NOTE TO USERS

University of Alberta

The role of receptor dimerization in epidermal growth factor receptor-mediated endocytosis and mitogenesis

by

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Abstract

Binding of Epidermal Growth Factor (EGF) to EGF receptor (EGFR) at the cell surface induces dimerization of EGFR, which results in the activation of EGFR intrinsic tyrosine kinase and trans-autophosphorylation of EGFR. Little is known whether ligand binding is needed for all post-binding events or simply required for EGFR dimerization and whether EGFR dimerization is sufficient to stimulate EGFR-mediated signal transduction and endocytosis. In this thesis, we dimerize the transmembrane and cytoplasmic domains of EGFR through the use of leucine zippers (LZ). We show that the chimeric LZ-EGFR-GFP is constitutively dimerized and activated as ligand-induced phosphorylated EGFR, localizing in both the plasma membrane and endosomes, suggesting endocytosis. Moreover, LZ-EGFR-GFP is able to activate several signaling proteins including SHC, PLCγ1, ERK and Akt. We conclude that non-ligand induced dimerization is sufficient to activate EGFR and initiate cell signaling and EGFR endocytosis.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

α-	Anti specific antibody
AG1478	EGFR specific tyrphostine tyrosine kinase inhibitor
Akt	Protein kinase B
AP-1	Activator protein 1
ATG	Start codon alanine-threonine-glycine
BrdU	Bromo-deoxy-uridine.
Cbl	Casitas B-lineage lymphoma
c-Fos	Subunit of AP-1 transcription complex
c-Jun	Subunit of AP-1 transcription complex
CT	Carboxyl terminal
DNA	Deoxyribonucleic acid
DsRed	Discostoma species red fluorescent protein
EEA1	Early endosomal autoantigen one
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFRvIII	Epidermal growth factor deletion variant III
ERK	Extracellular signal-regulated kinase
GFP	Green fluorescent protein
GH	Growth hormone
GHR	Growth hormone receptor
Grb2	Growth factor receptor bound protein 2
GTP	Guanine triphosphate
IB	Immunoblot
IP	Immunoprecipitation
kD	kilo Daltons
LZ	Leucine zipper
PI3K	Phosphatidyl-inositol 3-kinase
PLCγ-1	Phospho-lipase C gamma one
pY	Phosphorylated tyrosine
p85	85 kD subunit of phosphatidylinositol 3-kinase
Rab5	Rab monomeric GTPase
pEGFR	Phosphorylated epidermal growth factor
RTK	Receptor tyrosine kinase
pTyr	Phosphorylated tyrosine
SF	Serum free
SHC	Src homologous and collagen like protein
TGF-β	Transforming growth factor beta
TM	Epidermal growth factor receptor transmembrane domain
TK	EGFR intrinsic tyrosine kinase domain.
Wt	Wild type

1.0 Introduction

1.1 EGFR subfamily of receptor tyrosine kinases

Activated receptor tyrosine kinases (RTKs) induce cell proliferation and differentiation by stimulating various signaling pathways (Schlessinger and Ullrich, 1992). The activated RTKs may also be rapidly internalized into the endocytic compartments and degraded in lysosomes (Carpenter, 1987). All RTKs have a similar molecular signature, possessing a large glycosylated extracellular ligand binding domain, a single hydrophobic transmembrane region, and a cytoplasmic domain that contains a tyrosine kinase catalytic domain. The receptor for epidermal growth factor (EGF) is the prototype for a subfamily of structurally related proteins; termed class I/ErbB receptors, that mediate the proliferation and differentiation of normal cells (Schlessinger, 1994; Carraway and Cantley, 1994). Following the identification of the EGF receptor (EGFR), three other members of the ErbB receptor subfamily were identified, these include: ErbB2 (Bargmann *et al.*, 1986; Yamamoto *et al.*, 1986) ErbB3 (Kraus *et al.*, 1989), and ErbB4 (Plowman *et al.*, 1993). The ErbB receptors play important roles in the development of various cancers, especially breast cancer (Blume-Jensen and Hunter, 2001).

EGFR is a 170 kD transmembrane glycoprotein of a single polypeptide chain. The heavily glycosylated 622-amino extracellular domain containing two cysteine rich regions is responsible for ligand binding. The transmembrane

domain is a single 23 residue α-helical transmembrane peptide. The 542-residue intracellular cytoplasmic domain contains a 250-amino acid conserved protein tyrosine kinase core followed by a 229-residue carboxy-terminal tail with regulatory tyrosine residues (Coussens *et al.*, 1985; Martin-Fernandez *et al.*, 2002; Schlessinger, 2002).

1.2 Regulation of EGFR-mediated signal transduction

Binding of EGF at the cell surface induces dimerization of EGFR, which results in the activation of EGFR tyrosine kinase activity and receptor trans-autophosphorylation (Carpenter, 1987; Schlessinger and Ullrich, 1992). Phosphorylated carboxy-terminal tyrosine residues in activated EGFR interact with downstream signaling proteins to form large signaling complexes. The receptor-signaling protein complexes then initiate the activation of various signaling pathways and eventually stimulate cell proliferation and survival.

The signaling proteins that bind EGFRs often contain related sequences of 50-100 amino acids in length referred to as Src-homology 2 (SH2), Src-homology 3 (SH3), phosphotyrosine binding (PTB) and Pleckstrin-homology (PH) domains. Each of these domains can fold into a compact and functional module independent of surrounding sequences (Mayer *et al.*, 1988; Pawson, 1995); (Sadowski *et al.*, 1986; Tyers *et al.*, 1988). SH2 and SH3 domains recognize short

peptide motifs bearing phosphotyrosine (pY) or polyproline PxxP core motifs, respectively. PH domains may associate with phospholipids or bind other specific proteins (Pawson, 1995). Various combinations of SH2, SH3 and PH domains are frequently found in the same signaling proteins. SH3 and PH domains can collaborate with SH2 domains in forming signaling complexes downstream of EGFRs.

Among the signaling proteins, Grb2 is an adapter protein that includes one SH2 and two SH3 domains. Grb2 activates Ras via a direct physical linkage with the activated receptor and the Ras guanine nucleotide exchange factor Sos (Lowenstein *et al.*, 1992; Buday and Downward, 1993; Egan *et al.*, 1993; Gale *et al.*, 1993; Li *et al.*, 1993). Grb2 binds directly to the EGFRs at Y1068, to a lesser extent at Y1086, and indirectly through SHC at Y992 and Y1173 (Batzer *et al.*, 1994) (Fig. 1). Activated EGFRs are also recognized by phosphatidy-inositol-3'-kinase (PI3K). PI3K is composed of a regulatory subunit, p85, and a catalytic subunit, p110. The regulatory subunit p85 is equipped with two SH2 domains and one SH3 domain and has been shown to co-immunoprecipitate with EGFRs in response to EGF stimulation (Soler *et al.*, 1994; Hu *et al.*, 1992; McGlade *et al.*, 1993). However, different from β-PDGF receptors (β-PDGFRs), no specific binding site on EGFRs has been determined yet (Panayotou *et al.*, 1992). PI3K phosphorylates the D3 position of

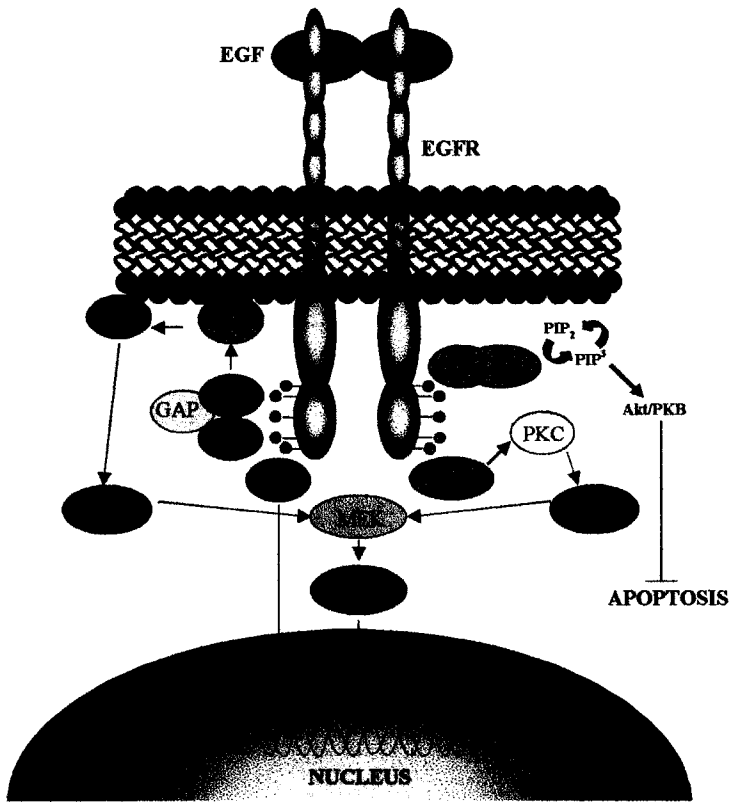


Figure 1. Model of activated EGFR-dependant signaling pathways. Signal transduction is initiated following EGF dependant dimerization and activation of EGFR. Tyrosine kinase domain dependant phosphorylation of five major C-terminal tyrosine residues act as docking sites for two predominant signaling proteins that will lead to the activation of downstream signaling pathways. These signaling pathways will eventually lead to the activation of distinct transcription factors within the nucleus of the cell.

phosphatidylinositol (PI), PI-4-phosphate (PI-4-P) and PI-4,5-P2 to produce PI-3-P, PI-3,4-P2 and PI-3,4,5-P3. Other signaling proteins, including SHC, PLC-γ and GAP also interact directly with EGFRs through their SH2 domains (Pelicci *et al.*, 1992; Wahl and Carpenter, 1991; Rotin *et al.*, 1992; Boguski and McCormick, 1993; Fantl *et al.*, 1992). Formation of the receptor-signaling protein complexes then initiates the activation of various signaling pathways (Fig. 1). For example, the interaction between EGFR and SHC/Grb2 results in the recruitment of Sos to the plasma membrane to activate Ras. Activated Ras mediates Raf activation which then phosphorylates and activates mitogen-activated protein kinase kinase (MEK) and extracellular-signal-regulated kinase (ERK), leading to the activation of the transcriptional factors c-Fos and c-Jun (Rotin *et al.*, 1992; Schlessinger and Ullrich, 1992; Soler *et al.*, 1994). This "Ras pathway" has been elucidated and is necessary for growth factor (GF)-stimulated DNA synthesis. Activated EGFR also stimulates a Ras-independent "Src pathway" that leads to activation of the transcriptional factor Myc and is also required for GF-induced DNA synthesis (Kraus *et al.*, 1987). In addition, microinjection studies with a dominant negative PLC-γ1with non-catalytic SH2-SH2-SH3 receptor-binding domains lead to a decreased mitogenic response upon EGF stimulation, emphasizing the requirement of PLC-γ1 (Wang *et al.*, 1998). Activation of PI3K by EGFR stimulates the Akt/PKB pathway promoting cell survival via the inactivation of a series of pro-apoptotic proteins (Gilman *et al.*, 1986).

1.3 Regulation of EGFR endocytosis

In addition to the activation of various signaling pathways, binding of EGF rapidly triggers the clustering and internalization of ligand-receptor complexes (Fig. 2). Receptor-mediated endocytosis is a multiple step event (Goldstein *et al.*, 1985) which in general, consists of two stages: 1) internalization, which covers the steps from targeting receptors to coated pits to the formation of coated vesicles and 2) intracellular trafficking, covering the steps after the receptors enter the cell. Several proteins or protein complexes, including adaptin AP-2, clathrin and dynamin participate in the internalization of all of receptors. Adaptin AP-2 is a cytoplasmic protein complex that interacts with the cytoplasmic tails of various receptors (Robinson, 1994). These interactions are thought to account for the ability of the cell to selectively direct receptors to clathrin-coated vesicles (Zaremba and Keen, 1983). The clathrin molecule is composed of three heavy chains and three light chains, joined together to form a three-legged assembly called a triskelion. The cell then utilizes these triskelion modules to form polygonal scaffolds. These scaffolds, when attached to a membrane cause the membrane to deform into a budding vesicle. Clathrin presumably binds to the membrane by interacting with membrane-bound adaptin AP-2 (Robinson, 1994). Dynamin has only recently been identified as a major player in the endocytic pathway (van der Bliek *et al.*, 1993; Chen *et al.*, 1991). It is composed of three GTP binding elements at the N-terminal and two overlapping consensus

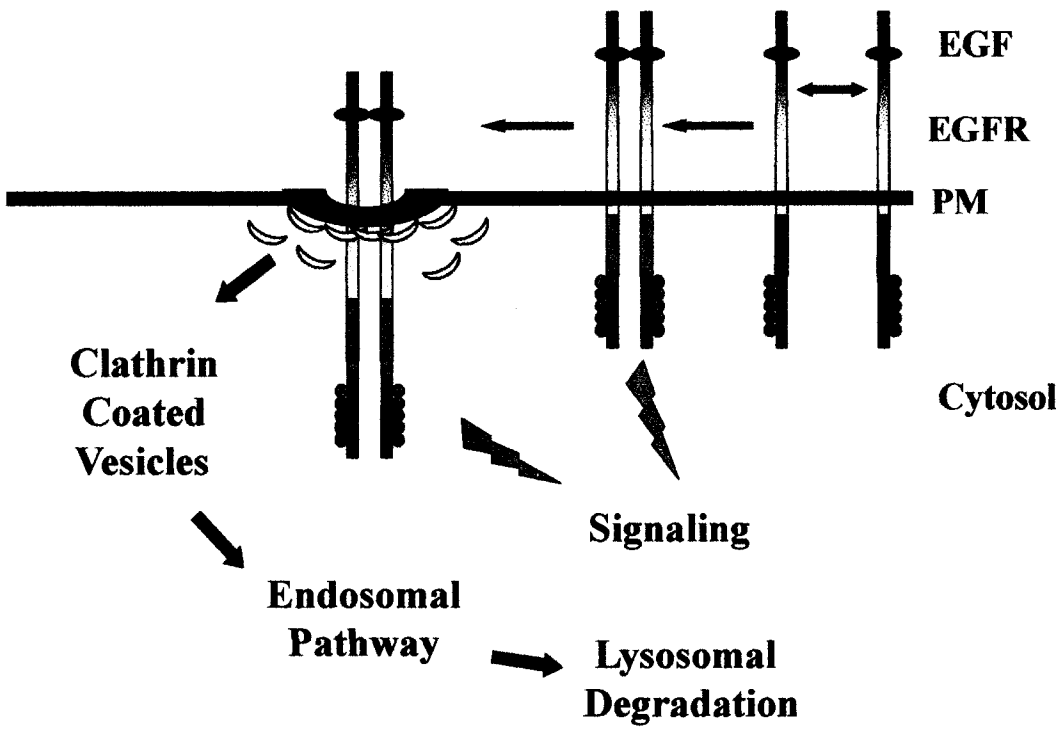


Figure 2. Model of EGFR receptor-mediated endocytosis. The endocytosis of the dimeric EGFR is mediated through clathrin coats (yellow) recruited to the plasma membrane and the formation of clathrin-coated vesicles (CCVs). The eventual fate of internalized dimeric complexes is their transport by early to late endosomes and fusion with lysosomes.

sequences for SH3 domain binding at the C-terminal, separated by a PH domain. Expression of mutant dynamin specifically blocks endocytic vesicle formation in a dominant-negative manner (Herskovits *et al.*, 1993; Damke *et al.*, 1994).

Intracellular trafficking of receptors involves a series of membrane budding and fusion events (Rothman, 1994). These are regulated by specific cytosolic and membrane-associated protein factors, including two groups of Ras-like small guanosine triphosphatases (GTPases) called Rabs (Valencia *et al.*, 1991) and adenosine diphosphate (ADP)-ribosylation factors (ARFs) (Serafini *et al.*, 1991; Clark *et al.*, 1993; Tsuchiya *et al.*, 1991). The 60 different Rab GTPases constitute the largest and most diverse group of the Ras-like small G-proteins. The current view is that Rabs are involved in the specification of the "correctness" of membrane-membrane interactions at the level of docking and fusion, or both (Zerial and Stenmark, 1993). For example, both Rab4 and Rab5 localize to early endosomes with Rab4 controling the function or formation of endosomes involved in recycling (van der Sluijs *et al.*, 1992; Bottger *et al.*, 1996) while Rab5 controls membrane traffic in the early endocytic pathway (Bucci *et al.*, 1992; Stenmark *et al.*, 1994; Rybin *et al.*, 1996). Another GTPase of interest is Rab7, which functions downstream of Rab5 and regulates membrane transport leading from early to later endosomes or to lysosomes (Feng *et al.*, 1995; Bottger *et al.*, 1996).

Binding of EGF results in the clustering and internalization of EGFR. The accumulation of EGF and EGFR can be detected in early endosomes after 1-5 min incubation with EGF at 37°C. EGF and EGFR accumulate in late endosomes after 10-20 min at 37°C. A substantial number of EGFR can be detected in organelles with typical biochemical and morphological features of mature lysosomes only after 40-60 min of continuous internalization at 37°C (Schlessinger, 1986; Carpenter, 1987; Sorkin and Carpenter, 1993). Since ligand binding is essential for the rapid internalization of EGFRs, the events resulting from ligand binding likely contribute to the regulation of ligand-induced EGFR internalization. Indeed, it is thought that the normal endocytosis and down-regulation of EGFRs require the activation of the intrinsic protein kinase and autophosphorylation (Chen *et al.*, 1987; Honegger *et al.*, 1987; Helin and Beguinot, 1991; Sorkin *et al.*, 1991; Sorkin *et al.*, 1992). The question of how the EGFR tyrosine kinase activity is ultimately required for its internalization remains unanswered (Glenney, Jr. *et al.*, 1988; Chen *et al.*, 1989; Wiley *et al.*, 1991; Honegger *et al.*, 1990; Felder *et al.*, 1990; Felder *et al.*, 1992; Sorkin *et al.*, 1993). Simultaneous point mutation of EGFR's five-tyrosine residues (Y992, Y1068, Y1086, Y1148 and Y1173) to phenylalanines reduces the internalization rate to a minimum (one quarter of the wild-type EGFR) and reduces receptor phosphorylation of the *in vivo* specific substrate PLC-γ1 to less than 50% compared to the wild-type receptor (Sorkin *et al.*, 1992). A 15-amino acid domain (residues 943-957) was found to be essential

for binding sorting nexin-1 (SNX1) that is involved in targeting EGFR to lysosomes (Kurten *et al.*, 1996). We have shown that binding of Grb2 to EGFR is required for the normal endocytosis and down-regulation of EGFR (Wang and Moran, 1996). While PI3K is required for β-PDGFR endocytosis and down-regulation (Joly *et al.*, 1995; Shpetner *et al.*, 1996), we recently suggested that activated EGFR endocytosis is controlled by a novel endosome fusion pathway regulated by Rab5 in the absence of PI3K activity rather than a Rab5-PI3K co-regulated endosome fusion pathway (Chen and Wang, 2001a; Chen and Wang, 2001b). A prominent role in mediating ligand-dependant downregulation of EGFR is the ubiquitin ligase Cbl. Over expression of the wild-type Cbl in Chinese hamster ovary cells leads to increased ligand-dependent EGFR ubiqitination, internalization and degradation (Levkowitz *et al.*, 1998). Amongst all Cbl functional domains, of interest is an evolutionary conserved RING finger domain that recruits ubiquitin-conjugating enzymes. It has been revealed that the RING finger regulates endocytic sorting of EGFR and desensitization of signal transduction (Jiang and Sorkin, 2003)

1.4 EGFR dimerization and its role in EGFR-mediated signal transduction and endocytosis

The extracellular domain of the EGFR is further divided into four subdomains I, II, III and IV. It was initially proposed that subdomains I and III

form the ligand binding region and the other two subdomains mediate receptor dimerization and interactions with other membrane proteins (Lax *et al.*, 1989). It has recently been suggested, based on crystal structures of the extracellular domain of the ErbB family, that domain IV regulates ligand-binding affinity by intramolecular interactions with domain II. These interactions between domains IV and II lead to the auto-inhibition of the receptor in the absence of ligand that constrains the relative orientation of domains I and III (Cho and Leahy, 2002).

The EGF receptor (EGFR) was the first RTK shown to dimerize after ligand binding. Dimerization of EGFR requires the binding of two molecules of EGF to two EGFR molecules in a 2:2 EGF: EGFR complex formed from stable intermediates of 1:1 EGF: EGFR complexes (Lemmon *et al.*, 1997). Recent crystallization studies of the entire extracellular domain of EGFR in complex with EGF, provide views of a 2:2 receptor: ligand complex (Ogiso *et al.*, 2002).

Little is known however, whether ligand binding is required for all post-binding events or if ligand binding is only required for dimerization and the dimerization is then sufficient to stimulate kinase activation, autophosphorylation and the binding of downstream proteins. It has been shown that inhibition of EGF-stimulated dimerization of EGFR via a receptor extracellular domain specific monoclonal antibody does not impair receptor

autophosphorylation or transmembrane signaling (Carraway and Cerione, 1993), which suggests that dimerization is not necessary for the activation of EGFR and downstream signaling. On the other hand, it has been reported that the ligand deficient oncogenic ErbB2, which is characterized by a single amino acid substitution (V664E) in its transmembrane domain, is constitutively dimerized and permanently active (Bargmann *et al.*, 1986; Stern *et al.*, 1988; Weiner *et al.*, 1989). It binds various downstream signaling proteins including Grb2 and Shc (Dankort *et al.*, 1997) and this activated oncogenic ErbB2 homodimer is internalized as ligand-activated EGFR (Gilboa *et al.*, 1995). We recently showed that in the presence of tyrphostine AG1478 to inhibit EGFR kinase activity, EGF is sufficient to stimulate EGFR endocytosis (Wang *et al.*, 2002). These results suggest that receptor dimerization is sufficient to activate the receptor and to mediate downstream events.

Studies of the type-III deletion variant of the EGFR (EGFRvIII) also yield controversial results regarding the role of dimerization on EGFR trafficking and signaling. EGFRvIII is devoid of amino acids 6-273 from the extracellular domain and is constitutively active. One study suggests that EGFRvIII is not dimerized, following crosslinking attempts with two different chemical crosslinkers with different chemical mechanisms and linker geometries. EGFRvIII however. is able to activate several signaling pathways and transform

cells (Chu *et al.*, 1997). On the other hand, other studies show that EGFRvIII is present as a higher molecular form implying dimerization, following sucrose sedimentation analysis of crosslinked WGA-agarose-purified receptors (Moscatello *et al.*, 1996; Fernandes *et al.*, 2001).

1.5 Leucine zipper structure, function and application

A leucine zipper is an alpha helix with leucine residues (generally five) presented at every seventh position along its length. Two opposing helices interdigitate, creating an interface that excludes water. In solution, strong hydrophobic forces from juxtaposed leucines aid in the formation of high affinity dimers between complimentary zippers. Leucine zippers are found as part of certain transcription factors, such as c-Fos and c-Jun, that form the AP-1 transcription complex (O'Shea *et al.*, 1989).

Recently, it was reported that dimerization of growth hormone receptor (GHR) with leucine zipper domain resulted in the constitutive activation of GHR. The entire extracellular domain of the growth hormone receptor (GHR) was replaced by the leucine zipper sequence of either the c-Fos or c-Jun (termed Fos-GHR and Jun-GHR, respectively). This replacement resulted in the forced dimerization of the transmembrane and cytoplasmic domains of the GHR in the absence of the extracellular domain leading to the constitutive activation of known growth hormone (GH) signaling end points (Behncken *et*

al., 2000).

Additional studies furthermore concluded that leucine zipper domains could functionally substitute the extracellular domains of the granulocyte-macrophage colony stimulating factor receptor subunits (GM-Rα/ GM-Rβ). They also concluded that homodimers of the cytoplasmic domain of the human interferon γ receptor β subunit (hIFNγRβ) maintained via an extracellular leucine zipper domain transduced a proliferative response in mouse cells (Patel *et al.*, 1996).

1.6 Rationales, hypothesis and objectives

The early events following ligand binding including receptor dimerization, autophosphorylation and association with various binding proteins are essential for EGFR to initiate cell signaling and for its endocytosis to lysosome for degradation. However, little is known whether the ligand binding is required for all of these post-binding events or ligand binding is only required for dimerization and the dimerization is then sufficient to stimulate the kinase activation, autophosphorylation and the binding of downstream proteins. Our hypothesis is that the dimerization of EGFR is sufficient to fully activate the receptor and initiates the trafficking and signaling of EGFR. To test this hypothesis, we seek to establish whether constitutive activation can be achieved simply by dimerizing the transmembrane and cytoplasmic domains

through the use of leucine zippers (LZ). We then determined if the constitutively dimerized LZ-EGFR-GFP is activated and localized in both the plasma membrane and endosomes, suggestive of endocytosis. We also determined if LZ-EGFR-GFP is capable of activating several signaling pathways including SHC, PLCγ1, ERK and Akt.

2.0 Materials and Methods

2.1 Materials

All chemicals, enzymes and kits were used according to the manufacturers' specifications and in accordance with protocols set out by the Environmental Health and Safety of the University of Alberta and WHMIS.

2.1.1 Chemicals and Reagents

acetic acid, glacial	BDH
acrylamide/Bis	Biorad
AEBSF	Sigma
ammonium persulfate	BDH
aprotinin	Sigma
agar	Gibco
agarose	Gibco
bacto- tryptone	DIFCO
N, N-bis[2hydroxyethyl]-2-aminoethanesulfonic acid (BES)	Sigma
disulfosuccinimidyl suberate (DSS)	Calbiochem
bromodeoxy uridine (BrdU)	Amersham
bromophenol blue	BioRad
calcium chloride	Sigma
coomasie brilliant blue, G250	BioRad
dimethyl sulfoxide	Fisher
dulbecco's modified eagle medium (DMEM)	Gibco
ethylene glycol-bis (β-amino ethyl ether tetra acetic acid) (EGTA)	Sigma
epidermal growth factor	Upstate
ethanol, 95%	Fisher
ethidium bromide	OmniPur
fetal bovine serum	Sigma
glucose	EM Science
glycerol	BDH
glycine	BioRad
hydrochloric acid	Fisher
isopropanol	Fisher
kanamycin	Sigma

Luria-Bertani media, broth base	Gibco
magnesium chloride	BDH
manganese chloride	Fisher
β–mercaptoethanol	Sigma
methanol	Fisher
nonidet P40	BDH
pepstatin A	Sigma
phosphate buffered saline, 10X	OmniPur
PIPES	Sigma
potassium chloride	BDH
skim milk	
sodium azide	Sigma
sodium chloride	BDH
sodium dodecyl sulfate (SDS)	BioRad
sodium fluoride	Sigma
sodium orthovanadate	Sigma
sucrose	BioBasic
tetramethylethylenediamine (TEMED)	Gibco
tris(hydroxymethyl)aminomethane	BioRad
Triton X-100	BDH
tween 20 (polyoxyethylene 20-sorbitan monolaurate)	Fisher
tyrphostine AG1478	Calbiochem
yeast extract, select	Gibco

2.1.2 Other Materials

Medical X-ray Film	Fuji
Transblot Nitrocellulose	BioRad
Whatman Chromatography Paper	Fisherbrand

2.1.3 Enzymes

DNA ligase, T4	GibcoBRL
Hot Start Taq, DNA polymerase	Qiagen
dNTPs	Invitrogen
Restriction endonucleases	GibcoBRL
RNAse	Sigma

2.1.4 Experimental Kits

QIAprep Spin Miniprep Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
HiSpeed Plasmid Midi Kit	Qiagen
SuperSignal ECL Western Blotting Detection Reagents	Pierce
TOPO-XL PCR cloning kit	Invitrogen

2.1.5 Plasmids

pCR-XL-TOPO	Invitrogen
pDsRed-C1	Clontech
pEGFP-N3	Clontech

2.1.6 Antibodies

2.1.6.1 Primary Antibodies

Rabbit anti-EGFR (1005)	Santa Cruz
Rabbit anti-phospho-EGFR (py992)	Santa Cruz
Rabbit anti-phospho-EGFR (py1068)	Santa Cruz
Rabbit anti-phospho-EGFR (py1086)	Santa Cruz
Rabbit anti-phospho-EGFR (py1148)	Santa Cruz
Rabbit anti-phospho-EGFR (py1173)	Santa Cruz
Rabbit anti-Shc	Santa Cruz
Rabbit anti-PLCγ1	Santa Cruz
Rabbit anti-phospho-PLCγ1	Santa Cruz
Rabbit anti-GFP (living)	Clontech
Rabbit anti-Akt	Santa Cruz
Rabbit anti-phospho-Akt (ser-473)	New England
Mouse anti-phospho-EGFR	Upstate
Mouse anti-phospho-tyrosine (py99)	Santa Cruz
Mouse anti- Erk1/2	Santa Cruz
Mouse anti- phospho-Erk1/2	Santa Cruz
Mouse anti-EEA1	BD Pharmigen
Mouse anti-BrdU	Amersham

2.1.6.2 Secondary Antibodies

Rhodamine-conjugated anti-mouse	Jackson ImmunoResearch
Horseradish peroxidase (HRP)-conjugated anti-rabbit	Bio-Rad
Horseradish peroxidase (HRP)-conjugated anti-mouse	Bio-Rad

2.1.7 Molecular Size Markers

1kb DNA ladder	Gibco
Prestained markers for SDS-PAGE	Sigma

2.1.8 Buffers and Other Solutions

A list of all buffers and solutions used in this study is provided in Table 2.1

2.1.9 Oligonucleotides

A list of all oligonucleotides and their manufacturers is included in Table 2.2

Table 2.1 Buffers and other solutions used in this study

Solution	Composition
BES buffer	N,N-bis [2hydroxyethyl]-2-aminoethane sulfonic acid)
Homogenization buffer	20mM Tris-Cl, pH7.0, 1mM $MgCl_2$, 4mM NaF
Immunoprecipitation buffer	20 mM Tris, pH7.5, 150 mM NaCl, 1% NP40, 0.1% sodium deoxycholate
Phosphate-buffered saline (PBS)	137mM NaCl, 2.7mM KCl, 10mM phosphate buffer
SDS-loading buffer	250mM Tris-Cl, 40% glycerol, 8% sodium dodecyl sulfate, 20% β-mercaptoethanol, 2% bromophenol blue
SOC medium	2% bacto tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose
Transfer buffer	48mM Tris-Cl, 39mM Glycine, 20% methanol, 0.03% sodium dodecyl sulfate
Transformation buffer	10mM Pipes, 55mM $MnCl_2$, 15mM$CaCl_2$, 250mM KCl
Triton X-100 lysis buffer	0.4% triton X-100, 140mM NaCl, 50 mM Tris-Cl, pH 7.2, 1mM EGTA

Table 2.2 Oligonucleotides used in this study

Name	Sequence	Manufacturer
c-Fos-1	5'-CTG ACA GAT ACA CTC CAA GCG GAGACAGAT CAA CTT GAA GAT GAG AAG TCT GCG TTG CAG ACT GAG ATT GCC AAT CTG CTG AAA GAG AAG GAA AAA CTG GAG TTT ATT TTG GCA GCC CAC G-3'	ACGT Corporation
c-Fos-2	5'-TCG ACG TGG GCT GCC AAA ATA AAC TCC AGT TTT TCC TTC TCT TTC AGC AGA TTG GCA ATC TCA GTC TGC AAC GCA GAC TTC TCA TCT TCA AGT TGA TCT GTC TCC GCT TGG AGT GTA TCT GTC AGG TAC-3'	ACGT Corporation
c-Fos-leading	5'-GCC AAG CTT GAC AGA TAC ACT CCA A-3'	GIBCO BRL.
c-Fos-lagging	5'-GAT GTC GAC GTG GGC TGC CAA AAT-3'	GIBCO BRL.
Start-EGFR-leading	5'-CTC GAG ATG CGA CCC TCC-3'	GIBCO BRL.
Start-EGFR-lagging	5'-GAA GCT TAG CCC GAC TCG-3'	GIBCO BRL.
EGFR-IC2	5'-GTA GGT ACC TGC TCC AAT AAA TTC ACT GCT TTG-3'	ACGT Corporation

2.2 Methods

2.2.1 Plasmids

All molecular cloning was performed essentially as described (Maniatis et al., 1989). A mutant of GFP, enhanced GFP (Clontech, Palo Alto, CA) was attached to the intracellular carboxyl terminus of human EGFR by standard recombinant techniques. Briefly, the chimeric EGFR-GFP vector was engineered by inserting in frame the complete EGFR (corresponding to amino acids –24- 1210 according to (Ullrich *et al.*, 1984)) into the pEGFP-N3 mammalian expression vector (Clontech, Palo Alto, CA). A XhoI site was introduced into the 5' end and a KpnI site was introduced into the 3' end of the complete EGFR, by polymerase chain reaction (PCR). The fragment was then ligated and inserted in frame into the pEGFP-N3 mammalian expression vector (Clontech, Palo Alto, CA). The chimeric LZ-EGFR-GFP receptor was engineered by joining the EGFR signal sequence (corresponding to amino acids -24-1 according to (Ullrich *et al.*, 1984) to the c-Fos leucine zipper domain (corresponding to amino acids 160- 200 according to (Van Beveren *et al.*, 1983)) to the EGFR transmembrane and intracellular domain (corresponding to amino acid positions 623-1210 according to (Ullrich *et al.*, 1984)), excised directly from the expression vector pEGFP-C2/HE-1 constructed by Mrs. Xinmei Chen, University of Alberta. A XhoI site was introduce into the 5' end and a HindIII site was introduced into the 3' end of the EGFR signal sequence. A HindIII site was introduced into the 5' end

and a SalI site was introduced into the 3' end of the c-Fos leucine zipper domain, by PCR. A SalI site was introduced into the 5' end and a KpnI site was introduced into the 3' end of the EGFR transmembrane and intracellular domain by PCR. Purified EGFR signal sequence, c-Fos leucine zipper domain and the EGFR transmembrane plus intracellular membrane domain fragments were then ligated and inserted in frame into the pEGFP-N3 mammalian expression vector (Clontech laboratories, Palo Alto, CA) (Schematic illustration in Figure 3).

A variant of DsRed, DsRed2 (Clontech, Palo Alto, CA) was attached to the amino terminus of Rab5 by standard recombinant techniques. The DsRed-Rab5 vector was then used in the indirect immunofluorescence colocalization studies. It was engineered by inserting in frame the complete Rab5 (corresponding to amino acids 1- 214 according to (Chavrier *et al.*, 1990)) into the pDsRed-C1 mammalian expression vector (Clontech, Palo Alto, CA), by Mr. Yang Li, University of Alberta. Following ligation of the PCR products into the pEGFP-N3 expression vector and DsRed-C1 expression vectors, all DNA constructs were amplified by transformation of competent bacterial cells.

2.2.2 Cell culture and treatment

Human embryonic kidney 293T cells were grown at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and were maintained in a 5% CO_2 atmosphere. Prior to activation of the expressed

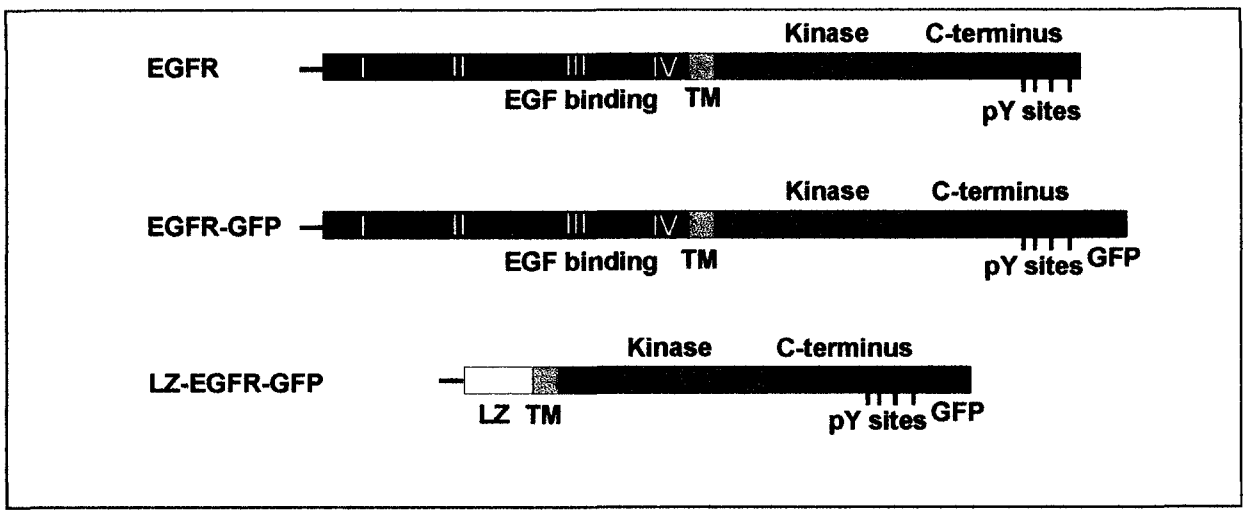


Figure 3. Schematic illustration of the chimeric protein constructs of EGFR-GFP (B) and LZ-EGFR-GFP (C) as compared to WtEGFR (A). The chimeric LZ-EGFR-GFP includes an ATG codon followed by the signal sequence of WtEGFR (dotted line). The complete extracellular domain of EGFR is replaced with a LZ sequence. The green fluorescent protein (GFP) tag is fused to the C-terminal tail in both the EGFR-GFP and LZ-EGFR-GFP. Denoted are the locations of the major carboxy terminal tail tyrosine residues (Y992, Y1068, Y1086, Y1148 and Y1173) phosphorylated upon intrinsic tyrosine kinase activation

chimeras, transfected cells were serum starved for 24 hours. Briefly, transiently transfected cultures were washed three times in phosphate-buffered saline (PBS) (137mM NaCl, 2.7mM KCl, 10mM phosphate buffer) and incubated in serum-free medium for the indicated time. EGF was then added to a final concentration of 100ng/ml for 30 min. After the treatment the cells were cooled down to 4°C. The cells were then harvested with a PBS buffer rinse down and collected. The cells were washed several times with repeated low speed centrifugation (4000 X g) and PBS resuspension cycles. Harvested cells were then retained for lysis. For cells treated with EGFR specific tyrosine kinase inhibitor tyrphostine AG1478, cells transiently transfected and expressing for 48 hours were treated with 0.5 μM AG1478 for 2 hours and EGF was added accordingly to a final concentration of 100 ng/ml in the last 30 min.

2.2.3 Transient Transfections

293T cells were transiently transfected with the various constructs, including EGFR-GFP, LZ-EGFR-GFP and/or DsRed-Rab5 by calcium phosphate precipitation. Plasmid DNA (8.6 μg) was dissolved in distilled H_2O with $CaCl_2$ at a concentration of 250mM to a final volume of 500μl. This mixture was then added dropwise to 500 μl of 50 mM BES (N, N-bis [2-hydroxyethyl]-2-aminoethanesulfonic acid) to a final volume of 1000μl. The mixture was then incubated at room temperature for 50 minutes, where it was then

added dropwise to cell cultures at 80% confluency in 100mm plates. Twenty-four to forty-eight hours after transfection, the cells were used for all *in vitro* assays.

2.2.4 Subcellular fractionation and total cell lysates

Subcellular fractionation isolation of plasma membrane (PM), endosomal (EN), and cytosolic (CY) fractions was carried out by our previously described method (Wang *et al.*, 1999). Briefly, following treatment, cells were scraped into homogenization buffer (HB) with 4mM NaF, 0.5 mM Na_3VO_4, 0.02% NaN_3, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml aprotinin, 1 μM pepstatin A and lysed by grinding in a Potter-Elvehjem homogenizer. The lysates were subjected to centrifugation at 200 X g for 5 min to remove cell debris and nuclei (P1). The postnuclear supernatant (S1) was then subjected to centrifugation at 1500 X g for 10 min to yield a supernatant S2 and a crude membrane fraction pellet P2. Next, P2 was resuspended in a 0.25 M sucrose homogenization buffer and transferred as a layer upon an equal volume of 1.42 M sucrose homogenization buffer and subjected to centrifugation at 82 000 X g for 1 h. The sediment at the 0.25-1.42 M interface was collected as the PM fraction. The S2 fraction was subjected to centrifugation at 100 000 X g for 30 min to yield the soluble CY fraction and a microsomal pellet. This pellet was resuspended in 0.25 M sucrose buffer and overlaid upon a discontinuous sucrose gradient containing equal volumes of homogenization buffer at 1.00 M and 1.15 M sucrose. The

resuspension was subjected to centrifugation at 200 000 X g for 1.5 h to obtain the enriched EN fraction at the 0.25-1.00 M interface. To examine EGFR in each subcellular fraction of 293T cells, aliquots containing 5μg of protein from each fraction were used in immunoblots.

For the total cell lysates, 293T cells transiently transfected were lysed with 0.4% Triton X-100 lysis buffer (0.4% triton X-100, 140mM NaCl, 50 mM Tris-Cl, pH 7.2, 1mM EGTA) in the presence of protease inhibitors (see table 2.3) for 1 hour at 4°C. Lysates were then cleared by subjection to centrifugation at 20 000 X g for 30 minutes. Following protein quantification the supernatant was then boiled in SDS-loading buffer (250mM Tris-Cl, 40% glycerol, 8% sodium dodecyl sulfate, 20% β-mercaptoethanol, 2% bromophenol blue) at 95°C for 5 minutes

Protein was quantitated using the Bradford protein dye assay, according to the method of Bradford (1976). Absorbance at λ = 595nm was measured by a Beckman DU 640 spectrophotometer (Beckman Instruments, Fullerton, CA). Bovine Serum Albumin (BSA) was used as a standard.

2.2.5 Gel Electrophoresis and Immunobloting

Aliquots of protein from each sample were used for SDS-polyacrylamide gel electrophoresis. Protein samples were separated by electrophoresis through 8% SDS-polyacrylamide gels (Laemmli, 1970) (37.5:1 Acrylamide/Bis, Tris-Cl; pH 8.8, TEMED, Ammonium persulfate). Prestained protein markers (Sigma) were

Table 2.3 Protease inhibitors (PINS) used in this study

Protease Inhibitor	Concentration
Aprotinin	10 μg/ml
4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF)	0.1 mM
Pepstatin A	1.0 μM
Sodium Orthovanadate (Na_3VO_4)	0.5 mM
Sodium Azide (NaN_3)	0.02%

used for molecular weight standards. Proteins were electrophoretically transferred onto transblot nitrocellulose membranes (BioRad, Hercules, CA) as described previously (Wang and Moran, 1996). Transfer was done using a semi-dry blotting apparatus (Model SD Transfer Cell, BioRad) at 15mA per minigel for 45 minutes in transfer buffer (see table 2.1). Blots were blocked with 3% skim milk in 0.05% Tween-PBS (blocking buffer) for 30 minutes. Membranes were then probed with the respective primary antibody (table 2.4) in blocking buffer overnight, washed twice with distilled H_2O, incubated with HRP-conjugated IgG secondary antibody (table 2.4) for 1 hour, washed twice with distilled H_2O, washed with 0.05% Tween-TBS for 5 minutes, rinsed twice with distilled H2O and washed with TBS buffer. Secondary antibodies were detected by enhanced chemiluminescence, with SuperSignal ECL Western Blotting Detection Reagents (Pierce Chemical) and exposure to x-ray film (Fuji Photo Film Co., Tokyo, Japan). Exposed film was visualized with a digital imaging system (Alpha Innotech Corporation). All antibodies used are listed in Table 2.4 with their respective dilutions.

2.2.6 Dimerization assay

Transiently transfected cells, following the appropriate treatment were harvested as described previously. Cell pellets were resuspended in PBS in the presence of 0.5 mM Na_3VO_4, 0.02% NaN_3, 0.1 mM AEBSF, 10 μg/ml aprotinin, 1 μM pepstatin A. Resuspensions were then lysed in a Potter-Elvehjem

Table 2.4 Antibodies and their dilutions used for Western Blotting

Antibody	Dilution
Rabbit anti-EGFR (1005)	1:2000
Rabbit anti-phospho-EGFR (py992)	1:2000
Rabbit anti-phospho-EGFR (py1068)	1:2000
Rabbit anti-phospho-EGFR (py1086)	1:2000
Rabbit anti-phospho-EGFR (py1148)	1:2000
Rabbit anti-phospho-EGFR (py1173)	1:2000
Rabbit anti-Shc	1:2000
Rabbit anti-PLCγ1	1:2000
Rabbit anti-phospho-PLCγ1	1:2000
Rabbit anti-GFP (living)	1:1 000,000
Mouse anti-phospho-tyrosine (py99)	1:1000
Mouse anti- Erk1/2	1:2000
Mouse anti- phospho-Erk1/2	1:2000
Rabbit anti-Akt	1:2000
Rabbit anti-phospho-Akt (ser-473)	1:1000
Mouse anti-EEA1	1:2000.
Horseradish peroxide conjugated-anti-rabbit IgG	1:2000
Horseradish peroxide conjugated-anti-mouse IgG	1:2000

**Manufacturers listed in subsection 2.1.6.1 and 2.1.6.2*

homogenizer and collected. To these lysates the crosslinker Disulfosuccinimidyl suberate (DSS), a non-cleavable, membrane impermeable and amine reactive cross-linker was added to a final concentration of 6mM. The mixture was then incubated at room temperature for 30 minutes after which the reaction was quenched with 250mM glycine for an additional 15 minutes at room temperature. The treated lysate was then subjected to ultra centrifugation at 100 000 X g for 1 hour. The pellet collected was then treated with 0.4% Triton X-100 lysis buffer in the presence of protease inhibitors (see table 2.3) overnight at 4°C. Lysates were then cleared by subjection to centrifugation at 20 000 X g for 30 minutes. The supernatant was then boiled in 4x SDS-loading buffer at 95°C for 5 minutes prior to SDS-PAGE.

2.2.7 Fluorescence Microscopy

293T cells were grown on glass coverslips and serum starved for 24 hours. After the according treatment, the cells were fixed by methanol (-20 °C) and mounted directly onto glass slides. To detect EGFR-GFP and LZ-EGFR-GFP alone, fluorescence excitation of the GFP tag was visualized with a Zeiss, Axiovert 200 (Carl Zeiss, Calgary, AB) and an AttoArc2, HBO 100W light source (Atto Instruments, Rockville, MD). Colocalization of the GFP tagged chimera with a DsRed tagged Rab5 was done following the co-transfection of both fluorescent tag-encoding vectors into 293T cells. Indirect immunofluorescence

was performed as described previously (Wang *et al.*, 1999). Cells were grown on glass coverslips and serum starved for 24 h. After treatment, the cells were fixed by methanol (-20 °C) and permeabilized with 0.2 % Triton X-100 without blocking. Next, the cells were incubated with indicated primary antibodies at room temperature for 1 h followed by fluorescence-labeled secondary antibodies for 1 h. To detect phosphorylated EGFR alone, the primary antibody was mouse anti-phospho-EGFR antibody (table 2.5), and the secondary antibody was rhodamine-conjugated donkey anti-mouse IgG (table 2.5). To detect the rhodamine label, fluorescence excitation of the rhodamine tag was visualized with a Zeiss Axiovert 200 microscope (Carl Zeiss Inc., Thornwood, NY) and an AttoArc2, HBO 100W light source (Atto Instruments, Rockville, MD).

A list of antibodies used for immunofluorescence and the concentrations at which they were used is given in table 2.5.

2.2.8 BrdU incorporation assay

DNA synthesis was assayed by bromodeoxyuridine (BrdU) incorporation. 293T cells were plated upon glass coverslips and transiently transfected with the chimeric constructs. Following expression for 48 hours, cells were washed three times with PBS and serum starved for 24 hours. For conditioned cells, coverslips were incubated with serum free or medium containing 100ng/ml EGF as needed. Unconditioned control cells were incubated with 0.5 μM AG1478 for 15 minutes,

followed by addition of serum free medium or 100ng/ml EGF as needed for 10 hours at 37 °C. Both conditioned and unconditioned cells were treated with BrdU (25 μM) in the final 4 hours of treatment. After treatment, cells were washed with 1X PBS and fixed with methanol (-20 °C) for 5 minutes. DNA was then denatured by incubation with 2 N HCl at room temperature for 30 minutes. Coverslips were then washed with PBS buffer twice and then incubated with mouse α-BrdU primary antibody (table 2.5) for 1 hour at room temperature. Coverslips were rinsed with PBS buffer and then incubated with rhodamine-conjugated α-mouse IgG for 1 hour. Coverslips were then mounted upon glass slides. Cell nuclei were visualized following fluorescence excitation of the rhodamine tag with a Zeiss Axiovert 200 microscope (Carl Zeiss Inc., Thornwood, NY) and a AttoArc2, HBO 100W light source (Atto Instruments, Rockville, MD).

Table 2.5 Antibodies and their dilutions used for immunofluorescence

Antibody	Dilution
Mouse anti- phospho-EGFR	1:100
Mouse anti-BrdU	1:100
Rhodamine-conjugated donkey anti-mouse IgG	1:200

**Manufacturers listed in subsection 2.1.6.1 and 2.1.6.2*

3.0 Results

3.1. Expression of GFP tagged wt-EGFR and LZ-EGFR

To determine whether the introduction of a leucine zipper into EGFR will result in the constitutive dimerization of EGFR transmembrane and intracellular domain, we transiently transfected 293T cells with the plasmid encoding LZ-EGFR-GFP. The 293T cells transfected with EGFR-GFP were used as a control. Expression levels were adjusted between the two chimeric proteins upon transient transfection by decreasing the incubation time of the EGFR-GFP - calcium phosphate precipitate during cell treatment. This was done due to the consistent higher expression rates observed for EGFR-GFP as compared to LZ-EGFR-GFP. Immunoblotting of the total lysates with anti-EGFR and anti-GFP antibodies showed the presence of a strong band of 100 kD for cells transfected with LZ-EGFR-GFP, and a 210 kD band for cells transfected with EGFR-GFP (Fig. 4A). This suggests that both LZ-EGF-GFP and EGFR-GFP were well expressed with expected molecular weight. The 293T cells transfected with pEGFP-N3 empty vector confirmed that endogenous levels of EGFR was at biochemically undetectable and thus very low levels.

3.2 Dimerization of LZ-EGFR-GFP

To determine whether the introduction of a leucine zipper results in the dimerization of EGFR, the 293T cells were transfected with LZ-EGFR-GFP or

EGFR-GFP and the cells were cross-linked with DSS (see section 2.2.6). Immunoblotting with anti-EGFR and GFP antibodies revealed the presence of a LZ-EGFR-GFP dimer with weight of approximately 200 kD for cells transfected with LZ-EGFR-GFP. We also observed a higher molecular weight protein band which may be the tetramer of LZ-EGFR-GFP. In the control cells transfected with EGFR-GFP, the EGFR-GFP formed dimers following EGF stimulation as expected (Fig. 4B).

3.3 Subcellular Distribution of LZ-EGFR-GFP

We next determined the subcellular distribution of LZ-EGFR-GFP by fluorescence microscopy. 293T cells were transfected with LZ-EGFR-GFP and the subcellular distribution of LZ-EGFR-GFP was determined by its intrinsic fluorescence. 293T cells transfected with EGFR-GFP were used as a control. As shown in Fig. 5, consistent with previous findings EGFR-GFP was localized to the plasma membrane without EGF stimulation and then localized to endosomes following EGF stimulation. However, LZ-EGFR-GFP was localized at both the plasma membrane and some vesicular structures (Fig. 5). To determine the localization of LZ-EGFR-GFP, 293T cells were co-transfected with both GFP-tagged LZ-EGFR and DsRed tagged wild-type Rab5 (Fig. 5). As shown, LZ-EGFR-GFP co-localized with DsRed-Rab5, which indicates that

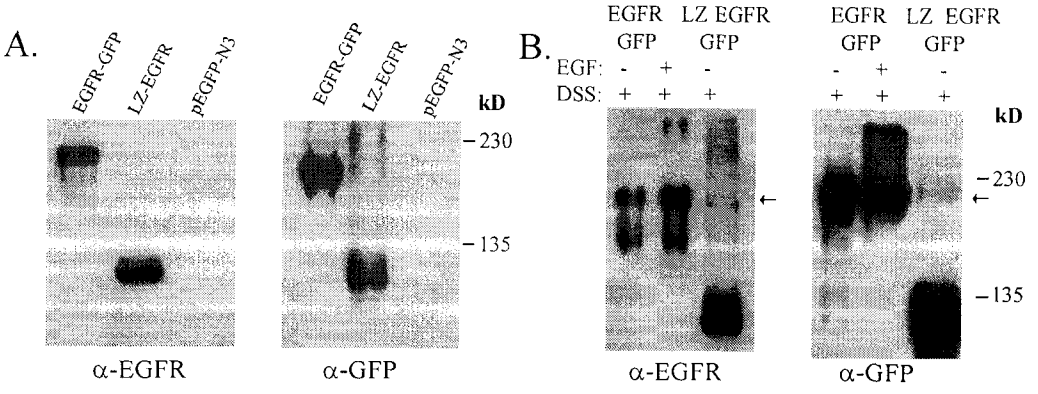


Figure 4. Immunoblot analysis of expression and dimerization of LZ-EGFR-GFP. (A) Expression of EGFR-GFP, LZ-EGFR-GFP and empty vector pEGFR-N3 in serum starved 293T cells. Total cell lysates were subjected to immunoblotting with anti-EGFR and anti-GFP antibodies. 293T cells transfected with EGFR-GFP or LZ-EGFR-GFP were either not stimulated or stimulated with EGF for 5 minutes at 37 °C. The cells were then cross-linked with DSS as described in Materials and Methods. Samples were subjected to immunobloting with anti-EGFR and anti-GFP antibodies to determine stable dimer forms (arrows).

LZ-EGFR-GFP was localized to endosomes. In the control cells transfected with both EGFR-GFP and DsRed-Rab5, EGFR-GFP was co-localized with DsRed-Rab5 following EGF stimulation for 30 min as expected (Fig. 5B). These results suggest that LZ-EGFR-GFP is localized to endosomes, which may suggest that LZ-EGFR-GFP is constitutively endocytosed. However, we are not to exclude the possibility that LZ-EGFR-GFP was targeted to endosomes from the trans-Golgi network (TGN).

The plasma membrane and endosome localization of LZ-EGFR-GFP was further analyzed by subcellular fractionation. Immunobloting showed that LZ-EGFR-GFP was indeed localized in both the plasma membrane and endosome fractions of unknown origin or destination (Fig. 6). The early endosome antigen 1 (EEA1) was used as a marker for endosomes, which is highly enriched in our endosomal fraction. The purity of the endosomal fraction, as assessed by the relative enrichment of EEA-1 were comparable to results obtained previously for this subcellular fractionation method (Wang *et al.*, 2002).

3.4. Kinase activation and phosphorylation of LZ-EGFR-GFP

We next determined whether the dimerization of EGFR by the leucine zipper resulted in the activation of EGFR. As shown in Figure 7, immunoblotting with anti-phospho-EGFR (p-EGFR) antibody revealed that LZ-EGFR-GFP was phosphorylated. Interestingly, we also observed a higher molecular weight protein

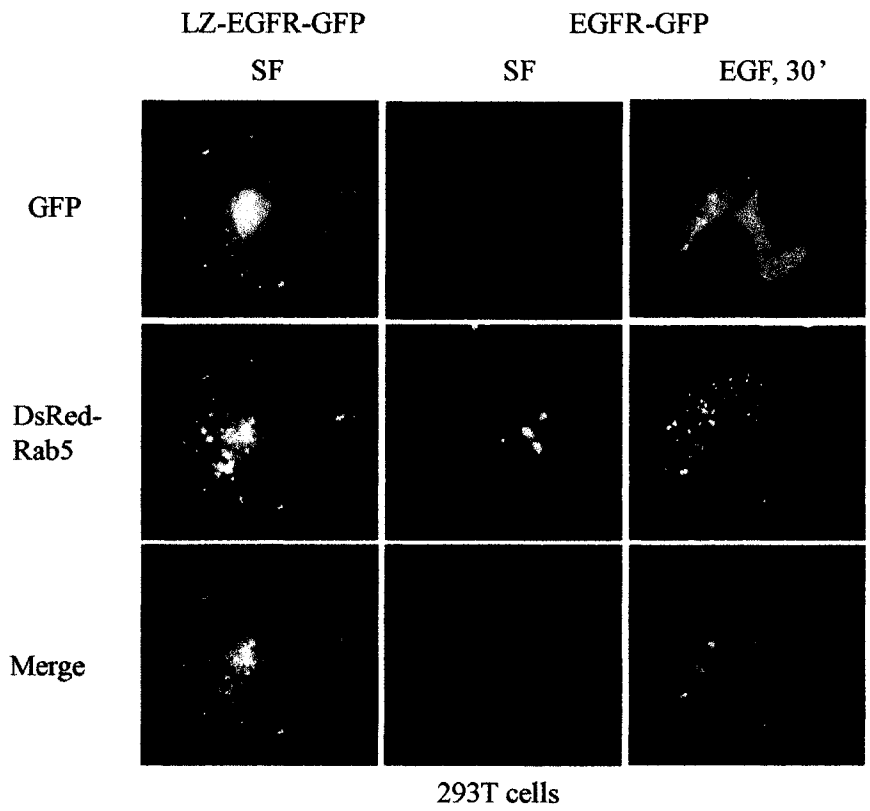


Figure 5. Fluorescence analysis of the subcellular localization of LZ-EGFR-GFP. Fluorescence microscopy of 293T cells transiently transfected with LZ-EGFR-GFP or EGFR-GFP. Transiently transfected 293T cells were either stimulated with EGF for 30 minutes at 37 °C or not stimulated. The localization of LZ-EGFR-GFP and EGFR-GFP was visualized by green fluorescence. 293T cells were cotransfected with DsRed-Rab5 and LZ-EGFR-GFP and EGFP-GFP. The cells were either stimulated with EGF for 30 minutes at 37 °C or not stimulated. The colocalization of DsRed-Rab5 (red) and LZ-EGFR-GFP or EGFR-GFP (green) was shown as yellow.

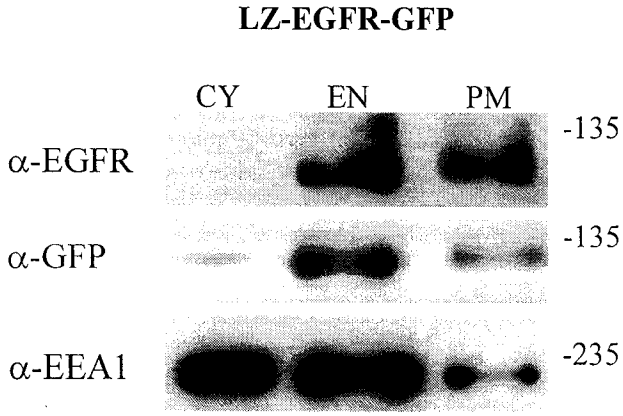


Figure 6. Subcellular fractionation. 293T cells transiently expressing LZ-EGFR-GFP were serum starved for 24 hours. The cells were then harvested and fractionated into the cytoplasmic (CY), endosomal (EN) and plasma membrane (PM) fractions. The subcellular fractions were subjected to immunoblotting with anti-EGFR and anti-GFP. Immunobloting with anti-EEA-1 antibodies was used as a marker for endosomal purity.

band that was presumed to be the LZ-EGFR-GFP dimer, as the endogenous EGFR should have a lower molecular weight.

To demonstrate that this phosphorylation is induced by the intrinsic kinase activity of EGFR, we blocked EGFR kinase activity with the EGFR tyrosine kinase inhibitor AG1478 and then examined the phosphorylation status of LZ-EGFR-GFP. We showed that the phosphorylation of LZ-EGFR-GFP was blocked following treatment with AG1478 (Fig. 7). This indicates that LZ-EGFR-GFP is phosphorylated by its functional intrinsic tyrosine kinase activity.

This finding was further supported by indirect immunofluorescence. 293T cells were transiently transfected with LZ-EGFR-GFP or EGFR-GFP and subjected to indirect immunofluorescence with anti-pEGFR antibody (Fig. 8A). We showed that LZ-EGFR-GFP was phosphorylated and localized to both the plasma membrane and endosomes. As expected for our control experiment, EGFR-GFP was not phosphorylated and localized at the plasma membrane prior to EGF stimulation, and phosphorylated and internalized into endosomes following EGF stimulation for 30 min. Moreover, inhibition of EGFR kinase activity by AG1478 blocked the phosphorylation of both LZ-EGFR-GFP and EGFR-GFP (Fig. 8B). This indicates once again that the phosphorylation of LZ-EGFR-GFP is due to its intrinsic kinase activity.

Together, our results indicate that fusion of a leucine zipper with the

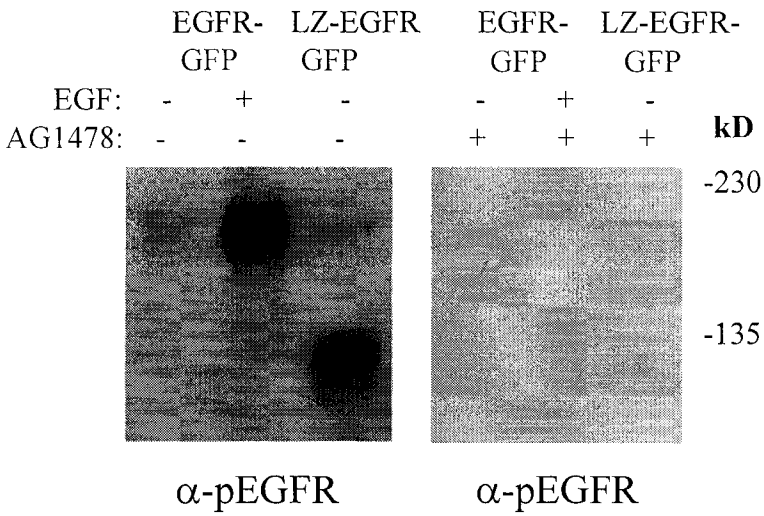
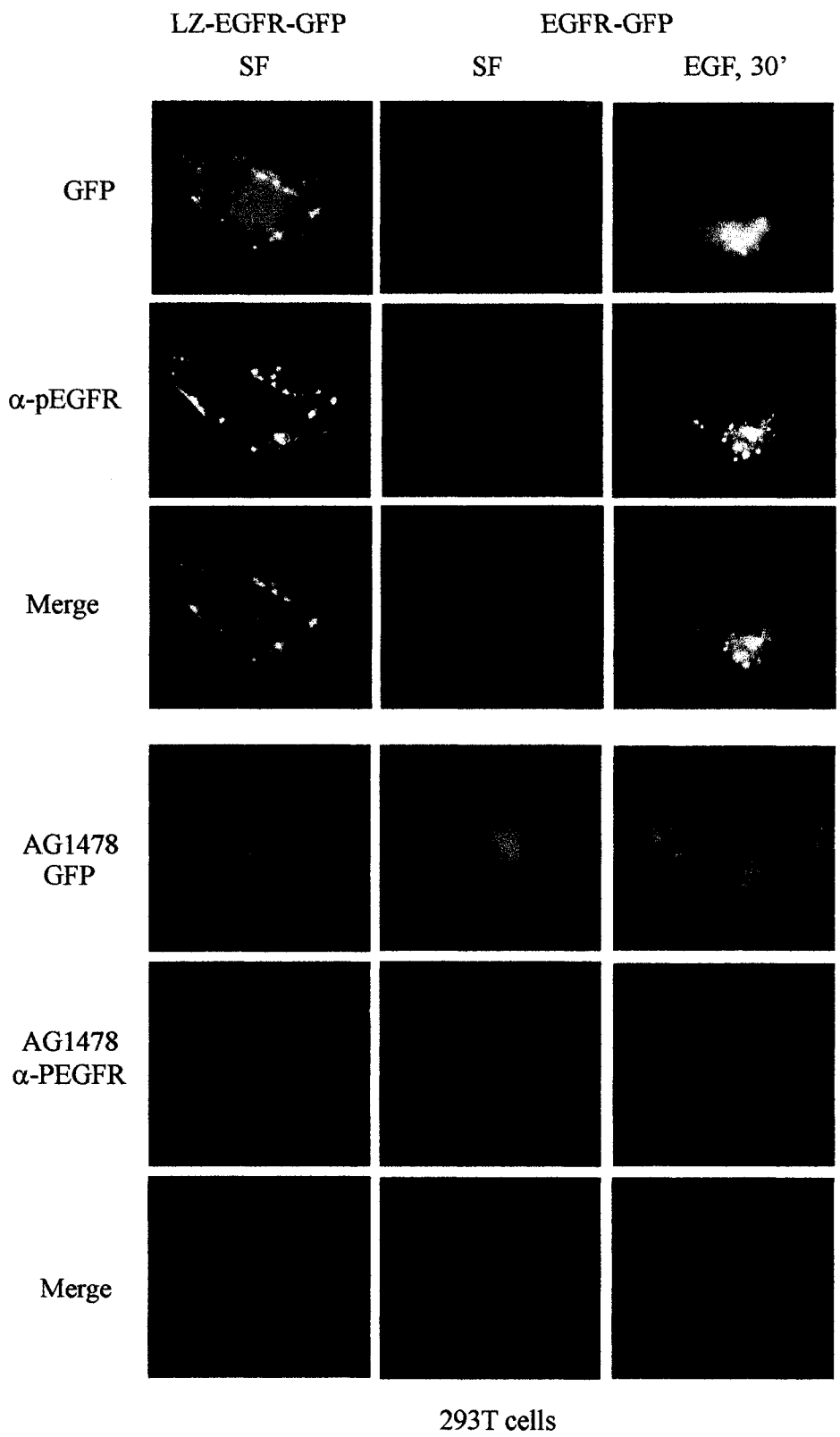


Figure 7. Intrinsic tyrosine kinase domain dependant receptor autophosphorylation and activation. 293T cells transiently transfected with EGFR-GFP and LZ-EGFR-GFP were serum starved for 24 hours. Some cells were treated with the EGFR tyrosine kinase inhibitor AG1478 for an additional 12 hours. EGFR-GFP transiently expressing cells were stimulated with EGF for 30 min at 37 °C. The cells were then washed with PBS three times and then harvested and lysed. Cell lysates were subjected to immunoblot analysis with the rabbit anti-pEGFR (pY992) antibody (Santa Cruz, CA).

Figure 8. Immunofluorescence analysis of LZ-EGFR-GFP phosphorylation and the dependance on its intrinsic tyrosine kinase activation. 293T cells transiently transfected with either LZ-EGFR-GFP or EGFR-GFP were serum starved for 24 hours and cells were treated with or without AG1478. Some of the EGFR-GFP expressing cells were then stimulated with EGF for 30 minutes at 37°C. Localization and activity were determined by indirect immunofluorescence. Colocalization of LZ-EGFR-GFP or EGFR-GFP (green) with p-EGFR (red) was determined.



transmembrane and intracellular domain of EGFR causes the dimerization of the chimera. The dimerization of the chimera then resulted in the constitutive activation of intrinsic tyrosine kinase and the phosphorylation of carboxyl terminal tyrosine residues.

3.5 The phosphorylation sites of LZ-EGFR-GFP

We next compared the phosphorylation status of LZ-EGFR-GFP with EGF activated EGFR-GFP. It is well established that EGF induces the phosphorylation of multiple tyrosine residues at the C-terminus of EGFR. These tyrosine residues include Y992, Y1068, Y1086, Y1148 and Y1173. Indeed, immunoblotting of 293T cells transfected with EGFR-GFP with the five specific antibodies against these phosphotyrosine residues showed that all of them were phosphorylated in EGFR-GFP following EGF stimulation. Similarly, all of these five tyrosine residues were also phosphorylated in LZ-EGFR-GFP (Fig. 9). These results indicate that constitutive dimerization induced by the leucine zipper domain fully activated the EGFR tyrosine kinase leading to the phosphorylation of its C-terminal tyrosine residues.

3.6. Activation of various signaling pathways by LZ-EGFR-GFP

Since these five phosphorylated tyrosine residues have been shown to bind and subsequently activate numerous signaling proteins including Grb2, SHC, and

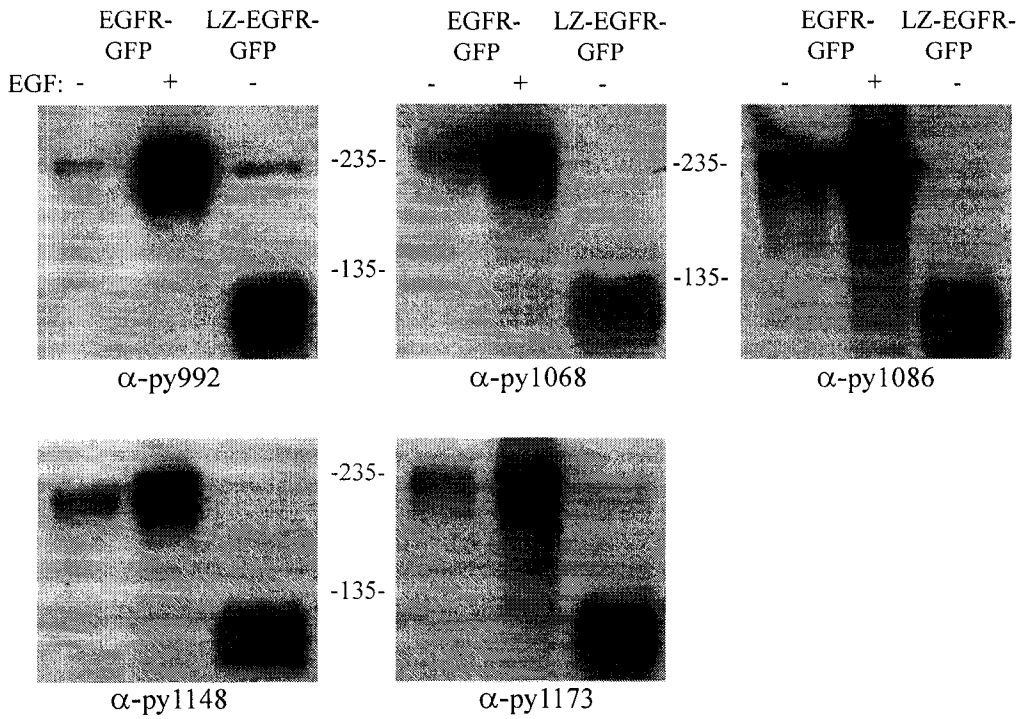


Figure 9. Phosphorylation of EGFR-GFP or LZ-EGFR-GFP at all five major C-terminal tyrosine residues. 293T cells transiently transfected with EGFR-GFP and LZ-EGFR-GFP were serum starved for 24 hours. Some of the EGFR-GFP transiently expressing cells were stimulated with EGF for 30 min at 37°C. The cells were then washed with PBS three times and then harvested and lysed. Cell lysates were subjected to immunoblot analysis with rabbit anti-pEGFR (tyrosine 992), rabbit anti-pEGFR (tyrosine 1068), rabbit anti-pEGFR (tyrosine 1086), rabbit anti-pEGFR (tyrosine 1148) and rabbit anti-pEGFR (tyrosine 1173) antibodies.

PLC-γ1 we next determined the phosphorylation status of these binding proteins in 293T cells transiently transfected with LZ-EGFR-GFP under serum starved conditions as compared to serum starved cells transiently transfected with EGFR-GFP. As shown in Fig. 10, in cells transfected with EGFR-GFP, stimulation with EGF resulted in the up-shift of the SHC p66 isoform, which indicates the tyrosine phosphorylation of SHC (Batzer *et al.*, 1994). An up-shift of a portion of the SHC p66 isoform was observed for the cells transiently transfected with LZ-EGFR-GFP under serum starved conditions. Especially noticeable when compared to serum starved cells transiently transfected with EGFR-GFP. This suggests that constitutively activated LZ-EGFR-GFP resulted in the activation of SHC under serum starved conditions. Immunoblotting with anti-phospho-PLC-γ1 antibodies showed that constitutively activated LZ-EGFR-GFP phosphorylates PLC-γ1under serum starved conditions. The constitutively activated LZ-EGFR-GFP appears to activate PLC-γ1 more effectively than EGFR-GFP under serum starved conditions (Fig. 10).

We next determined whether constitutively activated LZ-EGFR-GFP stimulated the downstream signaling proteins including ERK and Akt. Immunoblotting with anti-phospho-ERK (p-ERK) and phospho-Akt (p-Akt) showed that constitutively activated LZ-EGFR-GFP also activated ERK and Akt. This downstream activation by LZ-EGFR-GFP under serum starved conditions appears to be more effective than EGFR-GFP under similar conditions (Fig. 10).

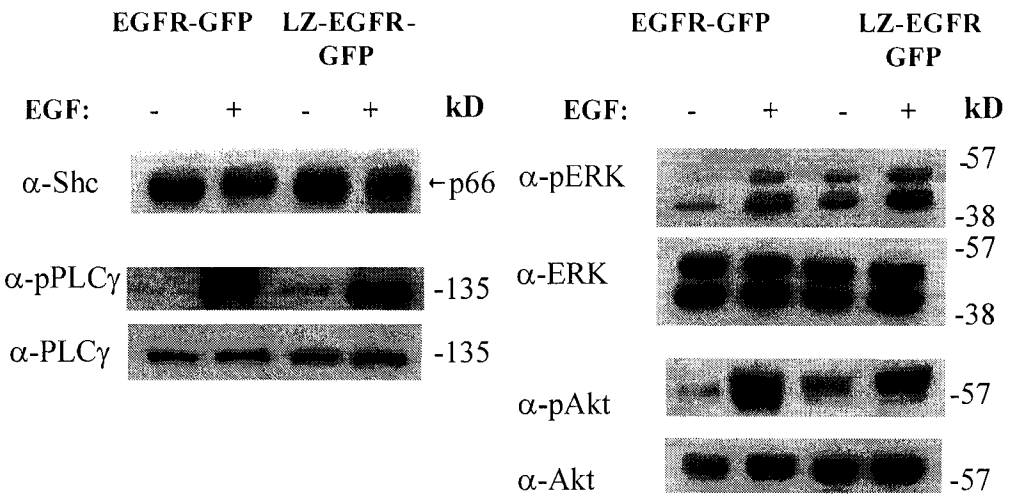


Figure 10. Stimulation of various signal transduction pathways by LZ-EGFR-GFP. 293T cells transiently transfected with EGFR-GFP or LZ-EGFR-GFP were serum starved for 24 hours. Cells were stimulated with EGF for 30 min at 37°C or not stimulated. Cell lysates were subjected to immunoblot analysis with rabbit anti-SHC, rabbit anti-phospho-PLCγ1, rabbit anti-PLCγ1, mouse anti-phospho-Erk1/2, mouse anti-Erk1/2, rabbit anti-phospho-Akt and rabbit anti-Akt antibodies.

3.7 Stimulation of cell proliferation by LZ-EGFR-GFP

Since constitutively activated LZ-EGFR-GFP stimulates numerous signaling proteins implicated in cell mitogenesis, we determined whether LZ-EGFR-GFP induced cell proliferation under serum starved conditions. 293T cells were transiently transfected with EGFR-GFP or LZ-EGFR-GFP and cell proliferation was determined by a BrdU incorporation experiment in transiently transfected and GFP- tagged protein expressing cells. Following serum starvation of transfected cells for 24 hours, cells were treated with EGF and/or AG1478 (section 2.2.8). DNA synthesis was assessed by BrdU incorporation in 300 cells per sample. Experimental data was collected and plotted as the mean of triplicate experiments totaling 900 cells per experiment. Percentage BrdU incorporation represented the number of GFP- tagged protein expressing cells that incorporated BrdU divided by all GFP- tagged protein expressing cells. As shown in Fig. 11, while in 293T cells transiently transfected with EGFR-GFP, EGF stimulates strong BrdU incorporation in 61% of all transiently transfected cells, the BrdU incorporation rate is very low at 17% of all transiently transfected cells without EGF stimulation. However, expression of LZ-EGFR-GFP stimulated strong BrdU incorporation under serum starved conditions at 42% of all transiently transfected cells. This suggests that LZ-EGFR-GFP functions as EGF-activated EGFR-GFP in promoting cell proliferation under serum starved conditions. Furthermore, to demonstrate that the strong BrdU incorporation is indeed due to the expression of

LZ-EGFR-GFP, we treated the cells with AG1478 to block the ligand independent tyrosine kinase activity of LZ-EGFR-GFP. We showed that AG1478 reduced the BrdU incorporation level to 9% in 293T cells expressing LZ-EGFR-GFP. As a control experiment, we also showed that AG1478 reduced the BrdU incorporation level to 11% in the cells expressing EGFR-GFP with EGF stimulation (Fig. 11). Together, our results indicate that expression of LZ-EGFR-GFP stimulates cell proliferation in a manner similar to EGF-activated EGFR-GFP.

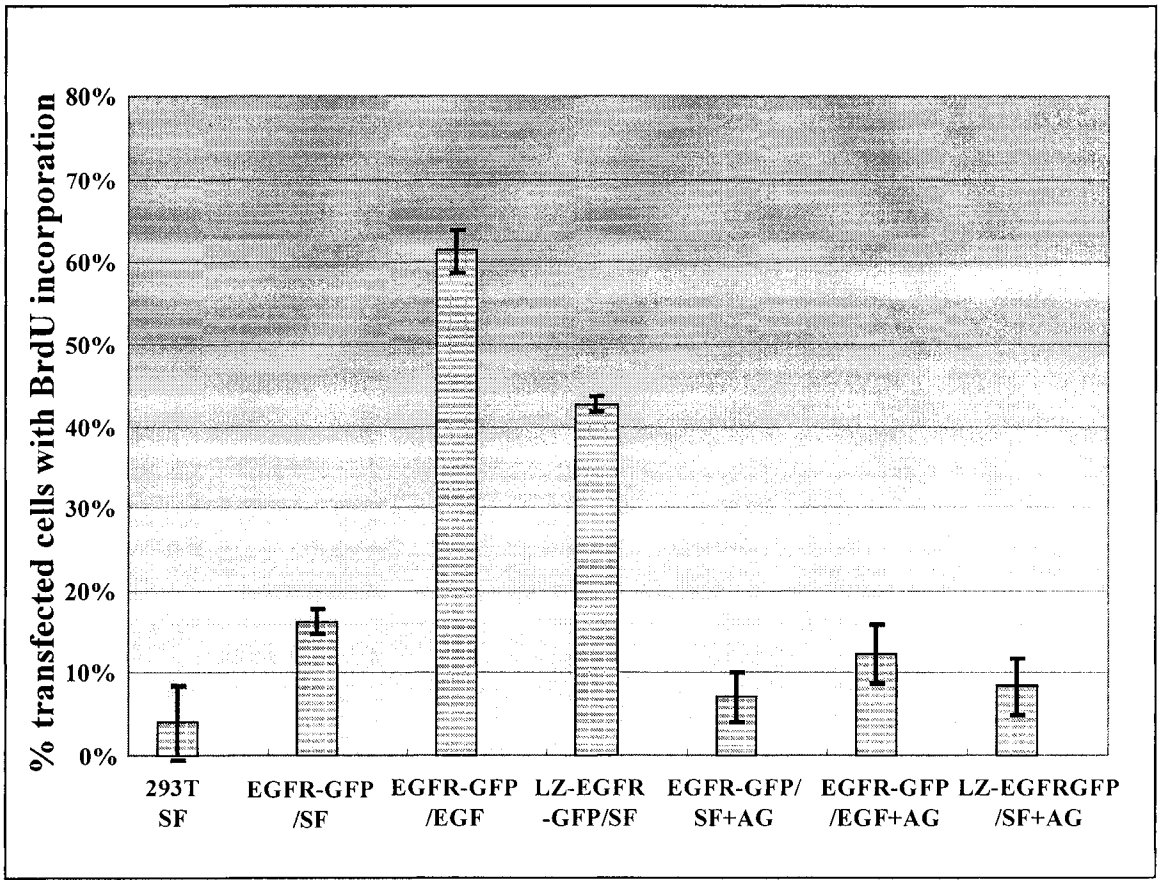


Figure 11. Stimulation of DNA synthesis by LZ-EGFR-GFP. 293T cells were transiently transfected with EGFR-GFP or LZ-EGFR-GFP. Following serum starvation for 24 hours, cells were treated with EGF and/or AG1478 as indicated. DNA synthesis was determined by BrdU incorporation as described in the Materials and methods. Cells were counted at 300 per sample and data was plotted as the mean of triplicate experiments.

4.0 Discussion

In this study we examined the role of ligand dependant dimerization in the activity of the EGF receptor. Essential processes including kinase activation, receptor autophosphorylation, binding of downstream signaling proteins and receptor endocytosis are all events that are initiated by ligand binding to the EGF receptor. Ligand binding however is immediately followed by receptor dimerization, a process which importance is often overshadowed by the preceding event of ligand binding. It remains unclear to date whether ligand binding is required to initiate all post-binding processes or whether it is required simply for receptor dimerization which is then sufficient to initiate the essential processes that follow receptor activation. The work presented in this study suggests that the formation of EGFR dimers is sufficient to initiate the processes of receptor activation, receptor signaling and receptor endocytosis, reflective of a fully functional dimeric receptor in the absence of ligand.

In order to provide evidence of a fully functional EGFR dimer we established a system to allow dimerization of EGFR without ligand binding through the introduction of a LZ domain to the N-terminus of EGFR. We chose this method of dimerization since previous studies have reported that high affinity dimers form when two complimentary leucine zippers are in close proximity to one another. Application of a leucine zipper domain was recently done with the complete substitution of the extracellular domain of the growth hormone receptor (GHR).

This substitution lead to the forced dimerization of the transmembrane and intracellular domains of the GHR and the constitutive activation of the GHR (Behncken *et al.*, 2000). Using a similar approach, we fused the c-Fos leucine zipper to the transmembrane and cytoplasmic domain of EGFR. We examined the expression of the chimeric receptor, its activation, localization and ability to induce cell proliferation through signaling.

To provide evidence of functional leucine zipper induced dimerization of EGFR we treated LZ-EGFR-GFP transiently transfected cells with the crosslinker Disulfosuccinimidyl suberate (DSS), a non-cleavable, membrane impermeable and amine reactive cross-linker. Upon treatment of LZ-EGFR-GFP expressing cells with crosslinker, high molecular weight protein bands were observed following immunoblot. These high molecular weight protein bands of the expected dimeric molecular weight were suspected to be that of leucine zipper induced dimers of EGFR. The protein bands that exceed the expected molecular weight of LZ-EGFR-GFP dimers may be tetramer of LZ-EGFR-GFP. However, the efficiency of the crosslinking by crosslinker DSS is very low. While the same crosslinking experiments have always generated less dimer band than monomer band in previously published studies regarding RTK dimerization, the yield of dimer band is even lower in our results for both LZ-EGFR-GFP and EGFR-GFP. The reason for the low efficiency is not clear, however it is possible that the interaction between receptor dimers and the crosslinker are somehow restricted

during our experimental conditions. It is also possible that we failed to find the optimal concentration of crosslinker and receptors. It is also possible, judging from the immunoblots we provided in Figure 4 that the dimer band of LZ-EGFR-GFP may be the spill over of EGFR-GFP protein sample in the adjacent lane.

LZ-EGFR-GFP dimers have also been observed without crosslinking in figures 7 & 9 (α-py992, α-py1173). This may suggest that the leucine zipper induced dimerization of EGFR is very stable after SDS-PAGE under reducing conditions, since similar phenomena were observed with the leucine zipper fused growth hormone receptor (Behncken *et al.*, 2000). On the other hand, the observation of dimer band in the absence of crosslinking further suggests that our crosslinking experiments are not very successful.

By using the leucine zipper-induced dimerization system, we first determined whether this dimerization of EGFR is able to activate EGFR kinase activity and result in the autophosphorylation of EGFR C-terminal tyrosine residues. We showed that LZ-EGFR-GFP was strongly phosphorylated and the phosphorylation of LZ-EGFR-GFP is dependent on its intrinsic kinase activity (Fig.7 & 8). Moreover, the phosphorylation pattern of LZ-EGFR-GFP is very similar to that of EGFR-GFP following EGF stimulation. It is well established that EGF stimulates the phosphorylation of 5 major tyrosine residues at the EGFR C-terminus including Y992, Y1068, Y1086, Y1148 and Y1173. We showed that all these five

tyrosine residues were phosphorylated in LZ-EGFR-GFP (Fig. 9). Although these results would have been further complimented with the use of the tyrosine kinase inhibitor AG1478, which would diminish the reactivity of the phosphotyrosine specific antibodies as done in figure 7, these results suggest that leucine zipper-induced dimerization of EGFR activates EGFR kinase activity, resulting in the phosphorylation of the EGFR C-terminus to the same extent as that induced by EGF.

We next determined whether leucine zipper-induced dimerization of EGFR stimulates EGFR endocytosis and EGFR-mediated cell signaling, in comparison to EGF-stimulated EGFR-GFP. We showed by both fluorescence microscopy and subcellular fractionation that leucine zipper-induced constitutive dimerization of EGFR leads to the colocalization and cofractionation of EGFR with endosomal markers in the absence of EGF (Fig. 5 & 6). Moreover, the LZ-EGFR-GFP remains phosphorylated at both the plasma membrane and endosomes (Fig. 8). While we do not have direct evidence, it is possible and logical to assume that the endosome-associated LZ-EGFR-GFP is internalized from the plasma membrane. Thus concluding that the plasma membrane associated LZ-EGFR-GFP is fully activated and capable of endocytosis. We are not to exclude however, the possibility that the endosome-associated LZ-EGFR-GFP is targeted directly from the trans Golgi network (TGN) following over expression of the chimera. One method to clarify the origin of endosomal LZ-EGFR-GFP would be to express the

gene of interest in an inducible vector expression system. This would allow controlled expression of the chimeric protein and a time-based observation of LZ-EGFR-GFP prior to and following the assumed activation at the plasma membrane.

The strong plasma membrane localization of LZ-EGFR-GFP is not in conflict with our suggestion that the plasma membrane localized LZ-EGFR-GFP is constitutively endocytosed. First, the plasma membrane localization of LZ-EGFR-GFP indicates that this protein is properly targeted to the plasma membrane. Second, the high level of plasma membrane localization may suggest that the endocytosis rate of LZ-EGFR-GFP is slower than the targeting rate of LZ-EGFR-GFP from ER/Golgi to the plasma membrane following the synthesis of the protein. It is also possible that LZ-EGFR-GFP may have a higher recycling rate.

A control should be added (Fig. 6) to support our finding about LZ-EGFP-GFP. 293T cells should also be transfected with EGFR-GFP and either stimulated with EGF or serum starved. The subcellular fractionation and subsequent immunoblotting of EGFR-GFP transfected cells will then be performed together with LZ-EGFR-GFP transfected cells.

We further showed that the constitutively activated LZ-EGFR-GFP is able to activate many signaling proteins including SHC, PLC-γ1, Erk and Akt (Fig.10) in the absence of EGF. And although an increase in activation of these downstream

signaling proteins is evident upon addition of EGF to cells expressing LZ-EGFR-GFP, due to low levels of endogenous EGFR, it is important to note that expression of the leucine zipper chimera under serum starved conditions leads to the activation of signaling proteins, unlike that of serum starved EGFR-GFP expressing cells (Fig.10). Moreover, expression of LZ-EGFR-GFP in 293T cells induced cell proliferation in the absence of serum as suggested from results of BrdU incorporation (Fig. 11). BrdU incorporation assays in nontransfected cells were not analyzed. These assays would have ensured incorporation consistency between culture transfections. However, the number of transfectants tabulated was sufficient thus normalizing the representative result. BrdU incorporation may also occur during DNA repair mechanisms that are continuous during the cell cycle, however this incorporation differs drastically from mitogenic induced BrdU incorporation. It is expected that the BrdU incorporation rate during DNA synthesis would be much higher than that of DNA repair, which involves different enzymatic reactions and is not restricted to the time limiting synthesis phase of the cell cycle. These results as a whole indicate that leucine zipper-induced dimerization of EGFR is sufficient to activate EGFR, induce EGFR endocytosis and stimulate various signaling pathways and eventually cause cell proliferation.

While the phosphorylation status of LZ-EGFR-GFP is similar to that of EGFR-GFP following EGF stimulation, LZ-EGFR-GFP activates downstream

signaling proteins and stimulates cell proliferation to a lower extent. The lower potency of LZ-EGFR-GFP in terms of cell signaling, as compared to EGF stimulated EGFR-GFP may be due to a couple of factors. First, constitutively activated LZ-EGFR-GFP is assumed to be endocytosed and targeted to lysosomes for degradation, which may result in the quick termination of LZ-EGFR-GFP signaling upon endocytosis. Second, downstream signaling proteins have constantly been activated by LZ-EGFR-GFP and thus, activation levels of these will likely reduce with time as inactive signaling proteins are depleted proximal to the plasma membrane. Therefore, it is very reasonable to see the sustained but low level of signaling protein activation in cells transfected with LZ-EGFR-GFP. Third, the truncation and substitution of the extracellular domain of EGFR may result in the slight conformation change of LZ-EGFR-GFP and thus reduce its potency.

It is very interesting to compare LZ-EGFR-GFP with the oncogenic ErbB2. The oncogenic ErbB2 has a single mutation at its transmembrane domain which leads to the constitutive dimerization and permanent activation of the oncogenic ErbB2 receptor without ligand-binding (Bargmann *et al.*, 1986; Stern *et al.*, 1988; Weiner *et al.*, 1989). Similar to LZ-EGFR-GFP, ligand is not required for the activation, trafficking and signaling of the oncogenic ErbB2 which suggest that the mutation-induced dimerization may also be the driving force behind the activation, trafficking and signaling of oncogenic ErbB2. It is clear from our study

that the transmembrane domain of EGFR does not impose restrictions, as the transmembrane domain of LZ-EGFR-GFP is retained. Dimerization is thus the determining event in the activation, trafficking and signaling of EGFR.

Other evidence supporting the role of dimerization in the activation of receptors come from the studies of a mutant EGFR, type-III deletion variant of the EGFR (EGFRvIII). EGFRvIII is devoid of amino acids 6-273 and is constitutively active. It was shown recently that EGFRvIII is constitutively dimerized (Fernandes *et al.*, 2001), which suggests that the dimerization is the driving force for the activation of EGFRvIII. In other studies, leucine zipper induced dimerization of human growth hormone receptor (GHR) also lead to the full activation of GHR in the absence of ligand (Behncken *et al.*, 2000). All of these results, in addition to the results of our study support an important role of receptor dimerization, instead of ligand binding in receptor activation to initiate cell signaling and receptor trafficking.

Various ligands including EGF and TGF-α are able to dimerize EGFR and activate EGFR tyrosine kinase activity. However, they result in different binding affinity to the downstream proteins and a different endocytic pathway of EGFR. These results may suggest that ligand binding is not only required for the dimerization of EGFR, but also regulates the events following dimerization. On the other hand, the different effects of EGF and TGF-α may also be due to the different dimer stability regulated by the two ligands. Indeed, it has been shown

that the binding between TGF-α and EGFR is less resistant to low pH than the binding between EGF and EGFR. In the early endosomes, the EGFR-TGF-α complex dissociates and EGFR recycles back to the plasma membrane (Lenferink *et al.*, 1997). It is very likely that the TGF-α-induced EGFR dimer dissociates following the dissociation of TGF-α from EGFR, and dissociation of the EGFR dimer then results in the altered trafficking and interactions with downstream signaling proteins.

Results from our leucine zipper induced dimerization study warrant further investigation. Although our results with LZ-EGFR-GFP clearly demonstrate that the dimerization of EGFR in the absence of ligand is sufficient to activate EGFR and stimulate its possible endocytosis and signaling, we did not address whether the dimerization is necessary for the endocytosis and signaling of EGFR. Such a study would require the disruption of EGFR and the subsequent examination of the effects on EGFR endocytosis and signaling.

In response to the low percentage of dimeric complexes evident in the crosslinker assay, future aim would be to perform the assay with other chemical crosslinkers that are available or that have become available during the completion of this study. One method to examine dimeric complexes induced by leucine zippers would be Size Exclusion Chromatography. This molecular weight determining method would provide evidence of low molecular weight monomeric receptors and high molecular weight dimeric receptors.

To clarify the origin of endosomal LZ-EGFR-GFP in the fluorescence analysis and colocalization studies, we could express the gene of interest in an inducible vector expression system. This would allow controlled expression of the chimeric protein and a time-based observation of LZ-EGFR-GFP prior to and following the assumed activation at the plasma membrane. Although it is assumed that the LZ-EGFR-GFP is endocytosed following activation at the plasma membrane, one method to confirm this would be to block the endocytosis of LZ-EGFR-GFP. This could be achieved by introducing a dominant negative mutant of dynamin (K44A) or a clathrin mutant. If the expression of dynamin or clathrin mutants results in the disappearance of endosome-associated LZ-EGFR-GFP, such an outcome would suggest that the observed endosomes-associated LZ-EGFR-GFP is internalized from the plasma membrane.

To enhance the outcome of our subcellular fractionation study, one future direction would involve the addition of a control fractionation of EGFR-GFP following EGF stimulation and under serum starved conditions. Such a result would confirm that indeed this well-established experimental method is capable of revealing the subcellular localization of LZ-EGFR-GFP. A supplemental immunoblot that would be informative is an anti-pEGFR as such an immunoblot would validate the presence of internalized and activated dimeric LZ-EGFR-GFP complexes under serum starved conditions. Furthermore, the application of the tyrosine kinase inhibitor AG1478, prior to subcellular fractionation would perhaps

provide suggestive evidence linking receptor activation and endocytosis.

In conclusion, we show in our study that substitution of the complete extracellular domain with a c-Fos leucine zipper domain results in the dimerization of EGFR. The non-ligand induced dimerization of EGFR results in the constitutive activation of EGFR tyrosine kinase and phosphorylation of the five major tyrosine residues in the C-terminus. This constitutively activated LZ-EGFR-GFP is localized to endosomes and is able to activate several signaling pathways that lead to the stimulation of cell proliferation under serum starved conditions. We conclude that receptor dimerization is a critical event in EGFR activation and the subsequent trafficking and signaling.

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