MECHANISM OF THE CROSS TALK BETWEEN GROWTH HORMONE RECEPTOR AND EPIDERMAL GROWTH FACTOR RECEPTOR

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ABSTRACT

EGF receptor (EGFR) is a receptor tyrosine kinase, mediating cell growth from ectodermal and mesodermal origin. The overexpression and aberrant function of EGFR are involved in a wide range of human carcinomas. Growth hormone receptor (GHR) co-exists with EGFR in various cell types and implicated in malignant tumor behavior. Previously in our lab, we found that via ERK activation GH can threonine phosphorylate EGFR and reduce EGF-induced EGFR downregulation. GH also synergizes with EGF in activating ERK. In this project, using reconstitution, we studied the mutation of the ERK phosphorylation consensus residue on EGFR, $T^{669}$. CHO-GHR cells, which lack EGFR and express GHR, were stably transfected to express human wild-type or T669A EGFR. GH or EGF caused phosphorylation of WT, but not T669A EGFR indicating that $T^{669}$ is required for this phosphorylation. Notably, EGF induced more rapid downregulation of EGFR in cells expressing EGFR T669A or WT EGFR with inhibited ERK activation, suggesting $T^{669}$ phosphorylation serves as a brake in EGF-induced receptor downregulation. In signaling experiments, EGFR T669A displayed enhanced acute EGFR tyrosine phosphorylation, suggesting that $T^{669}$ phosphorylation negatively modulates EGF-induced EGFR kinase activity. Similar findings were observed in a human fibrosarcoma cell line that harbors an activating Ras mutation that results in constitutive ERK activation. Collectively, these data indicate that ERK activation mediates threonine phosphorylation in the EGFR and modulates EGFR signaling. Then
we study the mechanism of signaling synergy between GH and EGF. We found that the signaling synergy existed specifically in MEK/ERK pathway, and at the level between Raf-1 and MEK. Previous reports suggest that KSR (Kinase Suppressor of Ras) is a major molecule modulating growth factor-induced ERK activation at Raf-1/MEK level; hence we examined KSR activation by detecting phosphorylation on serine 392. Notably, GH and EGF synergize in KSR activation, suggesting that KSR might be involved in the signaling synergy between GH and EGF. This study help us better understand GH’s effect on EGF signaling and EGF receptor behavior.
DEDICATION

I would dedicate this to my mother, Shulan Deng, and my father, Xingmao Li, for their love, encouragement and understanding; especially to my husband, Yu Yang, for his love and support. I would not be where I am today if it were not for them.
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# ABSTRACT

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INTRODUCTION

Epidermal Growth Factor:

Epidermal growth factor (EGF) was first discovered in 1962. At first, it was found as “tooth-lid factor”, because when salivary gland extract was injected into newborn mice, it led to eruption of incisors and precocious opening of the eyelids (1). Later, another stimulator, urogastrone was discovered. It was isolated from human urine and shown to inhibit gastric acid secretion from the intestinal mucosa (2-4). Both these factors were later identified as EGF(4). EGF distinguish itself by its ability to stimulate epidermal growth and keratinization (1, 2, 5). EGF also stimulates cell proliferation from ectodermal and mesodermal origin (2, 3, 5). Within the last decade, EGF related research has expanded at a tremendous speed, since it was found to be involved in numerous human cancers. Studies focusing on either the ligands or receptors are significantly impacting on the development of clinical cancer therapies.

EGF is a single strand polypeptide, composed of 53 amino acid residues. Among them are 6 cysteines (2, 3, 6). These cysteines serve important functions of maintaining biological activity of EGF by forming three intramolecular disulfide bonds (7). EGF is synthesized as a precursor, and undergoes proteolysis to generate the mature form (3). The EGF precursor, consisting of 1217 amino acid residues, is a glycosylated transmembrane protein. It has molecular weight of 140-150 kDa (2, 3, 8). This precursor is also functional. Like mature EGF, the precursor can also bind EGF receptor. The binding capacity is similar to mature EGF. EGF precursor can also stimulate the
proliferation of keratinocytes of mouse origin, similar to what was found with mature EGF. The EGF precursor was also observed to participate in the cell-cell interaction in vivo (3, 5, 9-11). More over, EGF precursor may also exert other biological effects locally, including cell growth, morphological changes and cell migration by interacting with cells expressing EGF receptors nearby.

EGF is one of the members of EGF family that is composed of several growth factors with similar structure as EGF. These other growth factors include: transforming growth factor-α (TGFα), amphiregulin (AR), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), epigen, epiregulin (EPR) and neuregulins. Each of these mature peptide growth factors is characterized by the consensus sequence consisting of the six spatially conserved cysteine residues, with the following interactions: C1-C3, C2-C4, C5-C6. This consensus sequence is known as EGF motif and is crucial for binding to EGFR receptor tyrosine kinase family. Among all the members, EGF, TGFα, and amphiregulin are further regarded as group one of the EGF family, since they bind and activate EGF receptor only (EGFR is also known to be erbB-1 and HER1). Two neuregulins that bind erbB-3 and erbB-4 are referred as group two. HB-EGF, betacellulin and epiregulin constitute group three, for their preference of binding both EGFR and erbB-4. Up till now, there is not direct ligand found for ErbB-2 yet, but it can heterodimerize with each of the other erbB members in the family, and hence is activated and initiates downstream signaling. (3, 9, 12, 13) (Figure 1).
EGFR family members are transmembrane glycoprotein having intrinsic tyrosine kinase activity. These receptors have similar structures. Their structure include: extracellular ligand binding domain; α-helical transmembrane pass; and intracellular domain, which has tyrosine kinase activity. After binding with its ligand, EGFR forms homodimer or heterodimer with other members in the family. ErbB2 hasn’t found to have a ligand. And erbB3 doesn’t have kinase activity. Upon stimulation, they form heterodimer to initiate downstream signaling.
Epidermal Growth Factor Receptor Structure:

Epidermal growth factor affects cell growth, differentiation, motility, and adhesion (14) by interacting with its receptors. EGF receptor has only one isoform and has intrinsic tyrosine kinase activity. The gene encoding EGFR is located on chromosome 7p11-13, and is composed of 26 exons of about 110kb. EGFR is synthesized as a precursor of 1210-residues. Then, the N-terminal sequence is cleaved leaving an 1186-residue protein inserted into the cell membrane (15). EGFR is also a glycoprotein. Before EGFR is inserted into the cell membrane, it undergoes N-linked glycosylation, which is critical for the cell membrane translocation and acquisition of function (16). EGFR is a type I single transmembrane protein. The amino-terminus of the molecule is located outside the cell membrane, and is composed of 622 amino acids; the carboxyl-terminus is located inside the cell, and consists 542 amino acids. The transmembrane part is an alpha-helix. (figure 1) The extracellular part serves the important function of ligand binding, which is carried out by two cysteine-rich domains. The intracellular part can be further grouped into three domains: juxtamembrane domain, tyrosine kinase domain and carboxyl-terminal tail. The juxtamembrane domain comprises about 50 amino acids, and regulates the receptor activation, downregulation and further biological effects initiated after receptor activation. The tyrosine kinase domain is composed of around 250 amino acids, serving the function of phosphorylation and activation of downstream signaling cascades. The carboxyl-terminal tail contains five autophosphorylation motifs, which serve as docking sites for SH2 or PTB (phosphotyrosine binding) domains of intracellular signal transducers. This tail is also the substrate
for auto-inhibition. These autophosphorylation motifs share similar structures (17), and are functionally redundant. (figure 4)

EGFR belongs to ErbB receptor super family, which consists three other ErbB receptors: ErbB2 (c-neu), ErbB3, and ErbB4. They share similar structures as EGFR. However, although they have similar extracellular domain, the ligands that bind and activate ErbB receptors vary. EGFR, ErbB3 and ErbB4 have their own ligands and no ligands are known for ErbB2, as stated above. All four members have intracellular tyrosine kinase domain, but only EGFR, ErbB2 and ErbB4 have tyrosine kinase activity. ErbB3’s tyrosine kinase domain is catalytically inactive. However, ErbB2 and ErbB3 can be activated and initiate downstream signaling by forming heterodimers with other members in the family. In addition to homodimers, heterodimers can be formed between: ErbB1-ErbB2, ErbB1-ErbB3; ErbB2-ErbB3, and ErbB3-ErbB4. ErbB2 is a preferred partner for heterodimerization. By forming a heterodimer with its partner, it can respond to ligand stimulation, and can augment ligand-induced signaling (18, 19) (Figure 1).

Epidermal Growth Factor Receptor Signaling:

After ligand binding, EGFR is activated resulting in activation of the tyrosine kinase domain, which in turn, phosphorylates multiple tyrosine residues in the more distal end of the intracellular domain (20). Up till now, there are ten tyrosine residues found to be phosphorylated during the process (21-23). The phosphorylation of these tyrosine residues serves as docking sites recruiting a number adaptor proteins, such as Grb2, Shc, PLC-γ, and et al (24, 25). The association of these adaptor proteins further complexes with and activates their unique downstream signaling molecules, which results in the
simultaneous activation of multiple pathways. There are three major signaling pathways initiated by EGFR activation, and eventually regulates gene expression and cell behavior.

**Shc, Grb2, and the Ras/MAPK pathway:**

The most studied pathway initiated upon EGF receptor activation is Ras-Raf-MAPK pathway. The commonly accepted starting point of this signaling cascade is believed to be the association of adaptor protein Grb2. It is considered to be the key player in EGF-dependent Ras activation (26). In quiescent state, Grb2 is localized in cytosol, and constitutively bound to Sos, which is the Ras exchange factor. After ligand stimulation, the tyrosine residues in the cytoplasmic tail are phosphorylated by the kinase domain from the dimerizing partner, and recruit Grb2. Two tyrosine residues mainly involved with Grb2 association are $^{1068}$Y and $^{1086}$Y (27). Grb2 associates with the receptor either directly by its SH2 domain, or indirectly, by binding to tyrosine phosphorylated Shc (28). Shc first associates with EGFR via its PTB domain, which, like the SH2 domain, binds phosphorylated tyrosine residues. Then, Shc is tyrosine phosphorylated and recruits Grb2 (29). In some cell systems, it is considered to be the main step in EGF-dependent induction of the Ras/MAPK pathway, however in other cellular systems Shc is not necessary for Ras activation by EGFR. Until now, it is still not fully understood whether the two modes of Grb2 recruitment have different functional roles, or they are simply part of cell-type specific variance. After Grb2/Sos translocates to the cell membrane and forms a complex with the receptor, they initiate interaction with membrane-associated Ras. The Ras-bound GDP is exchanged for Ras-bound GTP, which represents Ras activation. Activated Ras further activates Raf-1 (30). In turn, Raf-1 activation leads to the phosphorylation and activation of MEK and ERK. Upon activation, ERK translocates
into the nucleus and phosphorylates multiple nuclear transcription factors (31). Besides nuclear translocation, activated ERK also negatively feed back to this pathway, dissociates the Grb2-Sos complex, and terminates the signaling event. (28).

*PI3K/Akt Pathway:*

Phosphoinositide-3-kinase (PI3K)/Akt is another important pathway initiated upon EGFR activation and contributes to a variety of cellular processes including cell survival, and migration (32). PI3-Kinase is the key player in the pathway. It consists of a regulatory subunit (p85) and a catalytic subunit (p110). The regulatory subunit binds specifically to a pYXXM motif via its two SH2 domains. Quite a few cellular proteins contain this motif, such as PDGF (platelet-derived growth factor) receptor, IRS 1 (insulin receptor substrate 1), IRS2, and Gab1. However, EGFR does not have this p85 binding motif in its intracellular domain, hence EGF stimulated PI3-K activation is by an indirect mechanism. Two modes of EGF-induced PI3K activation have been reported, one via formation of EGFR/Erb3 hetherodimers (33) and the other mediated by Gab1. Unlike EGFR, the cytoplasmic region of ErbB 3 contains more than six of those motifs(34, 35). After ligand stimulation, ErbB3 can be phosphorylated by the activated kinase domain of EGFR, and is phosphorylated on the tyrosine residue in pYXXM motifs. Gab1 (Grb2-associated binder-1) is a docking protein. EGF stimulation leads to Gab1 association with the receptor. Gab1 associates with EGFR either by direct binding(36), or through Grb2. The direct binding is via an 83 amino acid-stretch on EGFR, termed the Met-binding-domain (MBD); while the majority of the association is believed to be by indirect binding of its proline rich region with SH3 domain on Grb2. (37-40) After Gab1 associates with the receptor, it get tyrosine phosphorylated, further recruits PI3K by its three pYXXM
motifs, and activates it (41). Akt is a major downstream mediator of PI3-K and is involved in several aspects of cell behavior, such as survival and proliferation. Akt may well be the major mediator of the anti-apoptotic effects of EGFR activation.

**PLCγ Pathway:**

PLCγ is the major molecule in another important pathway initiated upon EGFR activation. Although it has not been fully elucidated how PLCγ is activated by EGFR, there are reports saying that it requires direct association with the receptor (42). The binding sites on EGFR responsible for PLCγ association are Y992 and Y1173 (43). Then PLCγ is phosphorylated by the EGFR kinase on Y771 and Y1254 (44). Upon activation, PLCγ hydrolyses PtdIns(4,5)-P2 which generates two second messengers 1,2-diacylglycerol (DAG) and inositol 1,3,5-triphosphate (IP3). DAG helps mediate activation of protein kinase C (PKC), which further activates several signaling molecules such as MAPK, JNK (45, 46), and Na+/H+ exchanger (47). IP3 triggers calcium release inside the cells, which in turn regulates numerous calcium dependent enzymes. Hence, via IP3, EGFR regulates calcium dependent pathways such as NFκB and RaI (48, 49).
Figure 2. EGFR signaling pathways.
The EGFR activating signaling pathways are complicated. Major pathways involves Ras/Raf/ERK pathway, PI3 kinase pathway, and PLC-gamma pathway.
EGF Receptor Trafficking and Downregulation:

EGF receptor signaling and physiological effects are tightly modulated by negative regulatory mechanisms. One of the mechanisms that terminates the signaling events is degradation of activated receptors. EGFR degradation starts from ubiquitination of the receptor. Ubiquitination is formation of covalent bonds between ubiquitin, an evolutionarily conserved protein, and a substrate protein (50). Ubiquitination is a three steps processes, each step mediated by a specific enzyme, namely ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-ligase enzyme (E3). The covalent bond formed is an isopeptide linkage between the C-terminal glycine (Gly-76) of ubiquitin and the ε-amino group of a lysine in the substrate (50). There are two types of ubiquitination. One is monoubiquitination or multiple monoubiquitination; the other is polyubiquitination. Monoubiquitination is attachment of a single ubiquitin to a single lysine, and multiple monoubiquitination refers to several lysine residues of proteins being modified, each lysine residue attached to a single ubiquitin. (51). Monoubiquitination is found to be involved in endocytosis of plasma membrane proteins, lysosomal degradation and budding of retroviruses from the plasma membrane (51). Polyubiquitination is adding ubiquitin molecules to ubiquitin itself, thus forming a ubiquitin chain (50, 51). Polyubiquitination consists ubiquitin chain of at least four ubiquitins, and is found to be involved in proteasomal degradation (50). EGFR undergoes multiple monoubiquitination.

One of the key molecules mediating EGFR ubiquitination is E3 ligase, c-Cbl (52). Upon EGF stimulation, it is recruited to the plasma membrane, mediates ubiquitination, and is involved in intracellular receptor handling. Cbl is recruited to
ubiquitin-conjugating enzymes (E2) by its RING finger domain and then it directly binds to phosphorylated EGFRs with its TKB (tyrosine kinase-binding) domain and mediates the transfer of ubiquitin to the receptor (53). Cbl can be tyrosine phosphorylated by EGFR, but whether this relates to its function of mediating ubiquitination is not clear. The role of Cbl in ligand-induced EGFR degradation was supported by the observations that the ubiquitination and degradation of the activated EGF receptor can be enhanced by overexpression of Cbl. Studies about the detailed processes of EGFR ubiquitination suggest that EGFR ubiquitination occurs on cell surface, before it undergoes endocytosis. The above theory is supported by the observation that under conditions of low temperature, which impairs endocytosis, or by expression of a GTPase-defective dynamin, Cbl can still ubiquitinate EGFRs (54, 55). From endocytosis until the late endosome, Cbl is associated with the EGFR and mediates multiple cycles of ubiquitin transfer., (55-57). The exact number of ubiquitins added onto EGFR is yet unknown, but it is estimated to be a limited number (57), probably relating to the number of lysines present in the kinase domain of the receptor (58). Multiple monoubiquitination of EGFRs serves as a signal contributing to endocytic trafficking and sorting of receptors for degradation in the lysosome (54, 58).

Besides Cbl, there are several other groups of molecules involved in trafficking and degradation pathways of EGFR. During the process of internalization and endocytosis, the ubiquitinated receptor interacts with epidermal growth factor pathway substrate 15 (Eps15) and clathrin adaptor complex 2 (AP-2) (55). Cbl-interacting protein of 85 kDa (CIN85) also links to EGFR-Cbl complex.(59). Dynamin, a GTPase, then mediates the vesicle fission from the plasma membrane (60). After that, clathrins are shed,
enabling the vesicles to fuse with cytoplasmic vesicular structures and form early endosomes. The fusion process is mediated by another GTPase, termed Ras-association protein 5 (Rab5) (61).

Although the importance of Cbl-mediated EGFR ubiquitination in receptor downregulation is widely accepted, its role in receptor endocytosis is under debate. First, it was observed that truncated EGFR, which lacks the Cbl binding site, internalized at a similar rate compared with WT EGFR (62). Another phenomenon observed was that when overexpressing Cbl, the already endocytosed receptors degrades faster, while receptor internalization remains at the similar rate. Hence, it might be that Cbl’s principal task is facilitating EGFR intracellular trafficking rather than endocytosis.

During the process of intracellular processing, early endosomes invaginate and fuse with late endosomal compartment, which eventually forms multivasicular bodies (MVB) (64). This event ultimately ceases EGFR signaling by segregating the active receptor domain from its cytoplasmic transducers. There are three groups of proteins acting consecutively in MVB sorting, termed endosomal sorting complexes required for transport I-III (ESCRT-I-III). ESCRT-I includes: tumor susceptibility gene 101 (TSG101), human Vps28 (hVps28) and hepatocellular carcinoma related protein 1 (HCRP1). Among them, TSG101 is responsible for cargo recognition (65). The function of the other two components has not been specified. ESCRT-II includes: Vps22, Vps25, and Vps36 (66). They act downstream of ESCRT-I, are transiently recruited to the endosomal membrane, and help the formation of ESCRT-III, which is composed by Vps2, Vps20, Vps24 and sucrose non fermentor 7 (Snf7) (67). Hepatocyte growth factor regulated tyrosine kinase substrate (Hrs), in combination with signal transducing adaptor
molecules 1 and 2 (STAM1, STAM2) and the Golgi-localised, γ-carbonyl-containing, Arf-binding protein 3 (GGA3) connect EGFR and the ESCRT apparatus at endosomes (68, 69). Once the ESCRT machinery has completed cargo reception and concentration, the MVB fuses with the lysosome, and delivers the EGFR-carrying vesicles to the hydrolytic lysosome to be degraded.

Interesting findings suggest that EGFR after internalization can still signal and activate specific pathways. It was found that EGF receptor, after internalization is still associated with Grb2, Shc, and mSOS (70). A more interesting approach that blocks EGFR endocytosis results in upregulation of specific signal transduction pathways while others are attenuated (71), suggesting certain signals can arise from the endosomal compartment. PLC-γ and PI3-K pathways are only initiated from the cell surface, while the Ras pathway can be generated from both the cell surface and intracellular compartments (72, 73). These data reveal that trafficking can not only lead to receptor degradation, but can also modulate the specificity and the duration of the signal transduction.
Figure 3: EGFR trafficking and downregulation pathway:
EGFR downregulation starts from EGFR binding with its ligand. Upon ligand binding, EGFR are activated and dimerized. Activated EGFR recruits Cbl and get ubiquitinated. Then, they underwent endocytosis, and are sorted to early endosomes. A small fraction get sorted to recycling endosomes and recycle back to cell surface, while majority sorted to late endosomes and degraded in lysosome.

--- Adapted from Mol Biol Cell 12:1897-1910
Site Specific Phosphorylation of EGFR:

Site specific tyrosine phosphorylation of the EGFR:

Following ligand binding, EGFR undergoes enzymatic activation. These events result in the trans-(auto)-phosphorylation of multiple tyrosine residues in the COOH-terminal tail of the molecule. These site specific phosphorylated tyrosines serve as binding sites for cytoplasmic signaling proteins containing SH2 domains (74). There are five major sites of autophosphorylation that have been identified in EGFR: $^{1068}\text{Tyr}$, $^{992}\text{Tyr}$, $^{1045}\text{Tyr}$, $^{1173}\text{Tyr}$, and $^{1086}\text{Tyr}$ (26, 75-77). Each of these sites has its own preferred binding adaptor or signaling proteins, and exerts a unique contribution to subsequent signaling events. Phosphorylation of Tyr-845 in the kinase domain may stabilize the activation, maintain the enzyme in an active state and provide a binding surface for substrate proteins (78, 79). Phosphorylation of Tyr-992 provides the direct binding site for phospholipase C-γ, and contributes to activation of PLC-γ-mediated downstream signaling (80). Phosphorylation of Tyr-1045 creates a major docking site for c-Cbl (81). Phosphorylation of Tyr-1068 of EGFR forms a direct binding site for Grb2 (82). Grb2 is an important component in Ras/Raf/MAPK signaling pathway. Phosphorylation of Tyr-1173 provides a docking site for Shc (83). All these tyrosine residues are phosphorylated upon EGF stimulation, and serve as indicators of the receptor activation.

Serine/Threonine Phosphorylation of the EGFR:

Besides tyrosine phosphorylation, EGF receptor can also be modified by serine/threonine phosphorylation. In contrast to tyrosine phosphorylation, serine/threonine phosphorylation is more involved with the regulatory effects on the receptor itself. There are four major serine/threonine phosphorylation sites in the cytoplasmic domain of the
receptor, include: $^{654}T$, $^{669}T$, $^{1046}S$, and $^{1047}S$ (84-92). $^{1046/7}S$ located at the carboxyl-terminal region, which is an important effector domain of the EGFR that binds to SH2 regions of signaling molecules (26, 93, 94). It is a substrate for calmodulin-dependent protein kinase II (CAM kinase II) (90). Mutation of Ser$^{1046/7}$ by replacement with Ala residues results in enhanced EGF signaling, and markedly inhibits EGF-stimulated endocytosis and downregulation of cell surface receptors (90). Thr$^{654}$ phosphorylation is mediated by PKC (95). Phosphorylation at this site causes an inhibition of the receptor tyrosine kinase activity (84, 86, 96, 97). Site-directed mutagenesis of the EGF receptor demonstrates that the replacement of Thr$^{654}$ with Ala blocks the desensitization of protein-tyrosine kinase activity (91, 98). A recent study shows that phosphorylation at Thr$^{654}$ diverts internalized EGFR molecule from a degradative fate to a recycling pathway (99). Thr$^{669}$ is the major threonine phosphorylated after EGF stimulation in intact cells (85). The primary sequence surrounding Thr$^{669}$ is the proximity of two proline residues (-Pro-Leu-Thr$^{669}$-Pro-), which is a consensus sequence for ERK-mediated phosphorylation. In an in vitro assay, using a synthetic peptide corresponding to residues 663-681 of EGF receptor, it was demonstrated that Thr$^{669}$ can be phosphorylated by MAP kinase (88, 89, 100). Unlike the above sites, the impact of phosphorylation at Thr$^{669}$ on EGFR kinase activity and degradation has not been thoroughly studied.
Figure 4. EGFR structure and tyrosine and serine/threonine phosphorylation of EGFR:
EGF receptor is a single transmembrane protein, consist an extracellular domain, a transmembrane domain and a cytoplasmic domain. In cytoplasmic domain, there are multiple tyrosine and serine/threonine phosphorylation sites. Tyrosine phosphorylation sites are often related with activation and function of some specific signaling pathway, and serine/threonine phosphorylation involved in regulatory role of receptor activation and downregulation.

PKC pathway: threonine phosphorylate EGFR on T654;

CaM Kinase II: serine phosphorylate EGFR on S1046 and S1047;
Growth Hormone and Its Crosstalk with EGFR Family:

Growth hormone is a 22k Da peptide (101), the majority of which is released from the anterior pituitary (102). It has important physiological functions in longitudinal bone growth, anabolism and metabolic regulation. GH functions through binding and activating its receptor, GHR, and initiates three major signaling pathways, including Jak2/Stat5, PI3-K/Akt, and Ras/Raf/MAP Kinase pathways (103-105). Recent research has revealed interesting findings that GH can also be released in an autocrine/paracrine fashion. More interestingly, it was found that GH may have implications in malignant carcinomas, such as mammary neoplasia (106). Thus, studies about signaling aspects of GH crosstalk with the EGF receptor family may have an impact on studies involving malignant tumors in various types of tissues and might be a fruitful direction to explore.

Within the past decade, several discoveries have been made in this respect. In 1997, Yamauchi demonstrated that GH caused tyrosine phosphorylation of EGFR, and this phosphorylation is independent of EGFR kinase activity, but rather depends on Jak2 activation. This GH induced tyrosine phosphorylation is on Tyr1068 of EGFR, which is the binding site for Grb2, suggesting that EGFR serves as a docking site for the GHR-induced ERK activation (107).

Studies about another member of the EGFR family, ErbB2, showed that GH induces a lessening of both basal and EGF-induced overall tyrosine phosphorylation on ErbB2, and a decrease of ErbB2 tyrosine kinase activity. Meanwhile, GH induces retardation of ErbB2 migration on SDS-PAGE, which was found to be caused by serine/threonine phosphorylation induced by GH. Both GH induced decrease in ErbB2 tyrosine phosphorylation and retardation in migration can be reversed by a MEK inhibitor (108).
To better study the GH induced serine/threonine phosphorylation on ErbB2, a monoclonal antibody (PTP101) that specifically detects proteins phosphorylated at consensus sites for proline-directed protein kinases, including ERK, was employed (109, 110). GH-induced ErbB2 serine/threonine phosphorylation was indeed recognized by PTP101, suggesting that GH, via ERK activation, serine/threonine phosphorylates ErbB2, and lessens EGF-induced ErbB2 activation.

Similar studies were carried out in the same cellular system to study EGFR. It was found that GH also causes PTP101-reactive phosphorylation of EGFR. Although it did not cause reduced EGFR tyrosine phosphorylation, GH pretreatment substantially decreased EGF-induced EGFR degradation, which is consistent with the finding of a reduction in EGF-induced intracellular redistribution. Notably, the protection from EGF-induced EGFR degradation and the PTP101 reactive phosphorylation were both reversed by a MEK1 inhibitor, suggesting that GH, via ERK activation, regulates EGF-induced trafficking and downregulation (111). Signaling studies show that GH cotreatment leads to an augmentation of EGF-induced ERK activation and Cbl tyrosine phosphorylation. The augmentation of Cbl tyrosine phosphorylation can be eliminated by MEK1 inhibitor, suggesting GH, by activating ERKs, also modulates EGF-induced EGFR signaling(111).

The abundance, tyrosine phosphorylation state and downstream signaling events of EGFR family members are of significant importance in tumor studies. GH’s impacts in these important areas should be a promising area for future research.
MAP Kinase Signaling Module and Its Modulating Molecule, KSR:

ERK is a member of MAPK (mitogen-activated protein kinases) super family, which is well known for its tightly regulated signaling module. The module is a protein kinases cascade starting from MAPKKK, which phosphorylates and activates MAPKK. MAPKK, in turn, phosphorylates and activates MAPK (112). The protein kinases that form MAPK signaling modules may be organized into signaling complexes by scaffolding proteins, forming a functional MAPK module and allowing a series of sequential interactions to create a protein kinase cascade (113, 114). Using artificial scaffolds, it has been observed that these molecules act by increasing the local concentration of the protein kinases in the cascade; providing spatial and temporal control of MAPK signaling; and eventually allowing for the precise regulation of MAPK signaling (115, 116).

Recent studies have indentified a number of scaffolding proteins, which may contribute to the physiological regulation of MAPK modules. In the table below are those found to function in coordination of extracellular signal-regulated kinase (ERK) modules in mammals. (Table 1.)
Kinase suppressor of Ras (KSR) is a highly conserved intracellular protein that is a positive regulator of Ras/Raf/ERK signaling (117-119). KSR acts as a scaffold for the assembly and activation of the ERK module. There are five conserved domains among KSR family members, including CA1, a 40-residue region unique to KSR proteins; CA2, a proline-rich region; CA3, a cysteine-rich zinc finger domain; CA4, a serine/threonine rich area; and CA5, a putative kinase domain at the C-terminal region. (figure 5)

Although KSR has a putative kinase domain, it does not have kinase activity, but rather functions as a scaffolding protein.
In both quiescent and growth factor-treated cells, MEK1 and MEK2 bind directly to KSR in the CA5 domain (120, 121). ERK1 and ERK2 also bind directly to KSR mediated by an FxFP motif in the KSR CA4 domain (122) and their binding is induced by Ras activation (120, 121). The association between Raf and KSR is not as well understood and observations vary among different species (123-125). Whether or not Raf-1 pre-associates with KSR, Ras activation and membrane recruitment appears to be universally required for KSR-mediated ERK activation (125, 126). In quiescent cells, KSR is localized in cytosol, and constitutively phosphorylated at Ser\(^{392}\) and Ser\(^{297}\), which serve as the binding sites for protein 14-3-3 (126). The binding of 14-3-3 retains KSR in the cytoplasm and in an inactive state (126). In response to signal activation, the two serine sites are dephosphorylated and KSR releases 14-3-3, thereby facilitating KSR’s binding with ERK and membrane localization (120, 126). Eventually, KSR helps aggregate MEK with activated Raf-1 and provides a docking site for ERK at the plasma membrane. The complex it forms facilitates the sequential phosphorylation, and contributes to ERK activation. (Figure 5)

Regarding other adaptor molecules in the ERK signaling module, MP1 functions only to regulate MEK1-ERK1 (127). \(\beta\)- Arrestins mainly function in the G-protein-coupled receptor signaling pathway (128), and CNK and SUR-8 function between Ras and Raf-1 (49, 129). As KSR’s effects appear to be more related, we have chosen it as our candidate to study in this project.
A:

Figure 5: KSR structure and activation procedure:
A. KSR molecule contains 5 conservative domains: CA1, CA2, CA3, CA4 and CA5. Among them, CA5 is important in binding MEK, while CA4 is the site binding MAPK. Serine 297 and serine 392 serves regulatory role, modulating KSR activation.

B. In quiescent state, KSR bind with MEK and KSR is basally phosphorylated at serine 297 and serine 392. The phosphorylation of these sites enables protein 14-3-3 binding to KSR and keeps it in inactive state. Upon stimulation, these two serine dephosphorylated, and protein 14-3-3 fall off. KSR undergoes conformational change and associates with MAPK. Eventually, KSR helps aggregate MEK, MAPK with activated Raf-1, and contribute to MAPK activation.

--- Adapted from J. Cell Sci. (2001) 114, P1609-1612
**Objective of Dissertation**

Epidermal growth factor is a 53 amino acid mitogenic protein that is involved in normal cell growth, wound healing and oncogenesis. EGF exerts its biological function through activating EGF receptor, which is a single transmembrane glycoprotein, with intrinsic tyrosine kinase activity. The overexpression and aberrant function of EGFR and its ligands are involved in a wide range of human malignant tumors. Ligand activation of the EGFR leads to its rapid internalization and eventual delivery to lysosomes for degradation. It is one of the most important mechanisms to attenuate signaling. Previous work in our lab has shown that growth hormone (GH), which exists along with EGF *in vivo*, blunts EGF-induced EGFR downregulation through ERK activation. Meanwhile, also via ERK activation, GH causes threonine phosphorylation of EGFR. In this project, we examine: 1) which site of EGFR is required for Serine/Threonine phosphorylation by activated ERK; 2) how does the Serine/Threonine phosphorylation of EGFR retard EGF-induced EGFR downregulation, trafficking and receptor activation. The Ras/Raf/MAPK pathway is one of the most important signaling pathways carrying out functions of EGFR. Our previous work showed that GH synergizes with EGF in ERK activation. In this study, we further explored 1) the level at which the synergy might occur; 2) the synergy between GH and EGF might be correlated to the enhanced activation of KSR, which modulates RAF-MEK-MAPK pathway.
ERK-DEPENDENT THREONINE PHOSPHORYLATION of EGF RECEPTOR MODILATES RECEPTOR DOWNREGULATION and SIGNALING

by

XIN LI, YAO HUANG, JING JIANG, AND STUART J. FRANK

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ABSTRACT

Epidermal growth factor (EGF) signaling is critical in normal and aberrant cellular behavior. Extracellular signal-regulated kinase (ERK) mediates important downstream aspects of EGF signaling. Additionally, EGFR undergoes MEK1-dependent ERK consensus site phosphorylation in response to EGF or cytokines such as growth hormone (GH) and prolactin (PRL). GH- or PRL-induced EGFR phosphorylation alters subsequent EGF-induced EGFR downregulation and signal characteristics in an ERK-dependent fashion. We now use reconstitution to study mutation of the sole EGFR ERK phosphorylation consensus residue, 669T. CHO-GHR cells, which lack EGFR and express GHR, were stably transfected to express human wild-type or T669A (669T changed to alanine) EGFRs at similar abundance. Treatment of cells with GH or EGF caused phosphorylation of WT, but not T669A EGFR, in an ERK activity-dependent fashion that was detected with an antibody that recognizes phosphorylation of ERK consensus sites, indicating that 669T is required for this phosphorylation. Notably, EGF-induced downregulation of EGFR abundance was much more rapid in cells expressing EGFR T669A vs. WT EGFR. Further, pretreatment with the MEK1/ERK inhibitor PD98059 enhanced EGF-induced EGFR loss in cells expressing WT EGFR, but not EGFR T669A, suggesting that the ERK-dependent effects on EGFR downregulation required phosphorylation of 669T. In signaling experiments, EGFR T669A displayed enhanced acute (15 min) EGFR tyrosine phosphorylation (reflecting EGFR kinase activity) compared to WT EGFR. Further, acute EGF-induced ubiquitination of WT EGFR was markedly enhanced by PD98059 pretreatment and was increased in EGFR T669A-expressing cells independent of PD98059. These signaling data suggest that ERK-mediated 669T phosphorylation negatively modulates EGF-induced EGFR kinase activity.
We furthered these investigations using a human fibrosarcoma cell line that endogenously expresses EGFR and ErbB-2 and also harbors an activating Ras mutation. In these cells, EGFR was constitutively detected with the ERK consensus site phosphorylation-specific antibody and EGF-induced EGFR downregulation was modest, but was substantially enhanced by pretreatment with MEK1/ERK inhibitor. Collectively, these data indicate that ERK activity, by phosphorylation of a threonine residue in the EGFR juxtamembrane cytoplasmic domain, modulates EGFR trafficking and signaling.

INTRODUCTION

Epidermal growth factor (EGF) is a 53 amino acid peptide that has important roles in cell growth, differentiation, motility and adhesion (1). These effects are mediated by binding and activating EGF receptor (EGFR). EGFR belongs to ErbB receptor super family, a group of transmembrane glycoprotein receptors, consisting of four members: EGFR (ErbB-1), ErbB-2 (c-neu, HER2), ErbB-3 (HER3), and ErbB-4 (HER 4)(2-5). Except for ErbB-3, each has intrinsic tyrosine kinase activity in its cytoplasmic domain. EGF specifically binds to EGFR, promoting formation of either EGFR-EGFR homodimers or EGFR-ErbB-2 heterodimers and allowing the intracellular tyrosine kinase domains to approximate and undergo transautophosphorylation(6-10). Consequent to kinase activation and autophosphorylation, C-terminal receptor phosphotyrosine residues (ten identified thus far) are engaged by signaling molecules including SHC, Grb-2, SHP2, phospholipase-C-\(\gamma\), and others (11-16), leading to activation of downstream signaling by the ERK, PI3-kinase, and PLC-\(\gamma\) pathways. Deregulated function of EGFR family proteins, including EGFR, is significant in onset and behavior of many human cancers.
(17-21) and measures targeting EGFR downregulation may alter cancer behavior(19,22). Thus, it is important to understand mechanisms regulating EGFR signaling and trafficking.

The itinerary of EGF-induced EGFR trafficking has been intensely studied (23). After cell surface EGF binding, EGFR undergoes clathrin coated pit-mediated endocytosis at a much enhanced rate compared to the constitutive (ligand-independent) rate. Thereafter, the activated receptor enters the endosomal pathway. If not recycled to the cell surface (as in the absence of EGF stimulation), EGFR progresses from early to late endosomes and then to the multivasicular bodies, ultimately undergoing degradation in lysosomes in a process termed receptor downregulation. Previous views held that signaling emanated only from activated cell surface EGFRs and that internalization terminated signaling (24); more recent studies suggest that signaling in some measure emanates from EGFRs that are internalized, but not yet spatially reoriented in the MVB or degraded in the lysosome (25-30). Thus, altered post-endocytic trafficking of activated EGFR may quantitatively and/or qualitatively impact EGF signaling.

We previously examined interplay between growth hormone (31-33) and prolactin (34) signaling and EGF signaling in murine preadipocytes and human T47D breast carcinoma cells, respectively. We observed that GH caused tyrosine phosphorylation of EGFR; this tyrosine phosphorylation has previously been shown to be catalyzed by JAK2 and unassociated with EGFR kinase activation (35). In addition, we found that GH promoted serine/threonine phosphorylation of both EGFR and ErbB-2. Using a monoclonal antibody, PTP101, that detects (serine/threonine) phosphorylation at substrate consensus sites for extracellular signal-regulated kinase (ERK), we observed GH- and PRL-dependent PTP101-reactive EGFR and ErbB-2 phosphorylation that was prevented by
pretreatment of the cells with inhibitors of MEK1, the upstream ERK-activating kinase (32-34). For EGFR, this GH- or PRL-induced ERK-dependent phosphorylation retarded subsequent EGF-induced receptor downregulation and potentiated acute EGF-induced signaling(32-34). Furthermore, in T47D cells, EGF itself caused EGFR PTP101-reactive phosphorylation and blockade of MEK1 resulted in enhanced EGF-induced EGFR downregulation, suggesting that EGF-induced ERK-mediated threonine phosphorylation may serve as a brake on receptor downregulation(34).

In the current work, we extend these observations by studying EGF-induced EGFR PTP101-reactive phosphorylation, EGFR downregulation, and signaling in a reconstitution system. We map the EGFR threonine residue required for this ERK-dependent homologous phosphorylation and assess its impact on downregulation and signaling. Further, we investigate these issues using a human fibrosarcoma cell line that endogenously expresses EGFR and ErbB-2 and also harbors an activating Ras mutation. In these cells, constitutive PTP101-reactive phosphorylation of EGFR was detected and EGF-induced EGFR downregulation was modest, but was substantially enhanced by pretreatment with MEK1/ERK inhibitor.

MATERIALS AND METHODS

Materials

Recombinant human GH was kindly provided by Eli Lilly Co. (Indianapolis, IN). Recombinant human EGF was purchased from Upstate Biotechnology (Lake Placid, NY). The MEK inhibitors, PD98059 (New England Biolabs, Beverly, MA) and U0126 (Promega, Madison, WI), were purchased commercially.
Polyclonal anti-EGFR, monoclonal anti-EGFR, anti-ErbB-2, monoclonal anti-STAT5 (W-17) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal anti-EGFR used in co-immunoprecipitation with erbB2, monoclonal anti-phosphothreonine-proline antibody PTP101, polyclonal anti-phospho-EGFR antibodies anti-pTyr-992 and anti-pTyr-1068, polyclonal anti-phospho-AKT (recognizing phosphorylated serine 473), polyclonal anti-AKT antibodies (Cell Signaling Technology, Beverly, MA), anti-active mitogen-activated protein kinase affinity-purified rabbit antibody (anti-active ERK, recognizing the dually phosphorylated Thr-183 and Tyr-185 residues corresponding to the active forms of ERK1 and ERK2 (Promega, Madison, WI), monoclonal anti-phosphotyrosine antibody 4G10, anti-mitogen-activated protein kinase affinity-purified rabbit antibody (recognizing both ERK1 and ERK2) (Upstate Biotechnology, Lake Placid, NY), anti-ubiquitin monoclonal antibody (Stressgen Biotechnologies, Victoria, BC Canada) and anti-Flag antibody, anti-Flag affinity gel (Sigma, St Louis, MO) were all purchased commercially.

Cell culture and transfection

CHO-GHR cells (36) were cultured in Dulbecco’s modified Eagle’s medium containing 1.0 g/liter glucose (Cellgro, Inc. Hemdon, VA) supplemented with 5% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin (all from Biofluids, Rockville, MD), and 100 µg/ml hygromycin B (all from Biofluids, Rockville, MD). (Cell culture reagents were obtained via the UAB Comprehensive Cancer Center Media Core Facility.) For transient transfection, CHO-GHR cells were seeded in 10cm² dishes and used at 80% confluence. The plasmids pX-WT EGFR, pX-EGFR T669A, and pX-EGFR T654A, kindly provided by Dr. R. Davis (University of Massachusetts Medical School,
Worcester, MA) (37) were transfected into CHO-GHR cells using lipofectAMINE Plus (Invitrogen) according to manufacturer’s instruction. To generate stable CHO-GHR transfectants expressing the wild-type EGFR or T669A EGFR, cells were seeded in 10 cm² dishes and used at 50% confluence. The plasmids pX-WT EGFR and pX-EGFR T669A, from Dr. R. Davis were subcloned into the pcDNA (Invitrogen Corp, Carlsbad, CA) eukaryotic expression vector. pcDNA-WT EGFR or pcDNA-EGFR T669A was transfected into CHO-GHR cells using lipofectAMINE Plus (Invitrogen) according to the manufacturer’s instructions. CHO-GHR cells expressing WT EGFR or EGFR T669A were selected in 1 mg/ml of G418 (Invitrogen) and cloned. Transfectants were maintained in culture medium containing 200 µg/ml of G418. For transient co-transfection with EGFR and ErbB-2, CHO-GHR cells were placed in 10cm² dishes and used at 80% confluence. The plasmids pCS105-Flag-ErbB-2, kindly provided by Dr. Chenbei Chang (UAB), or pcDNA were transfected together with either pX-WT EGFR or pX-T669A EGFR into CHO-GHR cells using methods as mentioned above. The plasmid ratio between pCS105-Flag-ErbB-2 or pcDNA and pX-WT EGFR or pX-T669A EGFR was 3:1. C14 cells were maintained in cultured in Dulbecco’s modified Eagle’s medium containing 1.0 g/liter glucose supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml hygromycin B and 200 µg/ml of G418.

Cell starvation, inhibitor pretreatment, cell stimulation, and protein extraction

Serum starvation of CHO-GHR cells was accomplished by substitution of 0.5% (w/v) bovine serum albumin (fraction V: Roche Molecular Biochemicals) for fetal bovine serum in the culture medium for 16-20h prior to experiments. Pretreatments and
stimulations were carried out at 37°C in binding buffer (consisting of 25mM Tris-HCL (PH 7.4), 120mM NaCl, 5mM KCl, 1.2mM MgCl₂, 0.1% (w/v) bovine serum albumin, and 1mM dextrose). Serum-starved cells were pretreated with PD98059 (100 µM), U0126 (10 µM) or vehicle (as a control) for 60 min prior to treatment with EGF (concentrations indicated in figure legends), or vehicle as specified in each experiment. Stimulations were terminated by washing the cells once with ice-cold phosphate-buffered saline supplemented with 0.4 mM sodium orthovanadate (PBS-vanadate) and then harvested by scraping in PBS-vanadate. Cells were collected by brief centrifugation, and pelleted cells were solubilized for 30 min at 4°C in lysis buffer (1% (v/v) Triton X-100, 150 mM NaCl, 10% (v/v) glycerol, 50 mM Tris-HCl (pH 8.0), 100 mM NaF, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM benzamidine, 5 µg/ml aprotinin, and 5 µg/ml leupeptin). After centrifugation at 15,000×g for 15 min at 4°C, the detergent extracts (supernatant) were subjected to immunoprecipitation or were directly electrophoresed and immunoblotted, as indicated below.

**Immunoprecipitation and immunoblotting**

For immunoprecipitation, cell extracts (500-1000 µg) were mixed with 5 µl of monoclonal anti-EGFR antibody (1 µg) and incubated at 4°C overnight with continuous agitation. Protein A-sepharose beads (Amersham Biosciences, Piscataway, NJ) were added and incubated at 4°C for an additional hour. The beads were washed four times with lysis buffer adjusted to 0.5% (v/v) Triton X-100. Laemmli sample buffer eluates were resolved by SDS-PAGE an immunoblotted as indicated below.
Proteins resolved by SDS-PAGE were transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences). The membranes were blocked with TBST buffer (20mM Tris-HCl (pH 7.6), 150mM NaCl, and 0.1% (v/v) Tween 20) containing 2% (w/v) bovine serum albumin and incubated with primary antibodies (0.5-1 µg/ml) as specified in each experiment. After three washes with TBST, the membranes were incubated with appropriate secondary antibodies (1:10,000 dilution) and washed. The bound antibodies were detected with SuperSignal chemiluminescent substrate (Pierce Chemical Co., Rockford, IL). Membrane stripping was performed according to the manufacturer’s suggestions (Amersham Biosciences).

Degradation of EGFR after blockade of protein synthesis

CHO-GHR-WT EGFR and CHO-GHR-EGFR T669A were grown to 80% confluence in six-well dishes and serum starved overnight. Cells were then incubated with cycloheximide (20 µg/ml) together with MEK inhibitor or vehicle for 1 h prior to stimulation with EGF (10nM) for the indicated durations. Cell lysates were resolved by SDS-PAGE and blotted with anti-EGFR.

Densitometric analysis

Densitometric quantitation of ECL immunoblots was performed using a high-resolution scanner and the ImageJ 1.30 program (developed by W.S. Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD). Pooled data from several experiments are displayed as mean ± S.E. The significance of differences ($p$ value) of pooled results was estimated using paired $t$ tests.
RESULTS

*EGF causes PTP101-reactive phosphorylation of wild-type EGFR, but not T669A EGFR, in reconstituted CHO cells.*

EGFR can be phosphorylated at multiple sites in the intracellular domain, including serine, threonine, and tyrosine residues. Several reports have stressed the correlation between phosphorylation at certain serine or threonine residues and modulation of EGFR activity (37-40). Our previous work indicated that GH, PRL, and EGF induce EGFR serine/threonine phosphorylation that requires ERK activity and is detected by PTP101, a monoclonal antibody that reacts with phosphorylated serine or threonine residues that reside within ERK substrate consensus sites (32-34). To further pursue the mechanism and impact of such EGFR phosphorylation, we explored which EGFR residues are involved. Previous studies suggest that $^{669}T$ is phosphorylated following EGF stimulation (41). By using a synthetic peptide that encompasses $^{669}T$, it has been shown that this residue can be a substrate for MAP kinase (ERK) (42-44). Indeed the EGFR intracellular domain encodes only one ERK consensus phosphorylation site (PX(S/T)P), which resides within the juxtamembrane region and includes PL $^{669}$TP (Figure 1A).

To understand whether $^{669}T$ is required for PTP101 reactivity, we examined an EGFR mutant, EGFR-T669A, in which this residue is mutated to alanine. CHO-GHR cells, which express the rabbit GHR, but not EGFR (45), were used as a target for transfection with wild-type (WT) or mutant EGFR. Both WT EGFR and EGFR T669A were detected by immunoblotting at the expected Mr, and acute stimulation of each transfectants with EGF resulted in tyrosine phosphorylation of EGFR (Figure 1B). These
data indicate that each receptor was displayed at the cell surface and that EGF binds productively to each at the cell surface.

We next tested the ability of WT and EGFR T669A to undergo PTP101-reactive phosphorylation (Figure 2). Cells expressing vector control, WT EGFR, or EGFR T669A were treated with vehicle (-), GH 10min, or EGF for 15 minutes prior to detergent extraction. Each sample was immunoprecipitated with anti-EGFR and eluates were resolved by SDS-PAGE and immunoblotted with PTP101 (Figure 2A). As expected, both GH and EGF induced PTP101-reactive phosphorylation of WT EGFR. In contrast, neither GH nor EGF promoted such phosphorylation of EGFR T669A. Reprobing with anti-EGFR verified ample WT and EGFR T669A expression and the lack of endogenous EGFR in vector-transfected cells. Further, aliquots of extract from each sample were resolved and immunoblotted with anti-pERK, revealing that both GH and EGF promoted ERK activation in cells harboring either WT or EGFR T669A. Thus, the inability of EGFR T669A to undergo PTP101-reactive phosphorylation was not due to the inability of either GH or EGF to induce ERK activation in cells that express this mutant receptor.

ERK pathway involvement in EGF-induced PTP101-reactive EGFR phosphorylation was further examined in the experiment shown in Figure 2B. Cells expressing WT or EGFR T669A were pretreated with the MEK1 inhibitor, PD98059 (+), or vehicle (-) and then stimulated with EGF (+) or vehicle (-) for 15 min. As in Figure 2A, EGF promoted PTP101-reactive phosphorylation of WT, but not EGFR T669A. Additionally, EGF-induced WT EGFR PTP101 reactivity was blocked by PD98059, indicating that both ERK activation and an intact threonine-669 are required for this EGFR phosphorylation.
Figure 1.
Schematic structure of EGFR and expression of WT EGFR and EGFR T669A.  
A, Domain structure of EGFR. Highlighted are two cysteine-rich (CR) extracellular domain regions, the transmembrane (TM) domain, the juxtamembrane (Jx) cytoplasmic domain, and the kinase domain. The Jx domain region amino acid sequence including 669T that resides in an ERK phosphorylation consensus (PX(S/T)P) is indicated.  
B, Expression of WT EGFR and EGFR T669A in CHO cells. Stably transfected cells expressing either WT EGFR or EGFR T669A were serum-starved and stimulated EGF (1nM) or vehicle for 15 min. Detergent extracts (800 µg) were immunoprecipitated with anti-EGFR and elutes separated by SDS-PAGE and sequentially immunoblotted with anti-phosphotyrosine (anti-pTyr) and anti-EGFR (upper panel). Detergent extracts (30 µg) were resolved by SDS-PAGE and sequentially immunoblotted with anti-phospho Akt and anti-Akt (lower panel). The experiments shown are representative of three such experiments.
Li, et al, Figure 1

A.

ERK consensus site: PX(S/T)P

B.

EGFR expressed WT T669A

EGF - + - +

IP: EGFR

WB: pTyr

WB: EGFR

Extract

WB: pAkt

WB: Akt
As a test of specificity, we also evaluated the consequences of mutation of threonine-654, another known phosphorylation site in the EGFR juxtamembrane intracellular domain; $^{654}$T phosphorylation is believed to be catalyzed by protein kinase C (PKC) (46,47). As shown previously (37), we found that expression of EGFR T654A, in which $^{654}$T is mutated to alanine, yielded surface receptor expression and EGF-induced EGFR tyrosine phosphorylation (data not shown). However, in distinction to EGFR T669A, PTP101-reactive phosphorylation of EGFR T654A was detected basally at a low level and further induced by EGF treatment; furthermore, this PTP101-reactive phosphorylation was inhibited by PD98059 pretreatment (Figure 2C). This indicates that $^{669}$T, but not $^{654}$T, is required for EGF-induced ERK-dependent EGFR PTP101-reactive phosphorylation.

EGF-induced EGFR downregulation is enhanced in CHO cells expressing EGFR T669A compared to WT EGFR.

Our previous work indicated that PTP101-reactive EGFR phosphorylation correlated with diminished EGF-induced EGFR downregulation (33,34). We thus examined the effect of the T669A mutation on EGF-induced EGFR loss in reconstituted CHO cells expressing either EGFR T669A or WT EGFR (Figure 3). In these stably transfected cells, EGFR expression is governed by a CMV promoter-driven eukaryotic expression vector. To nullify any effect of EGF signaling on the level of EGFR expression (via CMV promoter activation), we pretreated serum-starved cells for 1h with cycloheximide (CHX) to prevent new protein synthesis and then treated with EGF for 0.5-3h (Figure 3A). CHX itself had no substantial effect on EGFR abundance in either transfectant, even when present for 5h (data not shown). In WT EGFR-expressing cells,
Figure 2.
EGF causes PTP101-reactive phosphorylation of wild-type EGFR and EGFR T654A, but not EGFR T669A in reconstituted CHO cells.
A, CHO cells stably expressing WT EGFR or EGFR T669A were serum-starved and stimulated with GH (500 ng/ml) for 10 min or EGF (1 nM) for 15 min or vehicle. Detergent extracts were immunoprecipitated with anti-EGFR and eluates were resolved by SDS-PAGE and immunoblotted sequentially with PTP101 and anti-EGFR (upper panel). Detergent extracts were resolved by SDS-PAGE and immunoblotted sequentially with anti-active ERK (anti-pERK) and anti-ERK (lower panel).
B, Serum starved cells were pretreated with PD98059 (100 µM) or vehicle for one hour prior to stimulation with EGF (1 nM) for 15 min. Detergent extracts were immunoprecipitated with anti-EGFR and eluates were resolved by SDS-PAGE and immunoblotted sequentially with PTP101 and anti-EGFR (upper panel). Detergent extracts were resolved by SDS-PAGE and immunoblotted sequentially with anti-pERK (lower panel). The experiments shown are representative of two such experiments.
C, CHO cells transiently expressing WT EGFR, EGFR T654A or EGFR T669A were serum-starved, pretreated with PD98059 or vehicle and then stimulated with EGF (1 nM) or vehicle for 15 min. Detergent extracts were immunoprecipitated with anti-EGFR and eluates were resolved by SDS-PAGE and immunoblotted sequentially with PTP101 and anti-EGFR. The experiments shown are representative of three such experiments.
Li, et al, Figure 2

A. EGFR expressed

<table>
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<th>Treatment</th>
<th>vector</th>
<th>WT</th>
<th>T669A</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH EGF</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GH EGF</td>
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<tr>
<td>GH EGF</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>

IP: EGFR

- WB: PTP101
  - 210

- WB: EGFR
  - 210

Extract

- WB: pERK
  - 50

- WB: ERK
  - 50

B. EGFR expressed

<table>
<thead>
<tr>
<th>EGF</th>
<th>WT</th>
<th>T669A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
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<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PD98059</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

IP: EGFR

- WB: PTP101
  - 214

- WB: EGFR
  - 214

Extract

- WB: pERK
  - 50

C. EGFR expressed

<table>
<thead>
<tr>
<th>EGF</th>
<th>WT</th>
<th>T654A</th>
<th>T669A</th>
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<tr>
<td>PD98059</td>
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<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

IP: EGFR

- WB: PTP101
  - 210

- WB: EGFR
  - 210
EGF induced detectable loss of receptor after 1h and further loss thereafter. Notably, this EGF-induced receptor loss was more profound in cells harboring EGFR T669A. Several such experiments were analyzed densitometrically and the degree of EGF-induced loss of WT EGFR vs. EGFR T669A was quantitatively compared (Figure 3B). This revealed that ligand-induced receptor loss was significantly greater for EGFR T669A than for WT EGFR at all EGF stimulation time points tested and that the degree of loss was progressively greater for EGFR T669A vs. WT EGFR with increasing duration of EGF treatment; 3h of EGF exposure caused nearly 50% loss of WT EGFR and >90% loss of EGFR T669A. (Regression analysis indicated receptor half-lives of 3.2 h for WT EGFR and 1.6 h for EGFR T669A in response to EGF.)

We further pursued EGF-induced EGFR downregulation by exploring the effect of pharmacological inhibition of the ERK pathway (Figure 3C). Cells expressing either WT EGFR or EGFR T669A were pretreated with the MEK1 inhibitor, U0126, prior to treatment for 2h with EGF (+) or vehicle (-). As expected, EGF treatment caused downregulation of EGFR T669A that was markedly enhanced compared to WT EGFR. However, when EGF-induced ERK activation was blocked, downregulation of WT EGFR was greatly increased, suggesting that ERK-mediated phosphorylation of the receptor lessens its downregulation. Indeed, pretreatment with U0126 did not affect EGF-induced downregulation of EGFR T669A. In concert with the results shown in Figure 2, these data indicate that EGF induces ERK-mediated phosphorylation of EGFR, likely at $^{669}\text{T}$, and this phosphorylation serves as a brake to receptor downregulation.
Figure 3.
EGF-induced EGFR downregulation is enhanced in CHO cells expressing EGFR T669A compared to WT EGFR.

A, Serum starved cells were pretreated with cycloheximide (20 µg/ml) for 1 h prior to stimulation with EGF (10 nM) for indicated durations. Detergent extracts were resolved by SDS-PAGE and immunoblotted sequentially with anti-EGFR and anti-STAT5.

B, Data in A along with those obtained from three other experiments, were subjected to densitometric analysis. EGFR mass at each time point was normalized to control (time 0; considered 100% in each experiment). Data are expressed as mean ± S.E. (n = 4). For comparison of WT EGFR vs. EGFR T669A, * - p < 0.05; ** - p < 0.01.

C, Serum starved cells were pretreated with cycloheximide and U0126 (10 µM) or vehicle for 30 min prior to stimulation with EGF (10 nM) for 15 min. Detergent extracts were resolved by SDS-PAGE and immunoblotted sequentially with anti-EGFR antibody (upper panel) and anti-pERK and anti-ERK (lower panel).
Li, et al, Figure 3

A. EGFR expressed

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EGF treatment (h)

WB: EGFR

WB: STAT5

B. Remaining EGFR (% of initial)

EFG treatment (h)

C. EGFR expressed

<table>
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<tr>
<td>U0126</td>
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WB: EGFR

WB: pERK

WB: ERK
Effects of EGF-induced EGFR $^{669}$T phosphorylation on EGFR tyrosine phosphorylation and ubiquitination.

Previous studies indicated that EGFR phosphorylation at sites other than tyrosine residues can affect receptor signaling. For example calmodulin-dependent protein kinase II (CaM kinase II) promotes phosphorylation at residues $^{1046}$S and $^{1047}$S; replacement of these residues by alanine results in decreased EGF-induced EGFR downregulation and enhanced EGF signaling (38,39). Activation of protein kinase C promotes phosphorylation of EGFR at $^{654}$T; mutation of this residue results in enhanced EGF-induced signaling and inhibits phorbol ester-induced blunting of EGFR kinase activity (37,40). In contrast to these EGFR phosphorylation sites, the impact of at $^{669}$T phosphorylation on EGF signaling in the context of intact cells has been relatively less clear. Hence, we sought to compare EGF-induced WT EGFR kinase activation with that of EGFR T669A (Figure 4).

Serum-starved cells harboring each of the receptors were treated with EGF (1 nM) for various durations from 15-180 min, after which proteins were extracted and immunoblotted with antibodies that detect phosphorylation of residues $^{992}$Y or $^{1068}$Y (Figure 4A). Phosphorylation of these residues is correlated with EGFR kinase activation (48,49). As expected, EGF caused phosphorylation at WT EGFR at both $^{992}$Y or $^{1068}$Y that peaked after roughly 30 min and persisted for up to 180 min. Likewise, EGF treatment induced phosphorylation of both residues in EGFR T669A; however, in this case the signals were maximal after 15 min and thereafter dissipated more rapidly than in WT EGFR. Notably, though, peak phosphorylation of EGFR T669A was substantially greater than that achieved for WT EGFR, such that even though it decayed more rapidly, the signals seen after 60 min for EGFR T669A were similar to those observed for WT
EGFR at the same time point. Blotting for EGFR verified that these differences in maximal phosphorylation and kinetics could not be explained by differences in EGFR abundance between the two cells.

The effects of pharmacologic ERK inhibition on this EGF-induced EGFR tyrosine phosphorylation were tested in Figure 4B. Cells pretreated with vehicle (-) or PD98059 (+) were then exposed to vehicle (0 min) or EGF for 5 or 15 min. As seen in Figure 4A, acute (both 5 and 15 min) EGF-induced phosphorylation of Y992 and Y1068 were enhanced in EGFR T669A compared to WT EGFR. Notably, pretreatment with the MEK1 inhibitor PD98059 led to marked enhancement of EGF-induced tyrosine phosphorylation of WT EGFR. In distinction, EGF-induced EGFR T669A tyrosine phosphorylation was not affected by PD98059 pretreatment. In concert with the findings in Figure 4A, these data strongly suggest that the ability of EGF to activate ERKs and thereby cause phosphorylation of EGFR T669 serves to dampen the degree of receptor kinase activation that ensues. EGF concentration dependence experiments (not shown) were consistent with these observations and indicated that EGFR tyrosine phosphorylation was easily detected with as little as 0.1 nM EGF (5 min) for EGFR T669A, but required 1 nM for WT EGFR.

EGFR is known to form dimers – homodimers (EGFR-EGFR) or heterodimers (EGFR-ErbB-2, for example; more below). We asked how coexpression of WT EGFR with EGFR T669A would compare with expression of each alone in terms of acute EGFR kinase activation in response to EGF (Figure 5). Cells were transiently transfected with expression plasmids encoding either WT EGFR, EGFR T669A, or an equal mixture of the two plasmids. After serum starvation, cells were treated with EGF for 0, 5, or 15 min, after which cell extracts were blotted with anti-pY-1068 and anti-EGFR (Figure 5A).
Figure 4.
EGF-induced EGFR tyrosine phosphorylation in CHO cells expressing EGFR T669A vs. WT EGFR.
A,B, Serum starved cells were stimulated with EGF (1 nM) for the indicated durations. Detergent extracts were resolved in parallel by SDS-PAGE and immunoblotted with anti-pY-992, anti-pY-1068, or anti-EGFR. In B, cells were pretreated with PD98059 (100 µM) or vehicle for 1 h prior to stimulation with EGF, as indicated. The experiments shown are representative of four such experiments.
Li, et al, Figure 4

A. EGFR expressed
   
<table>
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   WB: pY-992
   WB: pY-1068
   WB: EGFR

B. EGFR expressed
   
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</table>
   WB: pY-992
   WB: pY-1068
   WB: EGFR
EGF-induced EGFR tyrosine phosphorylation was enhanced in cells expressing EGFR T669A alone vs. WT EGFR, as expected. Notably, in cells harboring both wild-type and mutant receptors, the EGF-induced pattern of EGFR tyrosine phosphorylation was distinctly more similar to that seen in cells that express EGFR T669A alone than to those expressing WT EGFR alone. Densitometric quantitation with normalization for EGFR abundance of three similar experiments is displayed in Figure 5B and validates this observation. Thus, the propensity of EGFR T669A to enhanced EGF sensitivity is not dampened by coexpression with WT EGFR, consistent with the notion that in WT-T669A heterodimers the mutant exerts dominance regarding this phenotype.

Consequent to EGF-induced EGFR kinase activation, the tyrosine phosphorylated receptor is rapidly ubiquitinated by c-Cbl, an SH2 domain-containing ubiquitin ligase that binds EGFR; this ubiquitination promotes post-internalization EGFR sorting to lysosomes (50-52). Ubiquitinated EGFR can be detected after anti-EGFR immunoprecipitation, SDS-PAGE, and immunoblotting with anti-ubiquitin as a high-Mr smear. The smearing reflects multiple monoubiquitination, rather than polyubiquitination (53,54). Polyubiquitination signals proteasomal degradation, while monoubiquitination is involved in endocytic/endosomal-lysosomal trafficking (55-59). At each step of EGF-induced EGFR endocytosis and downregulation, multiply monoubiquitinated EGFR associates with ubiquitin interacting motif-containing proteins (60). As EGFR T669A underwent more robust acute tyrosine phosphorylation than did WT EGFR in response to EGF, we tested the effects of this mutation on EGF-induced EGFR ubiquitination (Figure 6).
Figure 5.
EGF-induced EGFR tyrosine phosphorylation in CHO cells expressing EGFR T669A, WT EGFR, or cotransfected with both EGFR T669A and WT EGFR. 

A, CHO-GHR cells were transiently transfected with plasmids encoding either WT EGFR, EGFR T669A, or both receptors, as indicated. Serum starved cells were stimulated with EGF (1 nM) for the indicated durations. Detergent extracts were resolved in parallel by SDS-PAGE and immunoblotted with anti-pY-1068 or anti-EGFR. 

B, Data in A, along with those obtained from two other experiments, were subjected to densitometric analysis. EGF-induced EGFR tyrosine phosphorylation was normalized by EGFR abundance and maximum signal achieved was considered 100%. Data are displayed as mean ± S.E. (n = 3). p values for indicated comparisons are listed.
Li, et al, Figure 5

A.

<table>
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<th>T669A</th>
<th>WT + T669A</th>
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<tbody>
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</tbody>
</table>

- WB: pY-1068
- WB: EGFR

B.

EGFR tyrosine phosphorylation (% of maximum)

- WT EGFR
- T669A EGFR
- WT + T669A EGFR

- EGF (min) 0 5 15
- p < 0.05
- NS
- p < 0.05
Serum-starved CHO transfectants were pretreated with PD98059 (+) or vehicle (-) prior to stimulation with EGF (+) or vehicle (-) for 15 min (Figure 6A). Detergent extracts were precipitated with anti-EGFR and eluates were immunoblotted with anti-ubiquitin. Aliquots of extract were resolved without precipitation and immunoblotted with anti-pERK to verify the effectiveness of the PD98059. As expected, EGF acutely induced WT EGFR ubiquitination. Notably, inhibition of ERK activation with PD98059 markedly enhanced the effect of EGF on WT EGFR ubiquitination. In contrast, the degree of ubiquitination of EGFR T669A induced by EGF was substantially greater than that seen for WT EGFR and was not affected by PD98059 pretreatment. Three such experiments were analyzed densitometrically, estimating the relative abundance of EGF-induced EGFR ubiquitination normalized in each case by the EGFR abundance in the same immunoprecipitate. The graphical display of these data (Figure 6B) indicates that blockade of ERK-dependent phosphorylation of threonine-669 of EGFR (either pharmacologically or mutagenetically) in response to EGF leads to statistically-significantly increased EGF-induced EGFR ubiquitination.

Endogenous EGFR PTP101 phosphorylation and its effects in a human fibrosarcoma cell.

The data outlined in Figures 1-6 derive from the study of EGFR (wild-type or mutant) expressed in the context of CHO cells, in which no ErbB family members are endogenously expressed. To further explore these issues in a more physiologically-relevant milieu, we used a human fibrosarcoma cell line that endogenously expresses EGFR and ErbB-2 and also harbors an activating Ras mutation. The C14 cell (61-63), is
a derivative of the HT1080 human fibrosarcoma cell line, in which ERKs are constitutively active due to a Ras mutation (64,65) and in which EGFR is endogenously

Figure 6. EGF-induced EGFR ubiquitination in CHO cells expressing EGFR T669A vs. WT EGFR.
A, Serum starved cells were pretreated with PD98059 or vehicle for 1 h prior to stimulation with EGF (10 nM) for 15 min. Detergent extracts were immunoprecipitated with anti-EGFR and eluates were resolved by SDS-PAGE and immunoblotted with anti-Ub and anti-EGFR. Detergent extracts were resolved by SDS-PAGE and immunoblotted with anti-pERK and anti-ERK.
B, Data in A, along with those obtained from two other experiments, were subjected to densitometric analysis. EGF-induced EGFR ubiquitination was normalized by EGFR abundance and maximum signal achieved was considered 100%. Data are displayed as mean ± S.E. (n = 3). *p* values for indicated comparisons are listed.
Li, et al, Figure 6

**A.**

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**IP:**

- WB: Ub
- WB: EGFR

**Extract**

- WB: pERK
- WB: ERK

**B.**

EGFR ubiquitination (% of maximum)

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<th>T669A EGFR</th>
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<tr>
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*p < 0.05*
expressed. Serum-starved C14 cells exhibited activated ERK, which was blocked by PD98059; immunoprecipitated EGFR from the same extracts was recognized by PTP101, but not if the cells were treated with PD98059, indicating substantial basal ERK-dependent PTP101-reactive EGFR phosphorylation in these fibrosarcoma cells (Figure 7). EGF treatment for 15 min enhanced both ERK activity and PTP101-reactive EGFR phosphorylation somewhat, effects prevented by PD98059 pretreatment. Importantly, these findings extend the correlation between ERK activity and PTP101-reactive EGFR phosphorylation found in mouse preadipocytes (32,33), human T47D breast cancer cells (34), and CHO cells reconstituted with human EGFR (Figure 2 of this manuscript) to include another cell line, in this instance a human fibrosarcoma cell. Further, they indicate that constitutive Ras/ERK activation characteristic of many human cancers can be associated with such receptor phosphorylation even in the absence of growth factor or cytokine stimulation.

We examined the effect of EGF on EGFR abundance in C14 cells. Serum-starved cells were treated with EGF (1 nM) for 0-4 h, as indicated, and EGFR abundance in the detergent cell extracts was monitored by anti-EGFR immunoblotting (Figure 8A). Pretreatment with PD98059 to inhibit both basal and EGF-induced ERK activity resulted in significantly enhanced EGF-induced EGFR loss compared to cells not pretreated (Figures 8A and 8B). ErbB2 levels in the same extracts were determined by immunoblotting as a loading control; these levels did not vary with either EGF or PD98059 treatment. Notably, EGF-induced EGFR downregulation in C14 cells was relatively blunted. Densitometric quantitation of several such experiments (Figure 8B) indicated that EGF treatment for 4 h yielded only ~40% decrease in EGFR levels. (Although comparison between different cell systems may not be entirely valid, EGF
Figure 7.
Constitutive Ras activation results in basal EGFR PTP101 phosphorylation.
Serum-starved C14 cells were pretreated with PD98059 (100 µM) or vehicle for 1 h prior to stimulation with EGF (1 nM) for 15 min. Detergent extracts were immunoprecipitated with anti-EGFR and eluates were resolved by SDS-PAGE and immunoblotted sequentially with PTP101 and anti-EGFR. Detergent extracts were resolved by SDS-PAGE immunoblotted sequentially with anti-pERK and anti-ERK. The experiment shown is representative of three such experiments.
Li, et al, Figure 7

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treatment of CHO cells stably expressing WT EGFR for 3 h caused a slightly greater
degree of receptor loss than that seen in C14 cells treated for 4 h.) Similarly enhanced
EGF-induced EGFR downregulation in C14 cells was observed when U0126, a distinct
ERK pathway inhibitor, was used instead of PD98059 (Figure 8C).

To assess EGFR activation, serum-starved C14 cells were exposed to EGF for 0.5-
2 h and extracts were immunoblotted with the antibody that detects phosphorylation of
\( ^{1068}Y \) (Figure 9A, left side). As expected, EGF treatment caused EGFR tyrosine
phosphorylation, although, interestingly, densitometric analysis of multiple experiments
(Figure 9B) revealed that peak activation was observed in these cells 1 h after EGF
exposure. (For comparison, peak EGF-induced \( ^{1068}Y \) phosphorylation was seen after 0.5
h in CHO cells that express WT EGFR (Figure 4A and data not shown).) Pretreatment of
C14 cells with PD98059 significantly enhanced \( ^{1068}Y \) receptor phosphorylation detected
after 0.5 h when compared to vehicle-pretreated cells (Figures 9A and 9B) and thereby
shifted the peak for this EGF-induced signal from 1 h to 0.5 h in these cells. Thus,
pharmacologic blunting of basal and EGF-induced ERK activity in C14 cells augmented
early EGF-induced EGFR activation; however, the overall \( ^{1068}Y \) phosphorylation signal at
later EGF stimulation time points in PD98059-pretreated cells was not enhanced, likely in
part because EGFR abundance was lessened at these later points in the inhibitor-
pretreated cells.
Figure 8.

EGF-induced EGFR downregulation in C14 cells.

A, Serum starved C14 cells were pretreated with PD98059 (100 µM) or vehicle for 1 h prior to stimulation with EGF (1 nM) for the indicated durations. Detergent extracts were resolved by SDS-PAGE and immunoblotted sequentially with anti-EGFR and anti-ErbB-2 (ErbB-2 is not downregulated in response to EGF and is thus used as a loading control.)

B, Data in A, along with those obtained from three other experiments, were subjected to densitometric analysis. EGFR mass at each time point was normalized to control (time 0; considered 100% in each experiment). Data are expressed as mean ± S.E. (n = 4). For comparison of PD98059 vs. DMSO pretreatment, * - p < 0.05; ** - p < 0.01.

C, Serum starved C14 cells were pretreated with UO126 (50µM) or vehicle for 30min prior to stimulation with EGF (1nM) for the indicated durations. Detergent extracts were resolved by SDS-PAGE and immunoblotted sequentially with anti-EGFR and anti-ErbB-2 antibodies.
Li, et al, Figure 8

A.

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WB: ErbB-2

WB: EGFR

B.

Remaining EGFR (% of initial)

EGF treatment (h)

DMSO

PD98059

C.

<table>
<thead>
<tr>
<th>EGF (h)</th>
<th>DMSO</th>
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WB: ErbB-2

WB: EGFR
Figure 9.
EGF-induced EGFR signaling in C14 cells.
A, Serum starved C14 cells were pretreated with PD98059 (100 µM) or vehicle for 1 h prior to stimulation with EGF (1 nM) for the indicated durations. Detergent extracts were resolved by SDS-PAGE and immunoblotted sequentially with anti-pY-1068, anti-EGFR, anti-pERK, and anti-ERK.
B, Data in A, along with those obtained from three other experiments, were subjected to densitometric analysis. pTyr-1068 EGFR mass at each time point was normalized to EGFR abundance. Maximal signal achieved was considered 100%. Data are expressed as mean ± S.E. (n = 4). For comparison of PD98059 vs. DMSO pretreatment, * - p < 0.05.
Coexpression of WT EGFR or EGFR T669A with ErbB-2.

The data in Figures 7-9 indicate that endogenous (wild-type) EGFR present along with endogenous ErbB-2 in fibrosarcoma cells undergoes EGF-induced PTP-101-reactive
phosphorylation and that in that setting pharmacologic prevention of ERK activity augments EGF-induced EGFR downregulation and EGF-induced EGFR tyrosine phosphorylation. In the experiments in Figure 10, we reverted to the CHO-GHR system (in which neither EGFR nor ErbB-2 is endogenously expressed) to probe the effects of coexpression or ErbB-2 on EGF-induced EGFR downregulation of EGFR T669A compared to WT EGFR.

Either WT EGFR or EGFR T669A expressing CHO-GHR cells were transfected with FLAG-tagged ErbB-2 or a vector control, as indicated (Figure 10A). After serum starvation and EGF treatment for 5 min, detergent extracts were immunoprecipitated with anti-FLAG and eluates were resolved and blotted sequentially for EGFR and ErbB-2. Both WT EGFR and EGFR T669A were specifically coprecipitated with ErbB-2 and EGF augmented this association. This indicates that the T669A mutation does not affect the ability of the EGFR to associate with ErbB-2. To examine EGF-induced EGFR downregulation (Figure 10B), CHO-GHR cells were cotransfected with FLAG-tagged ErbB2 or a vector control and either WT EGFR or EGFR T669A, as indicated, and treated with EGF for 3h in the presence of CHX, as in Figure 3. Notably, EGF-induced downregulation of EGFR T669A was substantially augmented in comparison to WT EGFR under these circumstances. This suggests that even though ErbB-2 is known to dampen EGF-induced EGFR downregulation (further discussed below), the difference between the WT EGFR and EGFR T669A is still maintained in the setting of ErbB-2 expression.
Figure 10.
Coexpression of WT EGFR or EGFR T669A with ErbB-2.

A, Either WT EGFR or EGFR T669A expressing CHO-GHR cells were transiently transfected with either Flag-ErbB-2 or pcDNA, as indicated. Cells were serum starved overnight and then stimulated with EGF (1 nM) for 5 min. Cell extracts were immunoprecipitated with anti-FLAG affinity gel and immunoblotted with anti-EGFR and anti-ErbB-2 antibodies sequentially.

B, CHO-GHR cells transiently cotransfected with Flag-ErbB-2 and either WT EGFR or EGFR T669A, as indicated, were serum starved overnight and pretreated with cycloheximide (20 μg/ml) for 1 h. Thereafter, cells were stimulated with EGF (10 nM) or vehicle for 3 h and detergent extracts were resolved by SDS-PAGE and sequentially immunoblotted with anti-EGFR and anti-ErbB2 antibodies.

The experiments in A and B are each representative of three independent experiments.
Li, et al, Figure 10

A.

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</table>

WB: EGFR

WB: ErbB-2

B.

| WT EGFR | T669A EGFR | ErbB-2 | EGF (h) | EGF (
<table>
<thead>
<tr>
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WB: EGFR

WB: ErbB-2
DISCUSSION

Regulation of EGFR activity is critical for normal cellular function. Receptor phosphorylation on several cytoplasmic domain tyrosine residues (including $^{845}Y$, $^{891}Y$, $^{920}Y$, $^{992}Y$, $^{1045}Y$, $^{1068}Y$, $^{1086}Y$, $^{1114}Y$, $^{1148}Y$, and $^{1173}Y$) occurs in response to EGF binding and is catalyzed by the intrinsic enzyme activity embedded in the cytoplasmic tyrosine kinase domain (residues 688-957) (11-14). Phosphorylation recruits a number of docking and signaling proteins, including Grb-2, SHC, PTP-1B, SHP-1, SHP-2, Cbl, and PLC-$\gamma$ to these sites (15,16), and influences several downstream signaling pathways to ultimately govern gene expression and cell behavior. Thus, the strength (stoichiometry/abundance), timing (pace, duration), and location (cell surface vs. intracellular) of ligand-induced EGFR tyrosine phosphorylation and the ligand-induced downregulation of receptor levels are believed to be key modulators of EGF action.

In addition to undergoing extensive cytoplasmic tail tyrosine phosphorylation, EGFR is also targeted for serine/threonine phosphorylation in response to several stimuli. Early studies indicated that four major non-tyrosine phosphorylation sites in the cytoplasmic domain include $^{654}T$, $^{669}T$, $^{1046}S$, and $^{1047}S$ (37-39,41,43,44,46,47,66,67). A more recent proteomic analysis (11) validated that EGF promotes phosphorylation of these four major sites and, in addition identified five other sites -- $^{671}S$, $^{967}S$, $^{1002}S$, $^{1057}S$, and $^{1142}S$. While the significance of phosphorylation at these latter sites is yet unknown, phosphorylation of residues $^{654}T$, $^{669}T$, $^{1046}S$, and $^{1047}S$ has been explored in some detail. $^{1046}S$, and $^{1047}S$, distal to the EGFR tyrosine kinase domain, were shown to be substrate phosphorylation sites for the serine/threonine kinase, calcium- and calmodulin-dependent protein kinase II (CaM kinase II) (38). Replacement of $^{1046/1047}S$ with alanines in the context of the full-length EGFR and comparison with WT EGFR in a CHO cell
reconstitution system revealed that EGFR S1046/1047A exhibited markedly decreased EGF-induced EGFR downregulation and enhanced EGF-induced signaling; moreover, a desensitizing effect of EGF on subsequent EGFR kinase activity found in WT EGFR was lost in EGFR S1046/1047A (38,39). Thus, it was concluded that phosphorylation of $^{1046}S$ and $^{1047}S$ in the receptor tail facilitated several negative regulatory events in the itinerary of the EGF-stimulated EGFR, including receptor internalization/downregulation and desensitization of the kinase to further EGF stimulation (38,39).

In contrast to $^{1046}S$ and $^{1047}S$, which reside in the distal tail, $^{654}T$ and $^{669}T$ are found in the juxtamembrane region of the EGFR cytoplasmic domain, proximal to the kinase domain. Initial studies of phosphorylation at $^{654}T$ focused on understanding the effects of the phorbol ester, PMA, which was shown to diminish EGFR tyrosine kinase activity by activation of protein kinase C (68,69). Mapping studies demonstrated that $^{654}T$ phosphorylation was promoted by PMA-induced PKC activity (46,47). Indeed, mutation of $^{654}T$ to alanine (EGFR T654A) resulted in enhanced EGF-induced signaling and reduced the inhibitory effect of PMA on EGFR kinase activity (37). Some studies with stimuli other than PMA have further linked such PKC-dependent EGFR phosphorylation to functional consequences. Sheffield and colleagues studied the effects of prolactin on EGFR function in normal murine mammary epithelial cells (70-73). In those cells, prolactin caused substantial PKC-dependent EGFR threonine phosphorylation (although the residue was not mapped) and subsequent desensitization of EGF-induced EGFR tyrosine kinase activity. A more recent study indicated that in vitro and in vivo effects of leutinizing hormone releasing hormone (LHRH) on prostate cancer cell growth were mediated via PKC and were not observed when EGFR T654A was expressed in the cancer cells, further supporting the potential role of this site in regulating EGFR kinase
activity(74). A very recent study (75) may provide mechanistic insights into the role of the juxtamembrane domain and, in particular, phosphorylation of $^{654}\text{T}$ in such EGFR kinase regulation. In that work (75), the juxtamembrane region was shown to be required for allosteric kinase activation and productive monomer interactions within the EGFR dimer that are believed important for EGF-induced kinase activation (10). Expression of an intracellular domain with mutation of $^{654}\text{T}$ to alanine (ICD T654A) augmented kinase activity of a coexpressed wild-type ICD, whereas introduction of a phosphomimetic mutation (ICD T654D) abrogated this activation and prevented allosteric interaction (75), thus implicating juxtamembrane domain phosphorylation in negative regulation.

$^{669}\text{T}$ is the only EGFR residue to reside in a consensus (PL$^{669}\text{TP}$) for phosphorylation by ERKs (76) and it is well-conserved among species. Indeed, studies using synthetic peptides that encompass $^{669}\text{T}$ showed that this residue is a substrate for MAP kinase [43, 44]; our data, derived using intact receptor in viable cells, support this finding by demonstrating PTP101 reactivity with WT EGFR, but not EGFR T669A. However, in contrast to $^{654}\text{T}$ and $^{1046/1047}\text{S}$, roles for $^{669}\text{T}$ in EGFR signaling and/or downregulation are less clear(37,77,78). We undertook the current studies because of our previous findings (32-34) that GH, PRL, and EGF each induced phosphorylation of EGFR that was detected with a monoclonal antibody (PTP101(79,80)) that reacts with serine or threonine residues contained within ERK consensus sites. In those studies, PTP101-reactive EGFR phosphorylation was prevented by pretreatment with ERK inhibitors; however, a PKC inhibitor failed to prevent GH-induced PTP101 reactivity (32-34). Furthermore, pretreatment of murine 3T3-F442A preadipocytes with GH (32,33) or human T47D breast cancer cells with PRL (34) blunted EGFR downregulation induced by subsequent EGF treatment and these effects of GH or PRL were also prevented by
ERK inhibitors. Similarly, we observed synergism between GH and EGF (in 3T3-F442A cells) and PRL and EGF (in T47D cells) for downstream signaling that was dependent on the abilities of GH or PRL to activate ERKs (33,34). Collectively, these findings suggested that (GH- or PRL-dependent) ERK-mediated phosphorylation of EGFR lessened the receptor’s downregulation and thereby contributed to enhanced EGF-dependent signaling. Finally, we observed that ERK inhibitor pretreatment alone enhanced EGF-dependent EGFR downregulation (34), strongly suggesting that ERK-mediated EGFR threonine phosphorylation, whether accomplished by PRL or GH before EGF stimulation or resulting from EGF-induced ERK activation, can modulate EGF-induced EGFR downregulation.

In the current study, we used both mutagenetic and pharmacologic methods to better assess ERK-dependent PTP101-reactive EGFR phosphorylation and its impact on receptor trafficking and signaling. We found in a CHO reconstitution system in which no endogenous EGFR is expressed that WT EGFR and EGFR T654A, but not EGFR T669A, underwent GH- and EGF-dependent PTP101-reactive phosphorylation. This effect was blocked by an ERK inhibitor, indicating that T669T is indeed required for the ERK-dependent PTP101-reactive phosphorylation of the receptor. Both T669A mutation and pharmacologic ERK inhibition substantially enhanced EGF-induced EGFR downregulation in this system; the ERK inhibitor effect is consistent with our observations (mentioned above) in T47D cells [34] and the T669A mutation result furthers the argument that it is the EGF-induced ERK-dependent phosphorylation at T669T that tends to negatively regulate the receptor’s own downregulation. Along these lines, our observations that both T669A mutation and pharmacologic ERK inhibition also allowed greater acute EGF-induced EGFR activation (as assessed by blotting of Y and
Y phosphorylation) and ubiquitination are of substantial interest. They suggest that the enhanced downregulation seen when EGF-induced ERK-mediated phosphorylation of T is blocked may be related to a tendency of this threonine phosphorylation to negatively regulate kinase activation (more below).

Importantly, we extended our examination of these issues to a human fibrosarcoma cell line in which ErbB-2, as well as EGFR, is expressed and which is known to harbor a mutated Ras that displays constitutive activation of the Ras/ERK pathway. In these cells, ERK was basally active under serum-starved conditions and EGF only marginally enhanced this activity. Again, the ERK activation state correlated with PTP101-reactive EGFR phosphorylation and both basal and EGF-induced PTP101 reactivity were prevented by pretreatment with PD98059 for 75 min. This highlights the reversibility of ERK-dependent EGFR phosphorylation and emphasizes that this modification is apparently transient, consistent with our previous observation in 3T3-F442A cells that the time courses for GH-induced ERK activation / deactivation and PTP101-reactive EGFR phosphorylation / dephosphorylation were highly correlated, albeit shifted in that loss of PTP101 reactivity lagged behind decline in ERK activity by roughly 15-60 min (32). Notably, PD98059 pretreatment of the fibrosarcoma cells also significantly hastened and enhanced EGF-induced EGFR downregulation and significantly augmented acute EGF-induced EGFR Y phosphorylation. These findings suggest that, as we inferred for GH and PRL treatment in previous studies, “loading” of the EGFR with ERK-dependent phosphorylation (presumably at T) prior to EGF stimulation modulates the receptor’s responses to EGF in terms of its ability to be activated and/or its post-EGF binding itinerary. More detailed studies will be required to understand the potential significance
of this regarding the threshold for and strength of EGFR triggering in cancers that are characterized by constitutive Ras/ERK activation.

EGFR is often found coexpressed in cells with the EGFR family member, ErbB-2, which itself has no ligand, but can heterodimerize with EGFR. In addition to potentiating effects on signaling offered by ErbB-2, heterodimerization of EGFR with ErbB-2 is known to dampen EGF-induced EGFR downregulation(81,82). These two receptors are coexpressed in C14 cells. Thus, it is conceivable that the relatively decreased EGF-induced EGFR downregulation seen in these cells compared to the CHO cell EGFR transfectants could be accounted for in part by the presence in C14 cells of ErbB-2. However, it is notable that even in the context of ErbB-2 coexpression, blockade of ERK activity in C14 cells enhanced EGF-induced EGFR downregulation significantly. This finding suggests that ERK-mediated phosphorylation of EGFR has effects on EGFR downregulation that are independent of effects of heterodimerization with ErbB-2. These conclusions are further bolstered by the cotransfection experiments in the CHO cell background in which ErbB-2 was coexpressed with either WT EGFR or EGFR T669A. In that setting, both wild-type and mutant EGFRs coimmunoprecipitated with ErbB-2, but EGF-induced EGFR downregulation was augmented for EGFR T669A compared to WT EGFR.

Our current findings also highlight apparent differences in the roles of phosphorylation of $^{669}$T vs. those of $^{1046/1047}$S and $^{654}$T described above. In particular, whereas EGFR S1046/1047A exhibited enhanced EGF-induced signaling and decreased EGF-induced EGFR downregulation and EGFR T654A manifested primarily enhanced EGF-induced signaling, EGFR T669A in our study displayed both enhanced acute EGF-induced EGFR activation and enhanced EGF-induced EGFR downregulation. In this
respect, our findings may indicate two separate roles for $^{669}T$ phosphorylation – negative regulation of kinase activity (perhaps mechanistically similar to that of $^{654}T$ phosphorylation referred to above) and negative regulation of EGF-induced EGFR downregulation. However, it is also possible that the enhanced downregulation seen in the T669A results from the enhanced kinase activation of this mutant, in that EGFR kinase activity is generally taken to be a prerequisite for EGF-induced EGFR ubiquitination and downregulation.

Along these lines, it is important to note that our previous studies did not indicate that GH or PRL pretreatment resulted in obvious changes in subsequent EGF-induced EGFR tyrosine phosphorylation compared with EGF treatment alone(33,34). Rather, the most prominent effect of GH- and PRL-induced ERK-mediated EGFR PTP101-reactive phosphorylation in those studies was to significantly diminish subsequent EGF-induced EGFR downregulation. Why would the two ERK-dependent effects (modulation of kinase activity and modulation of downregulation) be relatively uncoupled in our previous studies and so correlated in the current study? There could be many explanations, including that the effect of GH- and PRL-induced ERK activation on EGFR kinase activity (presumably a blunting effect) was present, but not detected, in our studies in 3T3-F442A and T47D cells.

In the aggregate, our current findings are important in that they implicate ERK-dependent phosphorylation of EGFR $^{669}T$ as a regulator of EGF-induced receptor kinase activation and downregulation. Whether these are entirely separable effects remains a question worthy of further investigation. Further, these data highlight the likely importance of the juxtamembrane region in EGFR function and suggest that this region and $^{669}T$ are important in both heterologous and homologous receptor regulation.
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REFERENCES


Mol Cell 12(3), 541-552

1137-1149


Mol Cell Proteomics 5(9), 1610-1627

13. Boeri Erba, E., Bergatto, E., Cabodi, S., Silengo, L., Tarone, G., Defilippi, P., and


Hematol 19(3), 183-232


Res 2(3), 203-210


GROWTH HORMONE ENHANCES EGF-INDUCED ERK ACTIVATION

by

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ABSTRACT

The epidermal growth factor (EGF) family of peptides signals through the erbB family of receptor tyrosine kinases and plays important roles in development and tumorigenesis. EGF exerts its effects by signaling through pathways including MEK/ERK, phosphatidylinositol 3-kinase/Akt and PLC-γ. Growth hormone (GH), which is important in anabolism and regulation of metabolism, is present along with EGF in the physiological milieu. We previously observed that GH pretreatment augments EGF-induced phosphorylation of ERK1/2 in murine 3T3-F442A preadipocytes, which endogenously express growth hormone receptor (GHR) and epidermal growth factor receptor (EGFR) (1). However, the mechanism(s) of this synergy has yet to be elucidated. In this study, we further explored this crosstalk in 3T3-F442A and other cellular systems, including 3T3-L1 preadipocytes stably transfected with EGFR and HEK-293 cells stably expressing GHR and Jak2. In each cell type, GH synergistically augmented EGF-induced ERK activation (in 3T3-F442A, 2.3-fold increase compared to the sum of individual GH and EGF stimulations). In contrast, GH did not enhance EGF-induced Akt activation nor pPLC-γ, suggesting that the GH/EGF synergy is selective for the Ras/Raf/MEK/ERK pathway. We further defined where this synergy may reside using 3T3-F442A cells. At the receptor level, we tested whether the GH/EGF synergy relates to altered EGFR activation, as assessed by immunoblotting to detect specific tyrosine phosphorylation of three EGFR residues (Y992, Y845, and Y1068) correlated with EGFR kinase activation. Notably, GH did not alter acute EGF-induced Y992, Y845 or Y1068 phosphorylation. Within the Ras/Raf/MEK/ERK cascade, we found that GH did not affect the level of acute EGF-induced Raf phosphorylation. Interestingly, GH pretreatment dramatically
augmented EGF-induced MEK1/2 phosphorylation (1.9-fold increase compared to the sum of individual GH and EGF stimulations). This prompted examination of the phosphorylation state of kinase suppressor of Ras (KSR), an adaptor that regulates Raf-to-MEK1 signaling; dephosphorylation of S-392 and S-297 of KSR corresponds to its activation. GH alone induced only marginal KSR dephosphorylation. In contrast, GH markedly enhanced EGF-induced KSR dephosphorylation, suggesting that KSR might be involved in the signaling synergy between GH and EGF. This finding suggests a novel mechanism for the crosstalk between GH and EGF.

INTRODUCTION

EGF and its receptor (EGFR) play a crucial role in cell proliferation, differentiation, motility and adhesion (1). Its biologic effects are mediated via EGFR, which is a 1186-amino acid, 170-kDa transmembrane protein that comprises a heavily glycosylated extracellular domain, a transmembrane domain, and a cytoplasmic domain (2, 3). Upon ligand binding, EGFR can form a homodimer or heterodimerize with other members of the EGFR super family including erbB2, erbB3, and erbB4 (4-6). After dimerization, the receptor undergoes inter- and intramolecular autophosphorylation on tyrosines in the C-terminal cytoplasmic domain (2, 7-9). These phosphotyrosines serve as docking sites for proteins containing the Src homology 2 domain such as Grb2, Shc, phosphatidylinositol 3-kinase (10). Upon binding of Src homology 2 proteins, there is a cascade of kinase activation. In the case of Grb2, binding results in the translocation of the Grb2/Sos complex to the plasma membrane. This translocation is thought to bring Sos into close proximity of Ras, leading to the activation of Ras(11). Upon activation, Ras
assists Raf-1 localization to the membrane where it initiates a cascade of phosphorylation events including phosphorylation and activation of the mitogen activating kinase kinase (MEK) and the mitogen activating protein kinase (MAPK) (12, 13). Activated MAPK can either phosphorylate cytoplasmic substrates such as p90\textsuperscript{rsk} (14, 15) or translocate to the nucleus to induce the production of transcription factors such as c-Fos (14, 16). MAPK phosphorylation and activation are shown to correlate with various cellular events, such as cell proliferation and differentiation (17).

Responsiveness to EGF can be regulated by hormones that coexist in the physiological milieu, such as growth hormone. It has been documented that GH inhibits chloride secretion, a process involving the transactivation of EGFR and consequent recruitment of ERK1/2 (18). Yamauchi et al. demonstrated that GH caused tyrosine phosphorylation of EGFR, both in vivo in the livers of mice and in cell culture system (19). This GH-induced EGFR tyrosine phosphorylation was shown to require JAK2, but not EGFR kinase activity. The phosphorylated residue is located in a consensus Grb-2 association motif and contributes to GH-induced ERK activation. This study suggests that EGFR may serves as a docking molecule in GH-induced ERK activation. Our previous study in 3T3-F442A cells found that GH caused EGFR threonine phosphorylation at the ERK consensus site. Also, GH pretreatment reduced EGF-induced EGFR downregulation and enhanced EGF induced ERK activation at early time point (20).

In this study, we further explore the crosstalk between GH and EGF signaling and the mechanisms involved. We observed that GH and EGF selectively initiate synergistic activation of the Ras/Raf/MAPK pathway in a variety of cell types that respond to GH and EGF. GH did not significantly boost EGF-induced EGFR tyrosine phosphorylation,
instead the synergy occurred at Raf-1/MEK level. Kinase suppressor of Ras (KSR), a scaffolding protein that translocates MEK1/2 to the cell membrane showed enhanced activation in cotreatment condition compare with EGF stimulation alone. This finding suggests a novel mechanism for the crosstalk between GH and EGF.

MATERIALS AND METHODS

Materials

Recombinant human GH was kindly provided by Eli Lilly Co. (Indianapolis, IN). Recombinant human EGF was purchased from Upstate Biotechnology (Lake Placid, NY).

Antibodies

Polyclonal anti-EGFR antibody, anti-PLC-γ antibody, anti-phospho-Raf-1 antibody, anti-Raf-1 antibody, anti-MEK 1/2 antibody, anti-KSR antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal anti-phospho-EGFR antibodies Tyr-845,Tyr-992 and Tyr-1068, anti-pPLC-γ antibody and anti-phospho-serine392 KSR antibody (Cell Signaling Technology, Beverly, MA), anti-active mitogen-activated protein kinase affinity-purified rabbit antibody (anti-active ERK, recognizing the dually phosphorylated Thr-183 and Tyr-185 residues corresponding to the active forms of ERK1 and ERK2 (Promega, Madison, WI), anti-mitogen-activated protein kinase affinity-purified rabbit antibody (recognizing both ERK1 and ERK2), anti-phospho-Raf-1 antibody (Upstate Biotechnology, Lake Placid, NY), anti-Phospho-MEK1/2 antibody (Biosource International Inc, Camarillo, CA) were all purchased commercially.

Cells and cell culture
3T3-F442A cells, kindly provided by Dr. H. Green (Harvard University, Boston, MA) and C. Carter-Su (University of Michigan, Ann Arbor, MI), were cultured in Dulbecco’s modified Eagle’s medium containing 4.5g/Liter glucose (Cellgro-Mediatech, Herndon, VA), supplemented with 10% calf serum, 100 units/mL penicillin, 100µg/mL streptomycin (Biofluids, Rockville, MD). 3T3-L1 cells from American Type Culture Collection (Manassas, VA) were grown in the above medium, supplemented with 10% fetal bovine serum (Biofluids) instead of 10% calf serum. After stably transfected with EGFR, cells were maintained in culture medium containing 200 µg/mL of G418.

Osteoblasts, were cultured in α -minimal essential medium (α MEM) (Cellgro, Inc.), and 10% fetal bovine serum (FBS). HEK-293-Jak2-GHR cells were grown in Dulbecco’s modified Eagle’s medium containing 1.0g/Liter glucose (Cellgro, Inc.), supplemented with 10% fetal bovine serum, 200 µg/mL of G418, and 100 µg/mL hygromycin B (Biofluids).

*Cell starvation, inhibitor pretreatment, cell stimulation, and protein extraction*

Serum starvation of 3T3-F442A cells was accomplished by substitution of 0.5% (w/v) bovine serum albumin fraction V (Roche Molecular Biochemicals) for fetal bovine serum in the culture medium for 16-20h prior to experiments. Pretreatments and stimulations were carried out at 37°C in binding buffer (consisting of 25mM Tris-HCL (pH 7.4), 120mM NaCl, 5mM KCl, 1.2mM MgCl₂, 0.1% (w/v) bovine serum albumin, and 1mM dextrose). Stimulations were terminated by washing the cells once with ice-cold phosphate-buffered saline supplemented with 0.4 mM sodium orthovanadate (PBS-vanadate) and cells were then harvested by scraping in PBS-vanadate. Cells were
collected by brief centrifugation and pelleted cells were solubilized for 30 min at 4°C in lysis buffer (1% (v/v) Triton X-100, 150 mM NaCl, 10% (v/v) glycerol, 50 mM Tris-HCl (pH 8.0), 100 mM NaF, 2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM benzamidine, 5 µg/mL aprotinin, and 5 µg/mL leupeptin. After centrifugation at 15,000×g for 15 min at 4°C, the detergent extracts (supernatant) were subjected to immunoprecipitation or were directly electrophoresed and immunoblotted, as indicated below.

**Immunoprecipitation and immunoblotting**

For immunoprecipitation, cell extracts (500-1000 µg) were mixed with 5 µL of polyclonal KSR antibody (1 µg) and incubated at 4°C overnight with continuous agitation. Protein G-sepharose beads (Amersham Biosciences, Piscataway, NJ) were added and incubated at 4°C for an additional hour. The beads were washed four times with lysis buffer adjusted to contain 0.5% (v/v) Triton X-100. Laemmli sample buffer eluates were resolved by SDS-PAGE and immunoblotted as indicated below.

Proteins resolved by SDS-PAGE were transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences). The membranes were blocked with TBST buffer (20 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% (v/v) Tween 20) containing 2% (w/v) bovine serum albumin and incubated with primary antibodies (0.5-1 µg/mL) as specified in each experiment. After three washes with TBST, the membranes were incubated with appropriate secondary antibodies (1:10,000 dilutions) and washed. The bound antibodies were detected with SuperSignal chemiluminescent substrate (Pierce Chemical Co.,
Rockford, IL). Membrane stripping was performed according to the manufacturer’s suggestions (Amersham Biosciences).

**Densitometric analysis**

Densitometric quantitation of ECL developed film was performed using a high-resolution scanner and the ImageJ 1.30 program (developed by W.S. Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD). Pooled data from several experiments are displayed as mean ± S.E. The significance of differences (p value) of pooled results was estimated using paired t tests.

**RESULTS**

*GH enhances EGF-induced ERK activation.*

Murine 3T3-F442A fibroblasts express both growth hormone receptor and the receptor for EGF (21). We and others have detected biochemical responses to both GH and EGF in this cell system (19, 21-26), therefore making it an appealing target for study of the cross-talk between GH and EGF signaling pathways. Our previous study indicated that GH treatment augments EGF-induced ERK1/2 activation in 3T3-F442A cells (20).

In Figure 1A, serum-starved cells were stimulated with EGF for 15min, or GH 10min followed by EGF pretreatment for 15min, or GH 25min or vehicle. The detergent extracts were evaluated with anti-active ERK immunoblotting. Consistent with previous reports, the level of ERK activation achieved by the combined stimulation was greater than the projected additive response to treatment with both EGF and GH (Fig. 1A, lane 4 versus lane 3 plus lane 2).
Figure 1. GH Enhances EGF-induced ERK activation in multiple cellular systems.
A. 3T3-F442A cells were serum-starved overnight and then stimulated with GH (500ng/mL) for 25min or EGF (1nM) for 15min or GH 10min followed by EGF 15min or vehicle as control. Detergent extracts were resolved by SDS-PAGE and immunoblotted sequentially with anti-active ERK (anti-pERK) and anti-ERK antibodies.
B. Data in A, along with those obtained from two other experiments, were subjected to densitometric analysis. The degree of ERK activation was normalized to the maximum signal achieved (considered 100%). Data are displayed as mean±S.E., n=3. p value for indicated comparisons are listed.
C. Primary osteoblast, HEK-293-Jak2-GHR, and 3T3-L1-WT EGFR cells were serum starved overnight, then stimulated with GH (500ng/mL) for 25min or EGF (1nM) for 15min or GH 10min followed by EGF 15min or vehicle as control. Detergent extracts were resolved by SDS-PAGE and immunoblotted sequentially with anti-active ERK (anti-pERK) and anti-ERK antibodies. Quantitative analysis by densitometry of the pERK in the experiment shown in the panel below.
Figure 1.

A. 3T3-F442A

WB: pERK

B. WB: ERK

*: p<0.05, n=3
C.

**Primary Osteoblast**
- G E G/E

**HEK-293-JAK2-GHR**
- G E G/E

**WB: pERK**
- 

**WB: ERK**
- 

**3T3-L1**

**L1-WT EGFR**
- GH EGF G/E

**WB: pERK**
- 

**WB: ERK**
- 

**Graphs**
To further confirm the synergistic effect between GH and EGF, as opposed to a cumulative effect of these combined ligands, we performed densitometric evaluation of three such experiments. Solid bars indicate the degree of ERK1/2 activation initiated under different stimulations Figure 1B, while the expected cumulative stimulation of both GH and EGF was calculated and represented as the dotted bar in panel B. There is a roughly 2.3 fold increase in the height of the last solid bar versus the dotted bar in Figure 1B. Statistical analysis shows a significant difference ($p<0.05$, n=3) between the actual synergistic activation induced by the combined stimulation (the last solid bar in panel B) versus the additive degree of activation predicted to occur based on the activation by either GH or EGF (the dotted bar in panel B). Thus, GH pretreatment appeared to sensitize EGF-induced ERK1/2 activation at an early time point.

We further confirmed these findings in several other cellular systems that had both GHR and EGFR receptors, either endogenously or stably expressed. Primary osteoblasts express both GHR and EGFR endogenously and both GH and EGF signaling contribute to osteogenesis (27). In Figure 1C, serum starved primary osteoblast cells were stimulated with EGF 15min, GH 25min, combined stimulation (GH 10min prior EGF 15min), or vehicle. Cell extracts were resolved by SDS-PAGE, and immunoblotted sequentially with anti-active ERK (upper panel) and anti-ERK antibody (lower panel). Consistent with our findings in 3T3-F442A cells, the ERK activation induced by combined treatment (the last solid bar) is greater compare than that induced by GH and EGF cumulatively (the dotted bar).

Similar results were obtained in 3T3-L1-EGFR cells and HEK-293-Jak2-GHR cells. 3T3-L1 cells are mouse preadipocyte that respond well to GH stimulation but lack
the EGF receptor. Cells stably transfected to express EGFR have been shown to respond to EGF stimulation. HEK-293 cells have endogenous EGFR and respond to EGF stimulation, but lack both GHR and Jak2, which are essential to GH signaling pathways. Stable expression of the Jak2 and GHR molecules enable these cells to respond to GH stimulation. In both of these cell systems, we also see the similar phenomena of the synergy between GH and EGF in terms of ERK activation (Figure 1C).

**GH synergizes with EGF specifically in MAPK pathway.**

There are several signaling pathways initiated by EGF receptor activation. The three major pathways are the MAPK, PI3K/Akt, and PLC-γ pathways. Having identified the synergistic effect of GH and EGF stimulation on the ERK pathway, we tested if GH and EGF stimulation resulted in the synergistic activation of these other signaling pathways. F442A cells were stimulated with vehicle (-), GH 25min, EGF 15min, or GH 10min followed by EGF 15min respectively after serum starvation overnight. Cell extracts were immunoblotted with antibodies directed against pAkt and total Akt sequentially (Figure 2A), or pPLC-γ and PLC-γ sequentially (Figure 2B). Interestingly, both Akt and PLC-γ phosphorylation were not stronger in combined treatment conditions compared with the predicted cumulative effect of both GH and EGF (compare lane 4 with lane 2 plus lane 3), suggesting that the synergistic effect of combined GH and EGF treatment does not occur in either the PI3K/Akt pathway or PLC-γ pathway, but specifically in the MAPK pathway.
Figure 2. GH does not synergize with EGF in PI3K/Akt or PLC-γ pathway. A. B. Serum starved F442A cells were stimulated with GH (500ng/mL) for 25min, or EGF (1nM) for 15min, or GH 10min followed by EGF for 15min, or vehicle. Detergent extracts were resolved by SDS-PAGE and immunoblotted sequentially with anti-phospho-AKT and anti-AKT antibodies (panel A) or anti-pPLC-γ and anti-PLC-γ antibodies (panel B).
Figure 2.

A.

- G E G/E

WB: pAKT

WB: AKT

B.

WB: pPLC-γ

WB: PLC-γ
GH enhances EGF-induced ERK activation at post-receptor level.

Previously in our lab, we found that acute treatment of 3T3-F442A cells with the combined stimulation of GH and EGF makes EGF-induced ErbB-2 activation less substantial compared with exposure to EGF alone, suggesting that GH signaling dampens the ErbB-2 activation mechanism (21). Further, it was found that GH both tyrosine and threonine phosphorylate EGFR. (19, 20). Also, phosphorylation of certain tyrosine residues was found to be correlated with some unique signaling pathways; pY1068 provides the docking site for Grb2 and is related with MAPK activation, pY992 binds the SH2 domain of PLCγ and contributes to its activation, and pY845 stabilizes the activation loop and maintains the enzymatic activity of the receptor. Therefore, we examined whether pretreatment with GH makes EGFR more susceptible to EGF-induced tyrosine phosphorylation using site-specific phospho-tyrosine antibodies.

Consistent with our prior treatment protocol, cells were treated with vehicle (-), GH 25min or EGF 15min or EGF 15min in the presence of GH (added 10min prior to EGF). We employed several site-specific tyrosine phosphorylation antibodies directed against Tyr-1068, Tyr-992, or Tyr-845. Total EGFR protein was blotted as a loading control. As expected, EGF induced strong EGFR tyrosine phosphorylation at all of these tyrosine phosphorylation sites. Treatment with GH induced slight EGFR tyrosine phosphorylation. Notably, we detected no significant increase of EGFR tyrosine phosphorylation in cells treated with the combined stimulation compared with EGF alone (Fig. 3, lanes 4 versus 3). Thus, GH pretreatment had no appreciable effect on the level of acute EGF-induced EGFR tyrosine phosphorylation, suggesting that the enhanced EGF-
induced ERK activation observed after GH pretreatment occurs at steps downstream of EGFR activation.

Figure 3. GH does not enhance EGFR activation. Serum starved F442A cells were stimulated with GH (500ng/mL) for 25min or EGF (1nM) for 15min or GH 10min followed by EGF 15min or vehicle. Detergent extracts were resolved by SDS-PAGE, and immunoblotted separately with anti-pY1068 EGFR, anti-pY992 EGFR, anti-pY845 EGFR, and total EGFR antibodies.
Figure 3

3T3 F442A

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We then examined the activation of signaling molecules along the MEK/ERK signaling pathway downstream of EGFR. We examined the effects of GH on the ability of EGF to activate the Shc molecule and Raf-1 kinase. Upon EGFR activation Shc binds to EGFR via its PTB domain, leading to Shc tyrosine phosphorylation and the recruitment of Grb2, which is the main step in EGF-dependent induction of the Ras/MAPK pathway\(^2\). Hence we used phosphorylation of Shc as an indicator for upstream activation in EGFR signaling pathway \((29)\). Phosphorylation of Ser-338 on Raf-1 (PS338-Raf-1) is believed to correlate with Raf-1 kinase activation\((30)\). Hence, we used anti-pShc and anti-PS338-Raf-1 antibodies to test the activation of signaling molecules upstream of ERK in the Ras/Raf/MEK pathway. As we expected, upon stimulation EGF induced potent activation of Shc and Raf-1. GH also induced a certain degree of activation of Shc and Raf-1. Notably, we detected no change in serine phosphorylation of either Shc or Raf-1 in cells treated with combined stimulation compared with EGF alone (Fig. 3, lane 4 versus 3). This suggests that the effect of GH on EGF-induced ERK activation occurs at a point further downstream in the signaling cascade.

\textit{GH enhanced EGF-induced MEK1/2 phosphorylation.}

EGF-induced ERK1/2 phosphorylation was enhanced by GH pretreatment, but the activation of EGF receptor and its downstream signaling molecule Raf-1 did not appear to be the cause of this enhancement. Hence, we examined the activation of MEK1/2 kinase, the signaling molecule directly downstream of Raf. Interestingly, the level of MEK1/2 activation achieved by the combination of EGF and GH was much greater than
that expected based on the activation achieved by stimulation with either EGF or GH alone (Fig. 5A, lane 4 versus lane 3 plus lane 2).
Figure 4. GH does not augment EGF-induced phosphorylation upstream of RAF.
A.B. Serum starved F442A was stimulated with GH (500ng/mL) for 25min or EGF (1nM)
for 15min or GH 10min followed by EGF 15min or vehicle. Detergent extracts were
resolved by SDS-PAGE and immunoblotted sequentially with anti-phospho-Shc and anti-
Shc antibodies (panel A) or anti-pRAF and anti-RAF antibodies (panel B).
Figure 4

A.

3T3 F442A

- G E G/E

WB: pSHC

WB: Total SHC

B.

- G E G/E

WB: pRAF

WB: Total RAF
To determine whether this augmentation reflected simple summation of GH and EGF-induced MEK1/2 activation or instead synergistic MEK1/2 activation by EGF in the presence of GH, we performed densitometric evaluation of three such experiments. The results for MEK1/2 activation under different treatment conditions are displayed as the solid bars in Fig. 5B. In this figure, the dotted bar indicates the calculated summation of the relative activation of MEK1/2 by EGF (corresponding to lane 3 of Fig. 5A) plus that caused by GH (corresponding to lane 2 in Fig. 5A). That summation is compared with the last solid bar which reflects the actual activation induced by combined stimulation with both EGF and GH (corresponding to lane 4 in Fig. 5A). To our excitement, significant synergistic augmentation was observed ($p<0.05$, n=3). There was a roughly 1.9 fold increase in the height of the last solid bar compared with the dotted bar suggesting that GH pretreatment sensitizes the EGF-induced MEK1/2 activation. This implicates that GH’s effect on EGF-induced ERK activation occurs at the level between Raf-1 and MEK1/2.

*GH enhanced EGF-induced KSR activation.*

We next investigated the mechanism by which GH facilitates EGF-induced MEK1/2 activation. Kinase suppressor of Ras (KSR) is a scaffolding protein that is basally associated with MEK1/2 and that aids in the translocation of MEK from the cytosol to the plasma membrane(31-34). In quiescent cells, KSR is basally phosphorylated at Ser-297 and Ser-392. After stimulation by extracellular stimuli, such as growth factors, Ser-297 and Ser-392 are dephosphorylated, which is essential for the translocation and accumulation of KSR at the membrane and activation of the molecule(35). Hence, we asked whether GH affected the activation of KSR by
investigating the serine phosphorylation state of KSR. We employed anti-ser392 KSR antibody to detect

Figure 5. GH dramatically enhances EGF-induced MEK phosphorylation. A. Serum starved F442A cells were stimulated with GH (500ng/mL) for 25min or EGF (1nM) for 15min or GH 10min followed by EGF 15min or vehicle. Detergent extracts were resolved by SDS-PAGE and immunoblotted sequentially with anti-phospho-MEK and anti-MEK antibodies. B. Data in A, along with those obtained from two other experiments, were subjected to densitometric analysis. The degree of MEK phosphorylation was normalized to the maximum signal achieved (considered 100%). Data are displayed as mean±S.E. (n=3). The p value for indicated comparisons is listed.
Figure 5.

A. 3T3 F442A

WB: pMEK

WB: Total MEK

B.

Degree of phosphorylation

<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>GH</th>
<th>EGF</th>
<th>G+E</th>
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*: n=3, p<0.05
KSR activation. Consistent with our prior treatment conditions, cells were treated with vehicle (-), GH, or EGF alone for 15 min or ERK for 15 min in the presence of GH (added 10 min prior to EGF). Cell extracts were immunoprecipitated with anti-KSR antibody and eluates were immunoblotted with anti-Ser 392 KSR antibody. As expected, KSR was basally serine phosphorylated. Consistent with previous reports EGF stimulation resulted in dephosphorylation of KSR at Ser392 (Fig. 7A, lane 1) while GH marginally induced dephosphorylation of Ser 392 on KSR (Fig. 7A, lane 2). Notably, combined stimulation resulted in a greater level of dephosphorylation than that observed with EGF stimulation alone (Fig. 7A, lane 4 versus lane 3).

The experiments were repeated four times. Densitometric analysis reveals an approximate 20% decrease in the phosphorylation level of KSR corresponding to EGF stimulation alone compared to an approximate 40% decrease with the combined EGF and GH stimulation (Fig. 7B, bar 3-4). Statistical analysis shows significant differences between basal levels of KSR phosphorylation and those seen with EGF stimulation alone or with the combined stimulation. There is also a significant difference between EGF stimulation alone and the combined stimulation suggesting that GH pretreatment makes KSR more susceptible to EGF-induced activation.
Figure 6. KSR structure and activation.
A. KSR contains 5 conservative domain: CA1, CA2, CA3, CA4 and CA5. Among them, CA5 is important in the binding of MEK, while CA4 binds MAPK. Serines 297 and 392 serve regulatory roles and modulate KSR activation.
B. In its quiescent state, KSR binds with MEK and KSR is basally phosphorylated at serines 297 and 392. The phosphorylation of these sites enables protein 14-3-3 to bind to KSR and to keep it in an inactive state. Upon stimulation, these two serines are dephosphorylated, and protein 14-3-3 fall off. KSR undergoes conformational changes and associates with MAPK. Eventually, KSR helps aggregate MEK and MAPK with activated Raf-1 and contributes to MAPK activation.
Figure 6.

Figure 7. GH pretreatment reduces EGF-induced KSR S392 phosphorylation. 
A. F442A cells were serum-starved overnight, then stimulated with GH (500ng/mL) for 25min or EGF (1nM) for 15min or GH 10min followed by EGF 15min or vehicle as control. The detergent extracts were resolved by SDS-PAGE and immunoblotted with anti-phospho-serine 392 (upper panel) and anti-KSR antibody (lower panel) separately. B. Date in A, along with those obtained from three other experiments, were subjected to densitometric analysis. pS392 KSR was normalized by KSR abundance and maximum signal achieved was considered 100%. Data are displayed as mean±S.E. The $p$ value for indicated comparison is listed.
Figure 7.

A.

WB: pS392 KSR

B.

Degree of phosphorylation

```plaintext
Con  GH  EGF  G/E
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*: p<0.05, n=4
DISCUSSION

EGFR and GHR belong to separate families of cell surface receptors. EGFR belongs to the ErbB family, which includes four receptor tyrosine kinases-EGFR, ErbB-2, ErbB-3, and ErbB-4. (36-39). EGFR has important effects on cell growth, differentiation, motility and adhesion (1). It has been especially implicated in the pathogenesis and progression of numerous cancers, including breast cancer(40, 41). GHR, on the other hand, belongs to the cytokine receptor superfamily. This family couples with nonreceptor tyrosine kinases, most notably Janus kinases, and function is to promote longitudinal growth, anabolism, and metabolic regulation. GH has also been implicated in mammary gland development (42-45) and, more importantly, in breast cancer pathogenesis and behavior(46-48).

Besides its common physiological response, GH can also initiate the phosphorylation of either tyrosine or threonine residues on EGFR. Yamauchi’s group demonstrated that treatment with GH resulted in tyrosine phosphorylation of the EGFR on tyr-1068. This phosphorylation required JAK2, but not EGFR kinase activity(19). Mutagenesis studies suggested that GH caused enhanced EGFR-Grb-2 association via this phosphorylated tyrosine that resides in the Grb-2 consensus binding motif and that EGFR contributes to GH induced ERK activation. In our studies, we also found that tyr-1068 can be phosphorylated by GH stimulation. However, although EGF-induced ERK activation is also mediated by Grb-2 association with EGFR, and enhanced Grb-2
association caused by GH stimulation may enhance EGF-induced ERK activation, GH-mediated EGFR tyrosine phosphorylation does not seem to account for the synergy observed upon dual treatment with GH and EGF. First, EGF-induced phosphorylation at tyr-1068 is much more dramatic than that induced by GH. Further, we observed little difference between levels of phospho-tyr1068 induced by either EGF stimulation or combined treatment. Additionally, the phosphorylation of RAF, which is downstream of Grb-2, was not synergistically increased with dual treatment of GH and EGF, further ruling out the possibility that the synergistic effect of combined GH and EGF treatment on ERK activation is based on GH-induced tyrosine phosphorylation on 1068.

Another important fact of GH-induced EGFR tyrosine phosphorylation is that the phosphorylation does not result from transactivation of the EGFR, in contrast to some G-protein coupled receptor ligands that activate EGFR kinase activity and hence result in EGFR tyrosine phosphorylation(49). Nor did GH cause metalloprotease-catalyzed release of EGFR ligands from their cell-surface precursor unlike those ligands that stimulate the receptors for angiotensin II, thrombin, and carbachol (50, 51). This is consistent with our findings that none of the four commonly tested tyrosine phosphorylation sites on EGFR showed enhanced phosphorylation in combined stimulation as compared with EGF stimulation alone.

GH also causes EGFR serine/threonine phosphorylation. There are four major non-tyrosine phosphorylation sites in the cytoplasmic domain including, \(\frac{654}{669}\), \(\frac{669}{1046}\), \(\frac{1046}{1047}\) S (52-60). The latter two sites were shown to be CaM kinase II substrates and phosphorylation of \(\frac{654}{669}\) is likely mediated by protein kinase C. Based on our previous finding, we believe that \(\frac{654}{669}\) T is involved in the crosstalk between GH and EGF. \(\frac{669}{669}\) T can
be phosphorylated by ERKs (56, 57). In a previous study in our lab, we found that GH caused PTP101-reactive phosphorylation of EGFR by an ERK-mediated pathway. (PTP101 is a monoclonal antibody that specifically detects substrates phosphorylated at consensus sites of proline-directed protein kinases, such as ERKs.) (61-63) Also via ERK activation, the presence of GH substantially reduced EGF-induced EGFR degradation. Meanwhile, it was pretreatment with GH augmented EGF-induced ERK activation (20).

In this study, it was suggested that GH-induced ERK activation has a role in regulating the trafficking EGF-stimulated EGFR thus raising the possibility that intracellular redistribution may contribute to the augmentation of early aspects of EGF-induced signaling. However, using mutagenesis, we replaced thr-669 with an alanine, which abolished the effect of ERK mediated phosphorylation of EGFR; nonetheless, similar synergy between GH and EGF was observed (data not shown), suggesting that the synergistic activation of ERK is not mediated by threonine phosphorylation of EGFR in response to GH treatment.

According to our observations, the synergy between GH and EGF occurs at the level between Raf-1 and MEK1/2, which prompted us to look into the possible mechanisms modulating ERK signaling modules. To date, several adapting proteins have been found to play a role in regulating ERK signaling modules, including KSR, MEK-Partner 1 (MP1), β-Arrestins, MEK Kinase 1, CNK and SUR-8. Of these, we discounted MP1 because it only regulates MEK1-ERK1 (64), while in our observation, both ERK1 and ERK2 showed synergistic activation. β-Arrestins mainly functions in G-protein-coupled receptor signaling pathway (65), and both CNK and SUR-8 function at a level upstream of Raf-1, between Ras and Raf-1. Hence, we looked into KSR as a candidate
molecule and explored its involvement in GH and EGF synergy in ERK activation. KSR is a highly conserved molecule in the ERK module. In quiescent cells, KSR is predominantly localized in the cytosol, constitutively associates with MEK, and is constitutively phosphorylated on serines 392 and 297. These two phosphorylated serines serve as a docking site for 14-3-3 protein, which locks KSR in an inactive form (31). In response to signal activation, ser-392 is dephosphorylated thereby releasing 14-3-3 and allowing KSR binding with ERK, and translocation of this complex to the plasma membrane (31, 66). Consistent with previous findings, treatment with EGF activated Ras and induced dephosphorylation of KSR on serine 392. The enhanced dephosphorylation of KSR on ser-392 seen with GH pretreatment may be correlated with the enhanced activation of downstream MEK1/2 and ERK1/2. Similarly, the involvement of KSR in the synergistic activation of ERK by two different stimuli was also observed by Messina’s group (67). They observed that insulin enhanced GH-induced ERK activation also occurs at the level between Raf-1 and MEK. Further, there was an increase in tyrosine phosphorylation of the KSR molecule and translocation of MEK to the cell membrane after insulin pretreatment. We did not use tyrosine phosphorylation of KSR as an indicator since there is no direct evidence suggesting the tyrosine phosphorylation of the molecule correlates with the enhanced function of KSR. However, it has yet to be determined whether there is a reciprocal correlation between tyrosine and serine phosphorylation of the KSR molecule. We further tried to detect active, translocated KSR by immunofluorescent staining in our cell system but were unable to obtain satisfactory results since the preadipocyte plasma membrane is especially rich in lipids.
EGFR has a wide variety of important functions and is involved in cell proliferation, survival, migration, and adhesion. Its physiological functions are mediated by the activation of specific downstream signaling cascades. There are multiple signaling pathways initiated upon EGFR activation, however MEK/ERK pathway is one of the most important, because of the cross-talk between it and the other signaling cascades. For example, PLC-γ mediates hydrolysis of phosphatidylinositol (4,5) bisphosphate and mobilization of the actin-modifying proteins that are required for EGFR-mediated motility, but motility is blocked if MEK signaling is abrogated (68-70). Further, the MEK/ERK pathway contributes to multiple responses, including both proliferation and migration (69). Hence, the MEK/ERK pathway is very important in EGFR-mediated functions, such as cell movement during embryonic development, maintaining the cellular architecture of self-renewing tissues, wound repair, defense against infectious agents (71-73), and in pathological conditions such as tumor growth and invasion (68, 74-76). Our current findings suggest that the role of GH in may involve changes in MEK/ERK pathway in post-EGF signaling.

In summary, we found that 1) combined treatment with GH and EGF results in synergistic activation of the ERK signaling cascade at the Raf/MEK level; 2) KSR, an adaptor molecule that targets MEK to the cell membrane, appears to be important in mediating the effect of GH on EGF-induced ERK activation.

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REFERENCES


15. **Thomas G** 1992 MAP kinase by any other name smells just as sweet. Cell 68:3-6


Komuro I, Yazaki Y, Kadowaki T 1997 Tyrosine phosphorylation of the EGF receptor by the kinase Jak2 is induced by growth hormone. Nature 390:91-96


epidermal growth factor receptors and mediates Ras activation in intact cells. Mol Endocrinol 12:536-543


31. **Muller J, Ory S, Copeland T, Piwnica-Worms H, Morrison DK** 2001 C-TAK1 regulates Ras signaling by phosphorylating the MAPK scaffold, KSR1. Mol Cell 8:983-993


34. **Xing H, Kornfeld K, Muslin AJ** 1997 The protein kinase KSR interacts with 14-3-3 protein and Raf. Curr Biol 7:294-300


42. **Sternlicht MD** 2006 Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis. Breast Cancer Res 8:201


44. **Walden PD, Ruan W, Feldman M, Kleinberg DL** 1998 Evidence that the mammary fat pad mediates the action of growth hormone in mammary gland development. Endocrinology 139:659-662


52. **Hunter T, Ling N, Cooper JA** 1984 Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane. Nature 311:480-483


62. Marinissen MJ, Chiariello M, Gutkind JS 2001 Regulation of gene expression by the small GTPase Rho through the ERK6 (p38 gamma) MAP kinase pathway. Genes Dev 15:535-553


74. **Hudson LG, McCawley LJ** 1998 Contributions of the epidermal growth factor receptor to keratinocyte motility. Microsc Res Tech 43:444-455

SUMMARY

Epidermal growth factor (EGF) is a 53 amino acid mitogenic protein that is a key regulator in fetal development, wound healing, gastric acid secretion, and can contribute to oncogenesis (1, 2, 14). EGF is synthesized as a precursor inserted in the cell membrane, and the mature form is generated by proteolysis (3). EGF exerts its biological functions through interacting with its receptor, EGF receptor (EGFR). EGFR is a type I single transmembrane glycoprotein with tyrosine kinase activity. It consists of a ligand-binding extracellular domain, an α-helix transmembrane pass, and an intracellular domain (130). After EGF binds EGFR, EGFRs dimerize (131-134). The dimerization allows the intracellular tyrosine kinase domains to approximate and become activated by transautophosphorylation (135, 136). Activation of EGFR results in recruitment and phosphorylation of several docking proteins, such as Grb2, Shc, PLC-γ, which lead to activation of downstream signaling pathways, including Ras/Raf/MAPK pathway, PI3K/AKT pathway, and PLC-γ pathway (27-30, 93). The overexpression and aberrant function of EGFR are important factors in tumor transformation, and they are involved in almost all aspects of cancer behaviors, such as proliferation, survival, angiogenesis, and migration (14, 137).

Growth hormone, on the other hand, is mainly released from anterior pituitary (102). Its major functions are longitudinal bone growth, anabolism and metabolic regulation. Interestingly, within recent years, GH was also found to be released in autocrine/paracrine fashion, and has been implicated in malignant carcinomas (138). And there have been several important findings about the crosstalk between GH and the
EGF receptor superfamily (107, 108, 111). Previously in our lab, we found that GH induces threonine/serine phosphorylation of EGFR. This threonine/serine phosphorylation is mediated by ERK activation and the monoclonal antibody, PTP101, which specifically recognize proteins phosphorylated at consensus sites for proline-directed protein kinases, such as ERK, can detect GH-induced EGFR phosphorylation. Besides inducing EGFR phosphorylation, GH, via ERK activation also blunted EGF-induced EGFR downregulation. GH also synergizes with EGF in ERK activation (111).

As both the abundance of the EGFR and its signaling strength have important impact on tumor genesis, this dissertation further explores the mechanism of the crosstalk between GH and EGFR with regard to the regulation of both EGFR abundance and the EGFR signaling. First we confirmed by mutagenesis the site required for GH-induced EGFR PTP101 reactive phosphorylation, Thr-669. Then by comparing cells expressing either WT or T669A EGFR, we found that phosphorylation on Thr-669 serves as a brake on EGF-induced EGFR degradation. That is, inability of phosphorylation at this site makes the receptor more susceptible to EGF-induced EGFR downregulation. Further, we found that tyrosine phosphorylation and ubiquitination of the EGFR was dampened after EGFR became phosphorylated on Thr-669. Second, we explored the mechanism involved in GH and EGF’s synergy on ERK activation. We located the level of the synergy, which occurs between RAF and MEK, and found that the adaptor protein between these two components, KSR, might be involved in the mechanism of how this synergy happens.

In 1988, Gordon Gill’s group first discovered that threonine 669 is one of the major sites on EGFR phosphorylated after EGF stimulation (85). Since then, it was found that a wide variety of stimulators can induce phosphorylation at this site. Several groups
worked on finding the kinase of this site (88, 89, 100). In 1989, Roger J. Davis’s group employed a synthetic peptide corresponding to residues 663-681 of the EGFR as a substrate for protein kinase assays. In vitro experiments were performed measuring protein kinase and phosphatase activity present in homogenates prepared from cells treated with and without EGF. Their findings indicated that EGF’s action to increase the phosphorylation of the receptor at threonine 669 is the activation of a threonine protein kinase, not by inhibition of a protein phosphatase. Later, in 1991, Marsha Rich Rosner’s group carefully compared the characteristics of T669 peptide kinase, and MAP kinase. They found that T669 peptide kinase and MAP kinase have similar elution profiles, which suggest they are similar in molecular weight and hydrophobic property; also, both kinases can be tyrosine phosphorylated and both of them have the property of a “switch” kinase, in that they must be phosphorylated at both tyrosine and threonine residues to be active. Additionally, the T669 peptide shares a similar sequence as MAP kinase substrate, MBP. Thus, they concluded that a MAP kinase phosphorylates the T669 peptide (88). However, until now, there is no further direct evidence proving that ERK1/2 phosphorylates EGF receptor on T669, especially in the context of an intact receptor expressed in cells. In our study, we are interested in the mechanism of the crosstalk between GH and EGF. Since GH induces EGF receptor phosphorylation mediated by ERK activation, and Thr-669 is the only EGFR residue that located in an ERK consensus phosphorylation site (“PX(T/S)P’’), we needed to confirm that this site affected GH-induced, ERK-mediated phosphorylation on EGFR before we further characterized its impact on EGF receptor behavior.
We stably transfected plasmids encoding either WT EGFR or T669A EGFR into CHO-GHR cells. CHO-GHR cells do not have endogenous EGFR, and respond to GH stimulation, which make it an ideal system for our purposed study. Monoclonal antibody, PTP101, was employed to detect proline-directed phosphorylation, which includes ERK-mediated phosphorylation. We found that GH induces PTP101-reactive phosphorylation in WT EGFR, but not in T669A EGFR expressing cells, suggesting Thr-669 is required for GH induced threonine phosphorylation of EGFR. EGF also induces EGFR phosphorylation on Thr-669 on EGFR, which is consistent with previous findings by Gill’s group. Further, we utilized the MEK inhibitor, PD98059 to examine if Thr-669 is required for ERK-mediated phosphorylation. We also found that EGF induces PTP101 reactive phosphorylation of WT EGFR, but not T669A EGFR. Furthermore, ERK inhibition greatly reduces EGFR PTP101 reactive phosphorylation in WT EGFR. This supports our hypothesis that Thr-669 is required for ERK-mediated phosphorylation on EGFR induced by either GH or EGF itself. These results are consistent with previous findings made by other groups, and further develop the theory of how EGF induces EGFR threonine phosphorylation. Meanwhile, it set the foundation for our later characterization of the impact of this site-specific phosphorylation on EGF receptor behaviors.

Growth hormone, via ERK activation, induces EGFR PTP101 reactive phosphorylation; meanwhile, also via ERK activation, GH blunts EGF-induced EGFR downregulation. Do these two phenomena correlate or does GH affect EGF-induced EGFR downregulation by modulating other intracellular molecules? We wish to find out whether this modification on EGF receptor participates in the mechanism of the blunting
of EGF-induced EGFR downregulation. By mutation of Thr-669 to Ala, the ERK mediated threonine phosphorylation is abolished. It is exciting to find that timecourse studies indicate that EGF stimulation induces more profound receptor loss in cells harboring EGFR T669A compared with WT EGFR. Statistical analysis revealed significantly greater ligand-induced receptor loss at all EGF stimulation time points tested. And the degree of loss was progressively greater for EGFR T669A vs. WT EGFR with increasing duration of EGF treatment. 3h of EGF exposure caused nearly 50% loss of WT EGFR and >90% loss of EGFR T669A. This suggests that inability to become phosphorylated at Thr-669 makes the receptor more susceptible to EGF-induced receptor loss. To further confirm our finding, we employed the MEK1/2 inhibitor, U0126. Notably, when EGF-induced ERK activation was blocked, downregulation of WT EGFR was greatly increased; on the other hand, EGF-induced downregulation of EGFR T669A was not affected. These data suggest that threonine phosphorylation at T669 serves as a brake on EGF-induced receptor downregulation.

This finding furthers the theory that the EGF receptor can be regulated by serine/threonine phosphorylation of its intracellular domain, especially in the juxtamembrane region. Thr-654 is a well studied site originally found to be phosphorylated in response to platelet-derived growth factor (PDGF). This site specific phosphorylation was mediated by protein kinase C (PKC) (84, 139). The phosphorylation of Thr-654 reduces EGF-induced EGFR downregulation likely by enhancing EGFR recycling (99). Ser-1046/1047 is another site more towards C-terminal region. The mutation of this site decreases the EGF-induced EGFR downregulation. Thr-669, like Thr-654, resides within juxtamembrane region, but its role in ligand-induced receptor downregulation is not well
studied yet. Our finding not only helps to explain how GH retards EGF-induced EGFR downregulation, but also enriches our knowledge about role of threonine/serine phosphorylation in regulating EGFR behavior.

EGF receptor downregulation is usually closely related with ubiquitination, since ubiquitination serves as a tag, directing the modified receptor through endosomal pathways and degradation in lysosomes. So, we next looked at EGF-induced EGFR ubiquitination. We immunoprecipitated EGF receptor and immunoblotted the eluates with anti-ubiquitin antibody. As expected, EGF acutely induced WT EGFR ubiquitination. However it is notable that EGFR T669A showed marked increase in ligand-induced receptor ubiquitination. Inhibition of ERK activation with PD98059 markedly enhanced the effect of EGF on WT EGFR ubiquitination to a similar degree to EGFR T669A and EGF-induced EGFR T669A ubiquitination was not affected by ERK inhibition. These data suggest that inability to become phosphorylated on Thr-669 makes the receptor more susceptible to EGF-induced receptor ubiquitination.

Internalization is the first step in the trafficking of the activated EGFR. In our study, we did not examine internalization per se. However, in our previous study with GH and EGFR, we found that GH pretreatment, although it retards EGF-induced EGFR downregulation did not alter the internalization rate of the receptor (111). This discrepancy may be explained by the controversial opinions about the role of ubiquitination in receptor endocytosis. That is, recent evidence suggests that ubiquitination is more important in facilitating intracellular trafficking of EGFR, rather than its internalization (62, 140).
Since ubiquitination is an important driving force directing molecules through the endosomal pathway, we examined the trafficking of EGFR to early endosomes by tracking the colocalization of EGFR with the early endosomal marker, EEA1. Notably, EGFR T669A shows dramatic colocalization with EEA1 after 5 min EGF stimulation while WT EGFR and EEA1 are not colocalized at this time point. After 15 min EGF stimulation, WT EGFRs become heavily colocalized with EEA1. This finding is consistent with the results of receptor ubiquitination and ligand-induced EGFR downregulation.

Receptor ubiquitination and the rate of trafficking correlates with the receptor kinase activity and autophosphorylation. Hence, we looked into ERK mediated impact on EGF-induced EGFR activation. In searching the literature, we found several studies about the regulation of EGF-stimulated EGFR activation by phosphorylation of its intracellular domain, especially in the juxtamembrane region. Phosphorylation on Thr-654, which is mediated by PKC, causes an inhibition of the receptor tyrosine kinase activity (84, 86, 96, 97). Mutation of Thr-654 with Ala enhanced EGF-induced EGFR kinase activity and conferred resistance to the regulation mediated by PKC (77, 91). Ser-1046/47, which is located at more distal end of the receptor, if mutated to Ala, leads to enhanced EGF signaling and less desensitization of the EGF receptor tyrosine kinase activity (90). These findings implicate serine or threonine phosphorylation of EGFR in modulation of ligand-induced receptor activation.

Despite previous research, a modulatory role of Thr-669 has not been clarified. Neither phosphorylation nor mutation of this site was shown to affect EGF-induced EGFR kinase activation. In 1988, Gordon Gill’s group e first found that Thr-669, among
other serine/threonine sites can be phosphorylated after EGF stimulation (85). In the same year, Marsha Rosner’s group found that Thapsigargin, a PKC independent tumor promoter can negatively regulate EGF-induced receptor tyrosine kinase activity. They found that instead of Thr-654, Thr-669 was phosphorylated, raising the possibility that Thr-669 phosphorylation may mediate changes of the kinase state of EGFR (87). Later, in 1993, Marsha Rosner’s group employed mutagenesis, replacing either Thr-654 or Thr-669 with glutamic acid, mimicking the negative charge introduced by phosphorylation at these sites. In vitro kinase assays showed comparable tyrosine phosphorylation of Glu-654- and Glu-669-containing EGFRs compared with WT EGFR. In vivo kinase assays analyzing EGF stimulated cell extract, revealed that Glu-654- and Glu-669-EGFR incorporated almost 7-fold more tyrosine phosphate than WT EGFR, which was the opposite of what was found about the impact of Thr-654 phosphorylation on EGF-induced EGFR kinase activity. However, using another substrate of EGFR kinase, angiotensin II, all three forms of the receptor displayed similar EGF-induced angiotensin II phosphorylation. They concluded that introduction of glutamic acid residues at 654 or 669 does not alter intrinsic EGF receptor tyrosine kinase activity (141). The most recent published work about Thr-669 phosphorylation and its effect on EGF-induced EGFR kinase activity was also carried out by Marsha Rosner’s group, and published in 1996. They replaced Thr-669 with cysteine, which likes Ala, abolished the 669 phosphorylation site. They found no alteration of EGFR kinase activity mediated by PKC. Using MEK inhibitor PD98059, they failed to find an alteration in EGFR autophosphorylation. This group did find that MEK activity is necessary for PKC-mediated down-regulation of EGFR tyrosine phosphorylation and thus concluded that PKC down regulates EGFR.
tyrosine kinase activity by a MEK-dependent mechanism. However, they reasoned that inhibition by PKC is not a direct result of phosphorylation of the EGFR by PKC or MAPK and that activation of MAPK is not sufficient to regulate EGFR kinase activity (142).

We pursued whether ERK mediated phosphorylation of EGFR affects ligand-induced receptor activation, as we had at our disposal excellent reagents to detect the activation of EGFR, including site-specific phosphotyrosine antibodies, which are known to correlate with the receptor activation. We found that an EGFR with Thr-669 mutated to Ala shows more enhanced EGF-induced receptor activation compared with WT EGFR, as detected at all three sites examined, Tyr-992, Tyr-1068 and Tyr-845 (Tyr-845 data not shown) in a timecourse study with EGF stimulation up to 3h. MEK inhibitor pretreatment resulted in enhanced tyrosine phosphorylation of EGF-induced WT EGFR. EGF-induced EGFR T669A phosphorylation was not affected by MEK inhibition, and the enhanced EGF-induced WT EGFR phosphorylation was comparable to that in EGFR T669A-expressing cells. This finding supports our findings observed in EGFR downregulation, ubiquitination and trafficking experiments and it also reveals a novel finding about the regulatory role of Thr-669 phosphorylation.

The discrepancy of our findings with what was previously found by Rosner’s group is an interesting question. One explanation could be the anti-phosphotyrosine antibodies we used in these studies. At that time, there was no site specific antibody available, and the receptor kinase activation was detected by the antibody that detects all the tyrosines phosphorylated on the receptor. Some of those sites may not correlate with
receptor activation, and may account for a significant portion of the phospho-tyrosine detected, masking the effect.

Functional assays such as proliferation assay or wound healing assay would be other important aspects demonstrating kinase activity. However, the plasmid encoding the reconstituted EGFRs are driven by the CMV promoter, which by itself responds directly to EGF stimulation, leading to time dependent synthesis of new EGF receptors. In receptor downregulation experiments, we pretreated cells with cycloheximide to inhibit new protein synthesis. However, functional assays require a relatively long period of time; thus, it is not feasible for us to perform functional studies comparing kinase activity of EGFR T669A and WT EGFR.

ERK belongs to the family of mitogen-activated protein (MAP) kinases, and is activated in response to a wide variety of extracellular stimuli (143, 144). It is essential in cellular growth and prolonged activation of ERK1/2 is associated with carcinogenesis (145). Our C14 cell is a derivative from HT1080 human fibrosarcoma cell line which has constitutive ERK activation due to a Ras mutation (146, 147). It also endogenously expresses EGF receptor, making it possible for us to study whether basally activated ERK can also mediate PTP101 reactive phosphorylation on EGFR and retard EGF-induced EGFR downregulation. We pretreated the cells with or without PD98059, and compared both basal and inducible PTP101 phosphorylation of the receptor and ligand-induced receptor downregulation under these conditions. Interestingly, we found that basal ERK activation mediates substantial PTP101 reactive phosphorylation on EGFR, but not in cells that are pretreated with PD98059. Additionally, PD98059, by inhibiting both basal and EGF-induced ERK activity significantly enhanced EGF-induced EGFR loss. EGF
receptor abundance correlates with cell proliferation and high expression of EGFR correlates with tumorigenesis; hence, our findings in C14 cells may imply that the constitutive Ras/ERK activation characteristic of many human cancers can be associated with such receptor phosphorylation and contribute to their malignancy.

Another intriguing finding from the previous studies in our lab is the synergy between GH and EGF in terms of ERK activation. Since we also find that GH retards EGF induced EGFR degradation, our first instinct was that the synergy of the ERK activation is due to greater receptor availability in the cell, or due to the different pattern in receptor trafficking. To explore this possibility, we compared the cells expressing either WT EGFR or EGFR T669A, to see if the synergy exists only in WT EGFR expressing cells but not the cells expressing EGFR T669A. In CHO-GHR-EGFR cells, EGF induced ERK activation is too strong to see additional enhancement, so we stably introduced a plasmid encoding either WT EGFR or EGFR T669A to 3T3-L1 cells, which more resembles the 3T3-F442A cells where the synergy was originally observed. In 3T3-L1 cell transfectants, EGFR expression levels were low and EGF-induced ERK activation was less intense. Surprisingly, both WT EGFR and EGFR T669A showed similar synergy in GH and EGF-induced ERK activation. This suggests that the synergy between GH and EGF in ERK activation may not be explained solely by GH-induced PTP101 reactive phosphorylation on EGFR. Other mechanisms need to be explored.

To pursue this, we first examined at which level where the synergy occurs, using 3T3-F442A cells. We began by examining whether GH pretreatment makes the EGFR more activable by EGF by detecting site-specific phosphorylation on EGFR. Cells were stimulated with GH for 25min, EGF for 15min, GH for 10min followed by EGF for
Cell extracts were resolved by SDS-PAGE and immunoblotted with antibodies recognizing relevant signaling molecules. As we found previously, GH did not enhance EGF-induced EGFR tyrosine phosphorylation in all three phosphorylation sites we tested, $^{992}\text{Y}$, $^{1068}\text{Y}$, and $^{845}\text{Y}$. Furthermore, EGF-induced activation of two other pathways, (PI3-K/AKT pathway and PLC-γ pathway) was also not augmented by GH. The levels of phospho-AKT and tyrosine phosphorylated PLC-γ induced by EGF alone were similar signals compared with that elicited by combined stimulation with EGF and GH, suggesting the synergy we see in ERK activation is not a universal finding in all the EGF signaling pathways, and does not result from an overall enhanced activation of EGF receptor.

We next examined the downstream molecules in the MAP kinase pathway. SHC and RAF are two molecules in the upstream part of the pathway. We found that neither of them synergistically activated after combined stimulation. Interestingly, MEK, the molecule immediately downstream of RAF, was synergistically activated by combined EGF and GH stimulation compared with EGF stimulation alone. The pattern and degree of enhancement of RAF phosphorylation paralleled those observed for ERK, suggesting the synergy between GH and EGF occurs at the level between Raf and MEK.

In considering adaptor molecules regulating the Ras/Raf/MAPK pathway, we found KSR as the best candidate for further exploration. KSR modulates ERK activation at the level between Raf and MEK, and it functions in response to growth factors, such as EGF. KSR regulates activation of both ERK1 and ERK2, is consistent with our observations. Additionally, it is the most well studied adaptor molecule in the MAP kinase pathway, allowing us to study the possible regulatory events induced by GH and
EGF. KSR constitutively binds MEK in the quiescent state. Upon activation, it recruits ERK, and translocate to cell membrane where activated Raf localized, forming a complex and facilitating activation of the pathway. The regulation of the KSR activation is controlled by dephosphorylation at two serine sites, Ser-392 and Ser-297. In the quiescent state, these two sites are constitutively phosphorylated and serve as docking site for protein 14-3-3, which locks the molecule in an inactive state. Upon EGF stimulation, these two serine sites are dephosphorylated, releasing protein 14-3-3 and promoting signaling cascade. Hence, using pSer-392 of KSR as the indicator, we examined KSR activation in response to stimulation of GH, EGF, GH/EGF combined, and vehicle. As expected, EGF induced significant dephosphorylation of KSR. Interestingly, the phosphorylation state of KSR was further decreased under combined stimulation compared with EGF stimulation alone. This result suggests that GH pretreatment enhanced the activation of KSR, implying that KSR may play a role in mediating GH’s effect on EGF-induced ERK activation. Synergistic activation of KSR related to synergy of MAPK pathway activation was also observed between GH and insulin signaling pathways. In Dr. Messina’s group, it was found that pretreatment with insulin enhances GH-induced ERK activation by enhancing KSR function (148). Further in this project, we would like to gather more evidence relating KSR activation in response to combined stimulation, such as the release of 14-3-3 protein or KSR membrane translocation. In addition, we wish to find out by what mechanism GH enhances activation of KSR.

Taken together, the body of evidence presented in this dissertation further explored the mechanism of the crosstalk between growth hormone and epidermal growth factor. Also, it enriched current understanding about serine/threonine phosphorylation of
EGF receptor and its impact on the receptor behavior, such as ligand-induced receptor degradation and receptor kinase activation. The findings obtained, provide us more strategies to modulate EGFR abundance and activation, and to manipulate the EGFR signaling, perhaps to therapeutic advantage.

The above studies are mostly *in vitro* observations at the signaling level. Functional studies examining how the feedback phosphorylation of ERK on EGF receptor affects cell behaviors, such as proliferation and cell migration would be useful as well. As mentioned above, this would require that reconstituted EGFR be driven by a vector that, by itself, will not respond to EGF stimulation. Such findings might enable studies in animal models. Regarding GH/EGF synergy studies, we will first further confirm our findings by multiple approaches. For example, we will confirm the correlation between Raf-1 activation and Raf-1 phosphorylation by kinase assay, since the relationship between phosphorylation and activation is controversial. Further, more approaches could be employed to confirm the activation status of KSR by membrane translocation, ERK co-association, and protein 14-3-3 releasing experiments. More importantly, the mechanistic studies about how GH affects KSR activation are possible. One approach would be to first determine which domain or region of KSR is needed for the synergy to occur. Such a mutagenesis study will not only provide hints about the potential role for GH, but might also give us an idea whether KSR is the sole player mediating the synergy between GH and EGF. Further, since the synergy between GH and EGF in terms of ERK activation was also observed between PRL and EGF, we could examine if the same observations exist between PRL and EGF as well. By manipulating
breast cancer cell lines so as to disrupt the synergy between PRL and EGF, we will be able to explore whether cancer cell behavior could be modulated.


19. Graus-Porta D, Beerli RR, Daly JM, Hynes NE 1997 ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. Embo J 16:1647-1655


epidermal growth factor receptors and mediates Ras activation in intact cells. Mol Endocrinol 12:536-543


33. **Soltoff SP, Carraway KL, 3rd, Prigent SA, Gullick WG, Cantley LC** 1994 ErbB3 is involved in activation of phosphatidylinositol 3-kinase by epidermal growth factor. Mol Cell Biol 14:3550-3558


40. **Maroun CR, Holgado-Madruga M, Royal I, Naujokas MA, Fournier TM, Wong AJ, Park M** 1999 The Gab1 PH domain is required for localization of Gab1 at sites of cell-cell contact and epithelial morphogenesis downstream from the met receptor tyrosine kinase. Mol Cell Biol 19:1784-1799


43. **Chattopadhyay A, Vecchi M, Ji Q, Mernaugh R, Carpenter G** 1999 The role of individual SH2 domains in mediating association of phospholipase C-gamma1 with the activated EGF receptor. J Biol Chem 274:26091-26097


73. Haugh JM, Meyer T 2002 Active EGF receptors have limited access to PtdIns(4,5)P(2) in endosomes: implications for phospholipase C and PI 3-kinase signaling. J Cell Sci 115:303-310


84. Hunter T, Ling N, Cooper JA 1984 Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane. Nature 311:480-483


100. **Countaway JL, Northwood IC, Davis RJ** 1989 Mechanism of phosphorylation of the epidermal growth factor receptor at threonine 669. *J Biol Chem* 264:10828-10835


103. **Frank SJ, Gilliland G, Kraft AS, Arnold CS** 1994 Interaction of the growth hormone receptor cytoplasmic domain with the JAK2 tyrosine kinase. *Endocrinology* 135:2228-2239


110. **Marinissen MJ, Chiariello M, Gutkind JS** 2001 Regulation of gene expression by the small GTPase Rho through the ERK6 (p38 gamma) MAP kinase pathway. Genes Dev 15:535-553


116. **Levchenko A, Bruck J, Sternberg PW** 2000 Scaffold proteins may biphasically affect the levels of mitogen-activated protein kinase signaling and reduce its threshold properties. Proc Natl Acad Sci U S A 97:5818-5823


121. **Muller J, Cacace AM, Lyons WE, McGill CB, Morrison DK** 2000 Identification of B-KSR1, a novel brain-specific isoform of KSR1 that functions in neuronal signaling. Mol Cell Biol 20:5529-5539


126. **Muller J, Ory S, Copeland T, Piwnica-Worms H, Morrison DK** 2001 C-TAK1 regulates Ras signaling by phosphorylating the MAPK scaffold, KSR1. Mol Cell 8:983-993


143. **Lewis TS, Shapiro PS, Ahn NG** 1998 Signal transduction through MAP kinase cascades. Adv Cancer Res 74:49-139


