Specific epidermal growth factor receptor autophosphorylation sites promote mouse colon epithelial cell chemotaxis and restitution

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Yamaoka T, Frey MR, Disce RS, Bernard JK, Polk DB. Specific epidermal growth factor receptor autophosphorylation sites promote mouse colon epithelial cell chemotaxis and restitution. Am J Physiol Gastrointest Liver Physiol 301: G368–G376, 2011. First published May 26, 2011; doi:10.1152/ajpgi.00327.2010.—Upon ligand binding, epidermal growth factor (EGF) receptor (R) autophosphorylates on COOH-terminal tyrosines, generating docking sites for signaling partners that stimulate proliferation, restitution, and chemotaxis. Specificity for individual EGFR tyrosines in cellular responses has been hypothesized but not well documented. Here we tested the requirement for particular tyrosines, and associated downstream pathways, in mouse colon epithelial cell chemotactic migration. We compared these requirements to those for the phenotypically distinct restitution (wound healing) migration. Wild-type, Y992/1173F, Y1045F, Y1068F, and Y1086F EGFR constructs were expressed in EGF−/− cells; EGF-induced chemotaxis or restitution were determined by Boyden chamber or modified scratch wound assay, respectively. Pharmacological inhibitors of p38, phospholipase C (PLC), Src, MEK, JNK/SAPK, phosphatidylinositol 3-kinase (PI 3-kinase), and protein kinase C (PKC) were used to block EGF-stimulated signaling. Pathway activation was determined by immunoblot analysis. Unlike wild-type EGFR, Y992/1173F and Y1086F EGFR did not stimulate colon epithelial cell chemotaxis toward EGF; Y1045F and Y1068F EGFR partially stimulated chemotaxis. Only wild-type EGFR promoted colonocyte restitution. Inhibition of p38, PLC, and Src, or Grb2 knockdown, blocked chemotaxis; JNK, PI 3-kinase, and PKC inhibitors or c-Cbl knockdown blocked restitution but not chemotaxis. All four EGFR mutants stimulated downstream signaling in response to EGF, but Y992/1173F EGFR was partially defective in PLCγ activation whereas both Y1068F and Y1086F EGFR failed to activate Src. We conclude that specific EGFR tyrosines play key roles in determining cellular responses to ligand. Chemotaxis and restitution, which have different migration phenotypes and physiological consequences, have overlapping but not identical EGFR signaling requirements.

ErbB receptors; colon epithelial cell; phosphotyrosines; intestinal repair

Chemotaxis (movement of individual cells along a chemical gradient) and restitution (coordinated wound closure by the cell monolayer) represent two phenotypically distinct forms of migration with different roles in tissue homeostasis and disease. However, the specific intracellular signaling events that distinguish these two cellular responses from each other are not well understood.

Epidermal growth factor receptor (EGFR) is a 170-kDa receptor tyrosine kinase that plays key roles in cellular migration, proliferation, and differentiation. Also known as ErbB1/HER1, EGFR is the prototypic member of the ErbB family of growth factor receptors. It binds a panel of ligands including EGF, TGF-α, amphiregulin, betacellulin, heparin-binding EGF, and epipluerulin (21). Upon ligand binding, EGFR forms homodimers and heterodimers with other ErbBs (4), which activates the intrinsic protein tyrosine kinase activities of the receptor and leads to transautophosphorylation of specific tyrosine residues within the cytoplasmic domain (9, 20).

The six autophosphorylation sites in the COOH-terminal tail of EGFR are tyrosines 992, 1045, 1068, 1086, 1104, and 1173. Once phosphorylated, these residues serve as docking sites for intracellular signaling proteins containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains and provide a connection between the external stimulation and specific internal signal transduction pathways (30). Phospho-Y992 and Y1173 recruit and bind to the SH2 domain of phospholipase (PL) Cγ1, resulting in phosphorylation of PLCγ1 on tyrosines 771 and 1254 by EGFR (33, 36, 38) and increased phospholipase activity, which is required for EGFR-stimulated cell motility (44). Y1045 phosphorylation creates a docking site for c-Cbl, which mediates EGFR ubiquitinylation and degradation (40). Phosphorylation of EGFR on Y1068 and Y1086 creates binding sites for Grb2 (2, 26), leading to activation of the MAPK/ERK cascade, and a binding site for Gab1, which recruits the p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase), leading to AKT activation (25). Finally, when phosphorylated, Y1148 and Y1173 serve as docking sites for the adaptor protein Shc, which also increases activity of the MAPK/ERK cascade (28).

Numerous studies have demonstrated a role for EGFR in stimulating either chemotaxis or restitution, and a variety of intracellular signaling pathways such as PLCγ1 (6, 23), Src (18), p38 MAPK (12), ERK MAPK (44), and PI 3-kinase (23) have been implicated in EGF-induced cell motility. However, it is not clear whether any selectivity for chemotaxis vs. restitution exists in EGFR-initiated signaling. Whether specific EGFR autophosphorylation sites and their associated signaling

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cascades provide the opportunity for a cell to choose between chemotaxis and restitution as a migration phenotype is as yet unknown.

We have previously described a number of the signaling cascades required for EGF-stimulated restitution (11, 23) or chemotactic movement (24) of small intestinal and colonic epithelial cells. The goal of the present study was to determine whether there is a differential requirement for individual EGF-stimulated pathways in chemotaxis and restitution and to test the role of specific EGFR phosphotyrosines in these responses. We established tyrosine to phenylalanine mutations in the EGFR autophosphorylation sites in Y992/1173, Y1045, Y1068, and Y1086 and determined the roles of these residues in EGF-stimulated migration using modified Boyden chamber and scratch wound assays. Our results demonstrate that chemotaxis and restitution have overlapping but not identical EGFR signaling requirements, via selective activation of downstream signaling pathways.

MATERIALS AND METHODS

Growth factors, antibodies, and inhibitors. Human recombinant EGFR was provided by Carlos George-Nascimento (Chiron, Emeryville, CA). Human recombinant HGF, rat tail collagen type I, mouse collagen type IV, mouse laminin, and ITS + culture medium supplement were from BD Biosciences (Bedford, MA). Recombinant murine interferon (IFN)-γ and human fibronectin were from Intergen (Norcross, GA); other medium additives were purchased from Mediatech (Herndon, VA). Antibodies were sourced as follows: anti-active ERK1/ERK2 MAPK, Promega (Madison, WI); rabbit anti-ERK1/ERK2, phosho-Akt (Ser-473), Akt, phosho-p38, phosho-SAPK/JNK and phosho-EGFR (Y1045, Y1068 and Y1173), Cell Signaling Technology (Beverly, MA); rabbit anti-EGFR, HER2, phosho-HER2 (Y1248) and phosho-PLCγ1 (Y783), Upstate (Charlottesville, VA); mouse monoclonal anti-EGFR conjugated to phycoerythrin [EGFR (528) PE], Santa Cruz Biotechnology (Santa Cruz, CA). AG1478 and SB202190 were from Calbiochem (San Diego, CA); U73122, U73343, D-609, L-108, PP1, and PP2 were from Biomol (Plymouth Meeting, PA). Y294002 was purchased from Sigma Chemical (St. Louis, MO). All pharmacological inhibitors and agonists were dissolved in DMSO and added to the medium with the final concentration of DMSO <0.1%.

Cell culture. The conditionally immortalized young adult mouse colon (YAMC) and EGFR-null (EGFR−/−) mouse colonic epithelial (MCE) cells were generously provided by Dr. Robert Whitehead (Vanderbilt University, Nashville, TN). These cells express a heat-labile simian virus 40 large T antigen with an IFN-γ inducible promoter (35, 43). Cells were grown on culture dishes coated with rat tail collagen type I (5 μg/cm²) in RPMI 1640 supplemented with 5% FBS, 0.1% ITS+, 100,000 IU/l penicillin, 100 mg/l streptomycin, and 5 U/ml murine IFN-γ (Intergen, Norcross, GA). Cells were cultured under immortalizing (permissive) conditions at 33°C in the presence of IFN-γ until confluent, and then the medium was removed. The cells were immediately washed twice with PBS and cultured under nonpermissive conditions at 37°C in the presence of IFN-γ until confluent and, then the media was replaced. The cells were immediately washed twice with PBS and cultured under nonpermissive conditions at 37°C with 0.5% FBS in the absence of IFN-γ and ITS+ until ready for use.

Chemotaxis assays. Directed migration assays were performed by using a modified 48-well Boyden chamber apparatus (Neuroprobe, Cabin John, MD). Poretics polycarbonate membranes with 8-μm pores (Osmonics, Livermore, CA) were coated with 1 μg/cm² of human fibronectin in carbonate buffer (pH 9.4) overnight before use as previously described (24). In some experiments, mouse laminin, mouse collagen type IV, or rat tail collagen type I was used at 1 μg/cm² to coat the membrane. Growth factors were diluted in RPMI 1640 and placed into the lower wells of the chamber in triplicate. The wells were then covered with the matrix-coated polycarbonate membrane. These assays were performed following 16 h of culture under nonpermissive conditions. Cells were detached with 0.05% trypsin-EDTA for 1 min, neutralized with 10% FBS-RPMI 1640 for 10 min, washed twice with RPMI 1640 without FBS, and then pretreated with inhibitors for 45 min at 37°C. At the end of the incubation time, the chamber was disassembled and the cells were scraped off the top of the membrane. The remaining cells were fixed in 100% methanol (10 min), washed in water, stained with hematoxylin (10 min), and rinsed with water. The membrane was placed on a slide and coverslipped with Aqua Polymount (Polysciences, Warrington, PA). Three (×40) images were obtained of each well and counted by an investigator blind to experimental conditions. An example of a chemotaxis assay image is shown in Fig. 1A. Chemotaxis was calculated as the difference between the number of migrated cells in the presence of a gradient and in the absence (growth factor added to both the top and bottom wells) of a gradient.

Plasmid generation and cellular transfection. The Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was employed for generation of tyrosine-to-phenylalanine mutations in pcDNA 3.1(−) wild-type (wt) EGFR. For transfections, EGFR−/− MCE were plated at 350,000 cells per well in a six-well plate. After 16-h incubation at permissive condition, transfection was performed by using the Lipofectamine 2000 (Invitrogen) reagent using 2 μg of DNA/well following the manufacturer’s instructions. At 48 h after transfection, cells were selected with 200 ng/ml Zeocin (Invitrogen). Zeocin-selected pools of cells were stained with anti-EGFR 528-PE antibody (50 μl per 5 ×10⁶ cells). PE (EGFR)-positive cells were sorted at the Veterans Affairs Medical Center Flow Cytometry Special Resource Center (Nashville, TN) by using a Becton-Dickinson FACS Aria. Stable EGFR-expressing pools were maintained in 200 μg/ml Zeocin.

Cellular proliferation assays. Cellular proliferation was measured by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-h-tetrazolium (MTS)-based colorimetric proliferation assay kit (Promega) as previously described (46). Cells were plated in 96-well culture dishes (5,000 cells/well) and incubated at nonpermissive condition in the presence and absence of EGF for 24 h. The produced formazan was dissolved and absorbance was measured at 490 nm. Reported values reflect averages of at least 12 replicate wells.

Restituation assays. Wound healing of colon epithelial cell monolayers was performed as previously described (11). Briefly, cells were plated on fibronectin, grown to confluence, and shifted to nonpermissive conditions overnight. Multiple circular wounds were made in the monolayer cultures using a drill press-mounted rotating silicone rod. Wounds were photographed over time and % closure at 8 h was determined.

Cellular lysate preparation and Western blot analysis. Cells were washed twice with ice-cold PBS, scraped into 1% Nonidet P-40 buffer [1% Nonidet P-40, 120 mM NaCl, and 50 mM Tris·HCl, pH 7.4, protease inhibitor cocktail (Sigma), phosphatase inhibitor I and II (Sigma), and incubated in an ice bath for 20 min. Lysates were cleared by centrifugation and subjected to SDS-PAGE analysis as previously described (10).

siRNA knockdown experiments. Nontargeting small interfering RNA (siRNA) pools and pools specific for PLCγ1, GRB2, and c-Blk were purchased from Dharmacon (Lafayette, CO). Mouse colon epithelial cells were transfected with 20 nM siRNA pools by using the Turbofect siRNA cationic polymer agent (Fermentas, Glen Burnie, MD) following the manufacturer’s instructions. At 48 h after transfection, cells were shifted to nonpermissive conditions for 24 h and subjected to Boyden chamber or scratch wound assays. Knockdown was monitored by Western blot analysis of lysates from parallel cultures.

Statistical analysis. All data are representative of at least three independent experiments. Statistical analyses were performed by use of Prism software (GraphPad, La Jolla, CA). Statistical significance of
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Fig. 1. EGF receptor (EGFR) kinase activity is required for EGF-induced chemotaxis and proliferation of mouse colon epithelial (MCE) cells. A: representative image of chemotaxis assay using MCE cells, expressing wild-type (wt) EGFR, migrating toward EGF. B: young adult MCE (YAMC) cells or YAMC cells expressing wt EGFR, kinase inactive (ki; K721R mutation that blocks ATP binding) EGFR, or vector alone plated on fibronectin-coated polycarbonate membranes in a Boyden chamber and subjected to an EGF (10 ng/ml)-directed chemotaxis assay. HGF (50 ng/ml) was used as a positive control for EGFR-directed migration. C: cells were exposed to EGF for 24 h and counted via a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sul- fophenyl)-2 h-tetrazolium (MTS)-based colorimetric assay. FBS was used as a positive control. *P < 0.001 vs. control.

**RESULTS**

EGFR tyrosine kinase activity is required for chemotactic migration of colon epithelial cells toward EGF. We and others have shown a requirement for EGFR tyrosine kinase activity in intestinal cell migration (1, 19, 23). The majority of these studies have used pharmacological inhibitors of EGFR signaling, which could have off-target effects or show activity against other ErbBs. Therefore, to confirm the specificity of these results and establish a system for reexpressing EGFR on an EGFR-null background to test the effects of specific EGFR mutations, stable EGFR−/− MCE cells expressing vector, wt EGFR, or kinase inactive (ki; K721R mutation that blocks ATP binding) EGFR were established as described in MATERIALS AND METHODS. EGF-directed chemotaxis of these cells was studied in modified 48-well Boyden chambers. Chemotaxis toward EGF was observed in YAMC cells (which express endogenous EGFR) and wt EGFR- but not vector- or ki EGFR expressing EGFR−/− MCE cells (Fig. 1B). Untransfected control EGFR−/− MCE cells showed an identical response to vector (data not shown). Further validation of the cell system was provided by the observation that, in parallel restitution and proliferation assays, EGFR-stimulated cell wound closure (see Fig. 4) and proliferation (Fig. 1C) in EGFR−/− MCE cells are also dependent on reexpression of wt EGFR.

p38 MAPK, PLCγ, and Src are required for EGF-stimulated colon epithelial chemotaxis. EGF-stimulated wound closure of a cell monolayer involves a variety of downstream signaling cascades, including ERK MAPK (16), JNK/SAPK (15), p38 MAPK (12), PI 3-kinase (10), PLCγ (24), Src (12), and PKC (23). However, it is not clear that pathways required for restitution are necessarily involved in directed migration of individual cells along a gradient. To determine which of these pathways are required for the chemotactic response, we assessed MCE cell movement toward EGF in a Boyden chamber in the presence of pharmacological inhibitors. Blockade of p38 (with SB202190), PLC (with U73122, D609, L108), or Src (with PP1, PP2, CGP77675) inhibited colon epithelial cell chemotaxis toward EGF (Fig. 2A). Chemotaxis was not blocked by inhibitors to MEK (U0126; blocks ERK activation), JNK (SP600125), PI 3-kinase (LY294002), or PKC (bisindolylmaleimide-1) (Fig. 2A). In contrast, restitution of cell monolayers over 8 h, which models the coordinated cell sheet migration seen in wound healing, is sensitive to the JNK, PI 3-kinase, and PKC inhibitors (Fig. 2B) as well as to p38, Src, or PLC blockade (Fig. 2B and Refs. 10, 12, 23). These results indicate that EGF-directed chemotaxis and EGF-stimulated restitution, which are phenotypically distinct at the cellular level, have signaling requirements that only partially overlap.

PLCγ1 is the PLC isozyme required for chemotaxis multiple PLC isoforms are expressed in mammalian cells. To clarify which isoform is required for directed migration toward EGF, cells were transfected with shRNA for PLCγ1 and subjected to chemotaxis assays. PLCγ1 knockdown blocked EGF-stimulated chemotaxis (Fig. 3).

Mutation of the primary PLCγ interaction sites on EGFR blocks EGF-directed migration. Multiple tyrosines in the COOH-terminal portion of EGFR are phosphorylated upon ligand binding and receptor activation, but little is known about the relative contributions of individual sites to cellular responses. To begin to address this question in the context of cellular motility we expressed receptor with Y992F/Y1173F mutations at Y992 and Y1173 [the interaction sites between EGFR and PLCγ (5, 27)] in EGFR−/− MCE cells. The Y992F/Y1173F mutant was expressed (Fig. 4A) and became phosphorylated...
and stimulated downstream signaling in response to EGF (Fig. 4B) or tumor necrosis factor [which promotes EGFR transactivation in colon epithelial cells (46)], but the Y9F substitutions attenuated EGF-stimulated chemotaxis, similar to ki EGFR (Fig. 4C). This construct was also deficient in EGF-stimulated restitution when compared with wt EGFR (Fig. 4D). Surprisingly, however, EGF-stimulated PLCγ phosphorylation was only partially attenuated in these cells (Fig. 4E); densitometric analysis of four independent experiments indicates that EGF-stimulated PLCγ phosphorylation in Y992F/Y1173F EGFR-expressing cells is 56.3 ± 6.4% of that in wt EGFR-expressing cells. This response, possibly a result of Y992F/Y1173F EGFR heterodimerization with other ErbB family members such as ErbB2, suggests that either a high threshold level of PLCγ activity is required for chemotaxis and restitution, or additional cooperative signaling cascades affected by the Y992F/Y1173F mutant are also required.

Phenylalanine substitutions on EGFR Y1045, Y1068, and Y1086 inhibit EGF-stimulated chemotaxis and restitution. Phenylalanine substitution at Y1045, which is the primary Cbl binding site on EGFR and is a key feedback target of p38 signaling (11), partially blocked chemotactic migration toward EGF (Fig. 5A). Similarly, mutation of Y1068, one of the sites for Grb2 or Gab1 binding, attenuated but did not completely block chemotaxis. In contrast, phenylalanine mutation of the second Grb2 docking site, Y1086, completely blocked EGF-directed chemotactic movement of these cells. Interestingly, all three of these mutants, Y1045F, Y1068F, and Y1086F, were unable to support EGF-stimulated wound healing (Fig. 5B), but Y1045F and Y1068F mutants did not impair EGF-stimulated cell proliferation (Fig. 5C). Thus specific EGFR tyrosine residues have differential impact on chemotaxis, restitution, and proliferation.

To examine the signaling linked to these tyrosine residues, we prepared whole cell lysates from EGF-treated MCE cells expressing Y1045F, Y1068F, or Y1086F EGFR compared with wt and ki EGFR and performed Western blot analysis by using phosphospecific antibodies. All of the Y>F mutant constructs were activated by EGF, with no substantial altera-
tion in individual phosphosite modification except on the expected mutant sites (Fig. 6A). Similarly, wt (but not ki) EGFR and all three Y1045F mutant constructs promoted phosphorylation of ErbB2, a key heterodimer partner in colon epithelial cells.

We have previously shown that failure of restitution in Y1045F EGFR-expressing cells is associated with a loss of sensitivity to p38-induced feedback through Cbl, with no change in p38 activation (11). The defect in Y1045F chemotaxis and the inhibition by SB202190 in the present study may represent a similar mechanism.

In Y1068F and Y1086F EGFR-expressing cells compared with wt, we observed decreased Src phosphorylation in response to EGF (Fig. 6, B and C), indicating that decreased chemotaxis with this mutant may not be due to a simple defect in activating these targets. We have previously shown that failure of restitution in Y1045F EGFR-expressing cells is associated with a loss of sensitivity to p38-induced feedback through Cbl, with no change in p38 activation (11). The defect in Y1045F chemotaxis and the inhibition by SB202190 in the present study may represent a similar mechanism.

In Y1068F and Y1086F EGFR-expressing cells compared with wt, we observed decreased Src phosphorylation in response to EGF (Fig. 6, B and C). Thus these tyrosines are key for signaling through Src family members. As Src inhibition blocked chemotaxis in wt EGFR-expressing cells

Fig. 4. Y992/1173F mutation on EGFR blocks EGF-stimulated colon epithelial cell migration. A: wt, ki, and Y992/1173F EGFR were expressed in EGFR−/− MCE cells and relative expression was determined by Western blot analysis. B: EGFR−/− MCE cells expressing Y992/1173F EGFR were stimulated with TNF to transactivate, or EGF to directly activate, EGFR. Western blot analysis of whole cell lysates was performed to assess EGFR activation and phosphorylation of ErbB2, AKT, and ERK. C: wt, ki, and Y992/1173F EGFR-expressing cells were subjected to EGF-directed chemotaxis assays. D: cell monolayers were subjected to EGF-stimulated restitution assays. *P < 0.01 vs. control. Vec, vector. E: cells were stimulated with EGF for 5 min; PLCγ phosphorylation was determined by Western blot analysis using phosphospecific antibody. All blots are representative of at least 4 independent experiments.
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In this study, we report that p38 MAPK, Src, and PLCγ1 activity are required for colon epithelial cell chemotactic migration along an EGF gradient (Fig. 2) as well as for restitution/wound healing migration. Furthermore, either chemotaxis or restitution in response to EGF was inhibited by blockade of phosphorylation on EGFR Y992/Y1173, Y1045, Y1068, or Y1086 (Figs. 4 and 5). In Y992F/Y1173F EGFR-expressing cells, this correlated with reduced PLCγ activation, whereas decreased Src phosphorylation was observed with Y1068F and Y1086F EGFR constructs (Fig. 6). However, the phosphosite-specific responses and downstream signaling requirements for chemotaxis were not identical with the signals necessary for EGF-stimulated restitution. For example, Y1045F and Y1068F mutants completely blocked restitution in response to EGF while showing only a partial effect on chemotaxis, and restitution but not chemotaxis was sensitive to JNK, PI 3-kinase, and PKC inhibition. Furthermore, loss of Grb2, which binds Y1068/1086, decreased both forms of migration, whereas in contrast c-Cbl knockdown only affected restitution (Fig. 7). Thus, although the differences are subtle, EGF-driven chemotaxis and restitution responses are biochemically separable.

Following ligand-EGFR binding, rapid signal transduction to a number of downstream cascades including Ras/Raf/MEK/ERK, PLC/PKC, PI 3-kinase/AKT, and Src/p38MAPK results in regulated outcomes including proliferation, migration, and survival. The mechanisms by which a cell determines its behavior in response to stimulation are not well understood, though differential regulation of heterodimerization with other ErbBs and context-dependent interaction with downstream signaling molecules such as Src, PLC, and p38 are likely to play a major role. For example, expression of ErbB2 is critical for the corneal epithelial wound healing response to EGFR ligand (45), and proliferation vs. chemotaxis of melanoma cell lines in response to growth factor is dependent on the suite of ErbBs available (13). Additionally, we have previously shown that p38 activation and its ability to promote a feedback regulatory loop on EGFR, involving Cbl activation and intracellular receptor trafficking, is a key determinant of the proliferation/restitution switch in EGF-treated colonocytes (11). Interestingly, the observation that Y1045 is required for maximal chemotaxis (Fig. 5) but c-Cbl is not (Fig. 7) suggests the involvement of an additional EGFR binding partner that docks at Y1045.

It should be noted that the specific signaling cascades necessary for chemotaxis and restitution may be somewhat context specific. For example, whereas we observe no role for ERK in EGF-driven motility of colon epithelial cells (Ref. 12 and Fig. 2), ERK is required for chemotaxis of PC12 cells (17) or keratinocytes (41) in response to EGF. Furthermore, in many cell types the response is stimulus specific; for example, in BEAS-2B bronchial epithelial cells ERK is unnecessary for EGF- but required for TFF2-stimulated chemotaxis (7). Whether these differences represent tissue-specific signaling or...
reflect the particulars of different in vitro model systems remains an open, and important, question.

Previous studies using EGFR mutant constructs to identify potential site-specific responses have yielded mixed results. For example, the Carpenter laboratory found that in NIH3T3 cells expressing point-mutant human receptors, there was no strict requirement for individual phosphosites to mediate binding to downstream targets (31). The same group reported that Y992 is critical for EGFR activation and signaling only in the context of a truncated, but not full-length, receptor (32), suggesting that binding at some tyrosines can compensate for loss of others. On the other hand, Gotoh and colleagues (14) found that single autophosphorylation site mutants were additive in their effects on mitogenesis, indicating that each site makes a separable contribution. Similar to the discussion above, responses to EGFR mutation may well...
arise from cell- or tissue-specific context, and in particular the ErbB heterodimer partners available. This possibility is underscored by the observation that kinase-dead K721M EGFR is able to heterodimerize with ErbB2, but not ErbB3 or ErbB4, to promote activation of ERK and Akt and drive 32D cell survival (8). The requirement for phosphorylation on specific EGFR tyrosines for downstream signaling events might similarly depend on the panel of other ErbBs present in a given cell.

One signaling arm required for EGF-directed chemotaxis in this study was through Y992Y/1173-dependent activation of PLCγ1 (Fig. 4). In prior reports, PLC blockade inhibited cell migration independently of MAPK- and PI 3-kinase-driven proliferation (29, 44). Signaling through this cascade promotes cytoskeletal reorganization that enables cellular polarization (39, 42). Interestingly, in our results Y992F/Y1173F EGFR double mutation only partially inhibited the activation of PLCγ in response to EGF (Fig. 4E), although both chemotaxis and restitution were fully impaired by this mutant. As a number of cellular receptors, including the EGFR heterodimer partner ErbB2, can stimulate PLCγ2 (22), it may be that the residual activity in response to EGF in Y992F/Y1173F cells is a response to ErbB2 activation that, although insufficient for cell motility, is responsible for other cellular responses.

In this report we studied cross-communication between specific EGFR autophosphorylation sites with p38 MAPK, Grb2, Src kinase, and PLCγ signaling. A recent study of individual phosphosite activation in non-small cell lung cancer demonstrated a bias toward increased EGFR Y1068 and Y1148 phosphorylation in tumors (37); it is not yet clear whether there are ligand/receptor combinations that lead to differential phosphorylation of EGFR tyrosines in nonpathological conditions. Identification of PLC and Src as possible targets of phosphosite-specific signaling (Figs. 4 and 6) raises interesting possibilities for pathway-directed therapies. For example, the production of small peptides mimicking the binding site at EGFR phospho-Y1086 might be used to influence Grb2 binding and Src activation downstream of EGFR, as has been demonstrated for MUC1 interactions with β-catenin and EGFR (3). Such an approach could potentially selectively modulate chemotaxis or tumor cell invasion.

In summary, we have shown a requirement for p38 MAPK, Grb2, Src, and PLCγ1 activities for EGF-stimulated chemotactic migration and restitution in MCE cells. In contrast, inhibition of PI 3-kinase, PKC, and JNK/SAPK affected only restitution, whereas ERK MAPK blockade affected neither type of migration studied. These findings suggest that a limited set of EGFR downstream signal pathways are capable of regulating EGF-stimulated cell movement and are consistent with decreased chemotaxis and restitution in cells expressing EGFR with nonphosphorylatable mutations on Y992/1173, Y1086, Y1045, and Y1068, with restitution being more sensitive to substitution on the latter two sites. Further study of the interaction between individual EGFR autophosphorylation sites and cellular behavior may lead to the development of new experimental and therapeutic tools to manipulate the cell’s response to EGFR activation.

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