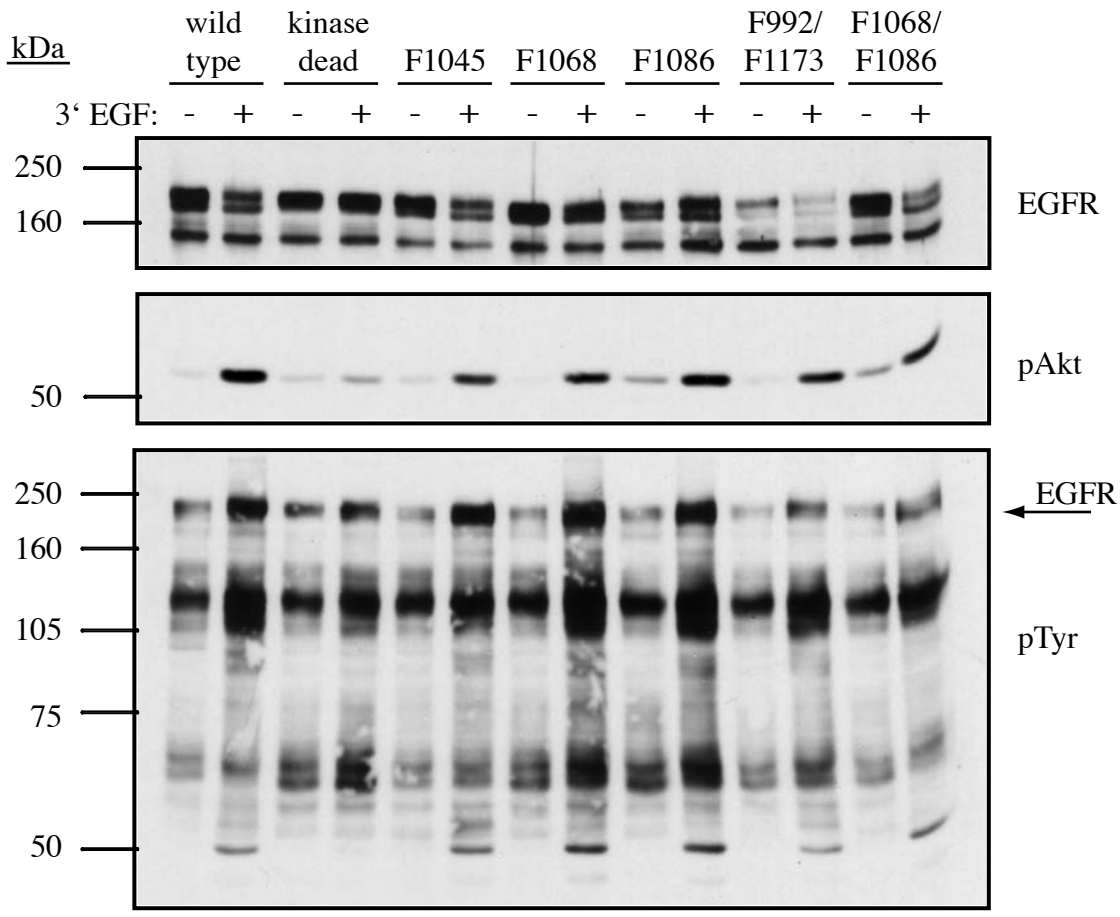


Figure 24. EGF treatment of $EGFR^{-/-}$ MCE stable cells expressing EGFR tyrosine to phenylalanine mutations. $EGFR^{-/-}$ MCE stable pools of wild type, kinase dead, or tyrosine to phenylalanine mutants F1045, F11068, F1086, F992/F1173, and F1068/F1086 cells were serum starved and then treated with or without 10ng/ml EGF for 3 minutes. Cell were lysed and Western blot analysis for total EGFR, phospho-AKT or phosphotyrosine was performed.

Based on the results from the signaling data, I hypothesized that the individual tyrosine mutations would not inhibit EGF stimulated migration. To determine the effect of these mutations on migration, YAMC, $EGFR^{-/-}$ MCE cells expressing vector, wild type, kinase dead, or tyrosine to phenylalanine mutations were subjected to wound healing assays. To allow comparison of the data from different wound healing assays, the fold increase in EGF stimulated wound closure over control was calculated. Using this calculation, the control wound closure is set at 1 and the change in wound closure with EGF is determined relative to control. Data from several different wound closure assays were then combined and the average fold increase in wound closure was calculated and graphed (Figure 25). Surprisingly, individual tyrosine mutations F1068, F1086, and F1045 significantly reduced EGF stimulated migration compared to wild type cells. Mutation of tyrosine 992 had no effect on EGF stimulated migration compared to control, suggesting that the inhibition is specific for individual tyrosines. F992/F1173 double mutation inhibited EGF stimulated migration, suggesting that tyrosine 1173 is also required for migration, but generation of the single F1173 mutant is needed to confirm this. These data suggest that although mutation of individual tyrosine residues does not inhibit EGF responsiveness (Figure 24), some of these tyrosine residues specifically contribute to cell migration (Figure 25), demonstrating the complexity of signaling that contributes to a migratory response.

Discussion

These experiments have dissected the pleiotropic effects of EGFR activation to identify specific pathways involved in EGF stimulated Rac activation and cell migration.

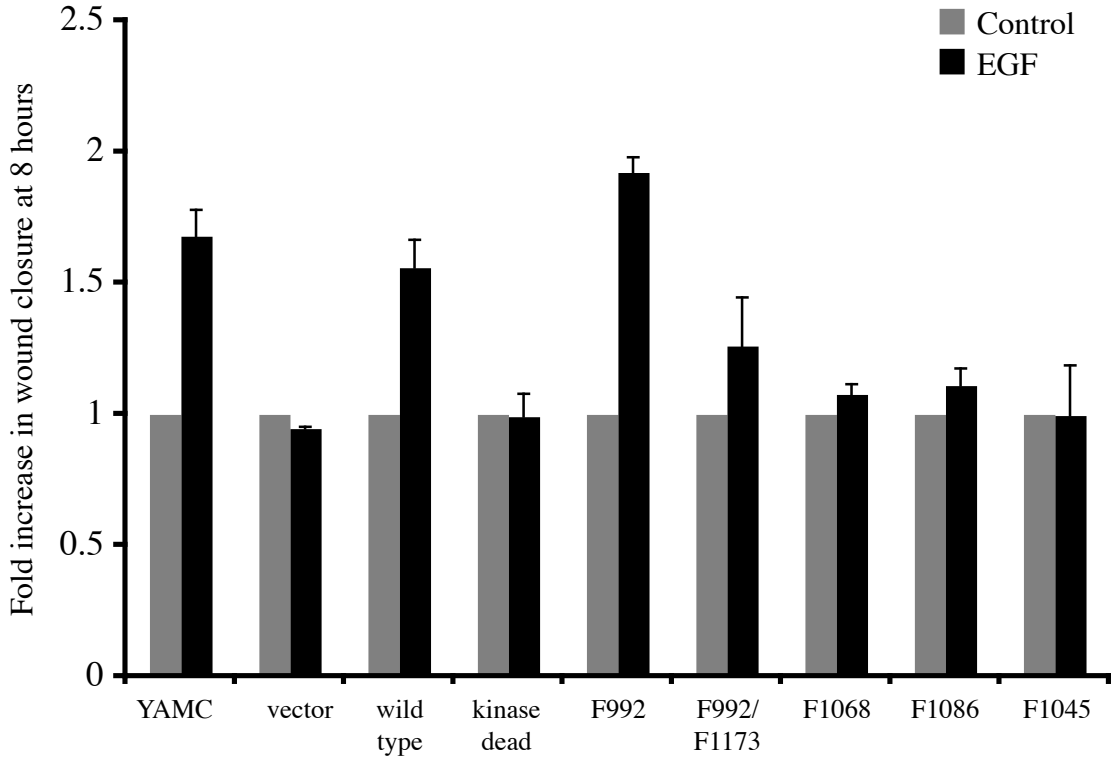


Figure 25. Migration of EGFR-/- MCE stable cells expressing EGFR tyrosine to phenylalanine mutations. YAMC cells or $EGFR^{-/-}$ stable pools expressing empty vector, wild type, kinase dead, or tyrosine to phenylalanine mutations F992, F992/F1173, F1068, F1086, and F1045 were serum starved, wounded in the absence or presence of 10 ng/ml EGF and the percent wound closure was calculated. The fold increase in migration was calculated by dividing the average percent wound closure in the presence of EGF by the average percent wound closure in the control cells. The control cell migration was set to a value of 1. Several different experiments were combined and the standard deviation was calculated to account for variability between experiments. The combined data from several different experiments is respresented.

The data presented in these studies demonstrate that both PI3K and Src family kinase activities are required for EGF stimulated Rac activation. However, PLCγ, PKC, Akt, and MEK 1/2 activities are not required for Rac activation, demonstrating the specificity of signaling through PI3K and Src activity to Rac. While several of the signaling pathways examined do not contribute to Rac activation, the complexity of signaling involved in EGFR-mediated cell migration is demonstrated by the inability of tyrosine to phenylalanine EGFR mutants to migrate in response to EGF. These data suggest that a complicated signaling network is required for EGF stimulated migration, of which Rac activation is one component. Because cell migration is a multi-step process that requires coordination of many different events to successfully more forward, inhibition of any of these steps is likely to result in decreased migration.

Previous data from our lab and others has shown that PLC activity and subsequent activation of PKCs are required for EGF stimulated migration (Chen et al., 1994b) (Polk, 1998). Consistent with these reports, mutation of the tyrosines on EGFR required for PLC binding, tyrosines 992 and 1173, results in decreased cell migration in response to EGF (Figure 25). However, my experiments using pharmacological inhibitors demonstrated that PLC and PKC are not involved in Rac activation in YAMC cells, suggesting that increased lamellipodia formation and Rac membrane translocation in response to EGF treatment occur independently of signaling pathways activated by PLC. Interestingly, a decrease in PtdIns 4,5-P_2 and an increase in IP_3 generation was observed at the leading edge of EGF stimulated fibroblasts and was proposed to be the result of increased PLC activity at the leading edge (Chou et al., 2002). Subsequent studies by the same group suggested that PLC activates Cdc42 to promote directed cell migration in

response to EGF (Chou et al., 2003). These data, in combination with my data, suggest a model in which PLC hydrolysis of PtdIns-4, 5-P_2 activates Cdc42 at the leading edge, while PI3K phosphorylation of PtdIns-4, 5-P_2 activates Rac at the leading edge, both promoting increased migration. Although both of these pathways are required for EGF stimulated migration, they appear to function in parallel because PI3K, but not PLC, is upstream of Rac activation. One possibility is that competition of these enzymes for a similar substrate, PtdIns-4, 5-P_2, at the membrane may determine the timing of which pathways are stimulated.

Since the initial studies in which EGF was described as a potent mitogen for cultured cells, much work has focused on characterizing the mechanism of EGFR-mediated proliferation. Recruitment and binding of the adaptor proteins Grb2 and Shc to the C-terminus of phosphorylated EGFR activates the small GTPase Ras, which in turn activates the canonical Ras-Raf-MEK-ERK kinase cascade, resulting in increased proliferation. Studies performed in fibroblasts and liver epithelial cells have demonstrated that increased cell motility induced by EGF occurs independently of increased proliferation (Chen et al., 1994a) (Geimer and Bade, 1991). Work from our lab has demonstrated that proliferation is reduced in EGF treated cells near the wound margin, suggesting that proliferation and migration are independent biological responses downstream of EGFR (Corredor et al., 2003). In agreement with these reports, pharmacological inhibition of the MAPK cascade using two different MEK 1/2 inhibitors, U0126 and PD98059, did not alter Rac activation in response to EGF (Figures 19A and 20), suggesting that the signaling pathways upstream of Rac activation are independent from proliferative signaling pathways. This is also consistent with the

kinetics of ERK 1/2 phosphorylation observed in Figure 11B, in which GTP-Rac stimulation occurs prior to ERK 1/2 activation. These data further support the idea that our cell culture model of wound healing mimics the *in vivo* response to injury in which restitution occurs rapidly, prior to and independent from the proliferative response observed during regeneration.

In these studies, I have demonstrated that PI3K is required for Rac activation, in a mechanism independent of Akt activation. When activated, PI3K generates a localized concentration of 3' phosphorylated phospholipids at the plasma membrane. The requirement for PI3K activity suggests that a PH-domain containing protein is required for Rac activation. While activity of the Rho GTPases is regulated by GDIs and GAPs, data suggest that the most important factor influencing activation is the regulation of GEFs (Schmidt and Hall, 2002). Currently 69 mammalian GEFs have been identified, most of which contain a characteristic pleckstrin homology (PH) domain and a Dbl homology (DH) domain, making them obvious candidates for the missing link downstream of PI3K (Rossman et al., 2005). However, the identity of the putative GEF involved in EGF stimulated Rac activation is currently unknown. Although a small number of GEFs have been well characterized, the remaining GEFs are either novel or their GTPase specificity has not been determined, making it difficult to predict which of the 85 family members are candidate GEFs for Rac. While tissue distribution of these proteins limits the number of GEFs present in any given cell type, we do not know which GEFs are expressed in YAMC cells. Microarray data from our lab confirms that mRNA for at least 15 different is GEFs is present in YAMC cells (data not shown). Additional

GEFs are likely expressed in YAMC cells because many of the known GEFs were not represented on the microarray chip used in this experiment.

The rapid and transient activation of Rho GTPases suggests that GEFs are tightly regulated; however, a common mechanism of regulation has not been established. Some of the GEFs, such as Vav-2, a GEF for Rho, Rac, and Cdc42 are activated by phosphorylation events, placing them at the top of the list for candidate GEFs involved in EGF stimulated Rac activation. In particular, Vav-2 has been shown to co-immunoprecipitate with activated EGFR and is tyrosine phosphorylated in response to EGF treatment of HeLa cells (Pandey *et al.*, 2000). Although Vav2 is expressed in YAMC cells, I was unable to detect either tyrosine phosphorylation of Vav2 or association of Vav2 with EGFR in response to EGF in YAMC cells (data not shown). GEF activation also requires its translocation to the plasma membrane through the GEF PH domain, which binds to 3' phosphorylated PtdIns generated at the plasma membrane when PI3K is activated. Based on my inhibitor studies, I propose that EGF stimulated PI3K activity recruits the GEF to the plasma membrane, bringing it into proximity with activated EGFR and Src, where it can be further activated through phosphorylation. The hematopoietic-specific GEF Vav1 is one example of a GEF that is regulated by Src family kinases. Phosphorylation of Vav1 at tyrosine 174 in the N-terminus by Src family kinases relieves an intramolecular inhibition and exposes the DH domain of the protein to facilitate its exchange activity (Aghazadeh et al., 2000). The synergistic effect of inhibiting both Src and PI3K suggests that these pathways independently contribute to activation of Rac (Figure 23). This supports a model in which generation of phospholipids at the plasma membrane can recruit and active the GEF to a small extent,

and likewise, Src phosphorylation can weakly activate the GEF. When both kinases are fully activated in response to EGF treatment, maximal GEF activation is achieved. Inhibition of PI3K does not inhibit activation of FAK phosphorylation (Figure 23), which is inhibited by all of the Src inhibitors used (Figure 21A), demonstrating that PI3K is not upstream of Src in this system. Similarly, inhibition of Src family kinases does not block Akt phosphorylation, demonstrating that PI3K activation is independent of Src (Figure 23).

An alternative model for these data is that phosphorylation of a Src-specific tyrosine residue within EGFR recruits and activates adaptor proteins required for Rac activation. EGF stimulated activation of Src results in phosphorylation of EGFR on tyrosines 845 and 1101 by Src (Biscardi et al., 1999a). It is possible that activation of Rac requires signaling through these tyrosine residues; however, generation of stable cells expressing a mutant F845/F1101 EGFR is necessary to test this. While mutational analysis of EGFR has revealed a number of tyrosines involved in EGF stimulated migration, these mutants have not yet been tested for their ability to activate Rac. Assessment of the ability of various mutants to activate Rac will further define the individual signaling pathways that contribute to EGF stimulated cell migration. For example, tyrosines 1068 and 1086 both bind to Grb2, suggesting that their binding partners are redundant (Figure 7). Surprisingly, mutation of these tyrosines individually inhibits EGF stimulated cell migration, suggesting a more complicated signaling network exists downstream of these tyrosine residues. As demonstrated in Figure 24, the signaling pathways disrupted by these tyrosine mutations have not been identified. Characterization of the specific signaling pathways downstream of these tyrosines will

further our understanding of the biological function of EGFR, as well as the role of these signaling pathways in growth factor stimulated motility.

CHAPTER V

FUTURE DIRECTIONS AND CONCLUSIONS

Future directions

Cell migration requires coordinated activation of several different Rho GTPases, namely, Rac, Rho, and Cdc42 (reviewed in (Raftopoulou and Hall, 2004)). In the experiments presented here, I demonstrate that the small GTPase Rac1 is required for EGF stimulated migration of intestinal epithelial cells. However, these studies do not address the role of Cdc42 and Rho in cell migration induced by EGF. While in some of my experiments weak Cdc42 activation was detected in response to EGF treatment (Figure 11A), I was not able to consistently detect this activation. The observation that filopodial extensions direct the formation of lamellipodia suggests that Cdc42 activation is upstream of Rac (Nobes and Hall, 1995). To address this question, I will utilize siRNA smart pools targeting Cdc42 to reduce the expression of Cdc42, followed by wound healing assays and Rac activation assays in the presence or absence of EGF. These experiments will determine whether EGFR can activate Rac in the absence of Cdc42 or if Cdc42 is required for EGFR to elicit these responses. Previous work in intestinal epithelial cells has demonstrated that the small GTPase Rho is required for EGF induced migration in a scratch wound assay (Santos et al., 1997). To determine if Rho is activated in YAMC cells, I will use a GST fusion protein of the Rhotekin Rho-binding domain to pull down active GTP-Rho from total cellular lysates after treatment with EGF

for various times (Ren et al., 1999). These studies will elucidate the contributions of other GTPases to EGF stimulated intestinal epithelial cell migration.

While we do not yet understand how the other GTPases contribute to EGF stimulated migration, I have demonstrated that Rac is rapidly and robustly activated in YAMC cells in response to EGF treatment. This activation likely occurs through the activation of a GEF downstream of EGFR; however, the identity of the GEF involved in EGF stimulated Rac activation in YAMC cells is unknown. A new method to identify activated GAPs and GEFs from total cell lysates has just been described (Garcia-Mata et al., 2006). In this assay, a GST-fusion protein encoding a mutated Rho GTPase specifically binds to activated GAPs or GEFs. The activated GEF or GAP is then identified either by Western blot analysis for candidate proteins, or by mass spectrometry. I will use this assay to identify Rac-specific GEFs that are activated in response to EGF treatment. If the GEF can be identified, further studies will be performed to characterize the Rho GTPase specificity of this GEF. Because my studies suggest that Src and EGFR kinase activity are required for Rac activation, I would also like to determine if the GEF is regulated by tyrosine phosphorylation. The role of this GEF in EGF stimulated cell migration will be determined using siRNA directed against the GEF to reduce the expression of this protein and wound healing assays will be performed. I can also use siRNA experiments to determine the role of this GEF in Rac activation in response to wounding, EGF treatment, or the combination of the two. These studies will determine if the same GEF is involved in basal and EGF stimulated migration, providing further insight into the mechanism by which EGF activates Rac.

As described in these studies, we have recently generated a panel of stable cell lines expressing tyrosine to phenylalanine EGFR mutants in cells devoid of endogenous EGFR. Much work needs to be done to further characterize the signaling pathways, if any, that are disrupted by these mutations. In particular, the inability of several different mutants to migrate in response to EGF suggests that signaling pathways downstream of these individual sites are required for cell migration. Determining the signaling pathways altered by these mutations will contribute to our understanding of signaling through EGFR as well as our knowledge of the how cell migration is regulated by EGFR. As many different pathways contribute to EGF stimulated migration, I will assess the ability of each of these mutants to activate Rac in response to EGF to parse out the tyrosines required for Rac versus other migratory signals. These mutants will also be assessed for their ability to proliferate in response to EGF treatment in an attempt to identify downstream signaling pathways required for migration, proliferation, or both processes. These experiments will expand my current studies to further characterize the mechanisms that regulate cell migration downstream of EGFR activation.

Conclusions

The process of cell migration is critical throughout the human life cycle, and in particular, tight regulation of cell migration is required to maintain a healthy gastrointestinal tract. In this study, I have extended our previous observations of EGF stimulated wound closure and identified a mechanism by which EGFR accelerates cell migration. I demonstrate that the small GTPase Rac is required for EGF stimulated cell migration in a wound healing assay that mimics the process of restitution in response to

injury. YAMC cells exposed to EGF rapidly activate Rac and exhibit increased lamellipodial extensions at the wound margin, resulting in increased cell motility. Two downstream targets of EGFR, Src family kinases and PI3K, are required to activate Rac in response to EGF treatment. These data suggest a novel signaling cascade downstream of EGFR activation leading to a rapid increase in cell motility that is independent of the proliferative (ERK 1/2) and cell survival (Akt) pathways stimulated upon EGFR activation. These data are consistent with the current model of intestinal repair in which restitution occurs immediately after wounding to restore the epithelial integrity, followed by a phase of regeneration to repopulate the damaged area (Okamoto and Watanabe, 2005). Together, these data demonstrate a novel pathway whereby EGFR activation increases wound closure through the synergism of two different kinase cascades that induce actin-based motility via the small GTPase Rac. These data contribute to our knowledge of the complex molecular mechanisms that control intestinal epithelial repair with implications for understanding the role of growth factor regulation of this small GTPase in intestinal cellular migration and differentiation programs *in vivo*. Elucidation of the mechanisms that regulate intestinal epithelial cell migration will allow the development of more specific therapeutic treatments for inflammatory bowel diseases.

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