Epidermal Growth Factor Receptor-mediated Cell Motility: Phospholipase C Activity Is Required, but Mitogen-activated Protein Kinase Activity Is Not Sufficient for Induced Cell Movement

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Abstract. We recently have demonstrated that EGF receptor (EGFR)-induced cell motility requires receptor kinase activity and autophosphorylation (P. Chen, K. Gupta, and A. Wells. 1994. *J. Cell Biol.* 124:547–555). This suggests that the immediate downstream effector molecule contains a *src* homology-2 domain. Phospholipase Cγ (PLCγ) is among the candidate transducers of this signal because of its potential roles in modulating cytoskeletal dynamics. We utilized signaling-restricted EGFR mutants expressed in receptor devoid NR6 cells to determine if PLC activation is necessary for EGFR-mediated cell movement.

Exposure to EGF (25 nM) augmented PLC activity in all five EGFR mutant cell lines which also responded by increased cell movement. Basal phosphoinositide turnover was not affected by EGF in the lines which do not present the enhanced motility response. The correlation between EGFR-mediated cell motility and PLC activity suggested, but did not prove, a causal link. A specific inhibitor of PLC, U73122 (1 μM) diminished both the EGF-induced motility and PLC responses, while its inactive analogue U73343 had no effect on these responses. Both the PLC and motility responses were decreased by expression of a dominant-negative PLCγ-1 fragment in EGF-responsive infectant lines. Lastly, anti-sense oligonucleotides (20 μM) to PLCγ-1 reduced both responses in NR6 cells expressing wild-type EGFR. These findings strongly support PLCγ as the immediate post receptor effector in this motogenic pathway.

We have demonstrated previously that EGFR-mediated cell motility and mitogenic signaling pathways are separable. The point of divergence is undefined. All kinase-active EGFR mutants induced the mitogenic response while only those which are autophosphorylated induced PLC activity. U73122 did not affect EGF-induced thymidine incorporation in these motility-responsive infectant cell lines. In addition, the dominant-negative PLCγ-1 fragment did not diminish EGF-induced thymidine incorporation. All kinase active EGFR stimulated mitogen-activated protein (MAP) kinase activity, regardless of whether the receptors induced cell movement; this EGF-induced MAP kinase activity was not affected by U73122 at concentrations that depressed the motility response. Thus, the signaling pathways which lead to motility and cell proliferation diverge at the immediate post-receptor stage, and we suggest that this is accomplished by differential activation of effector molecules.

CELL movement is essential for numerous normal biological and physiological events such as wound healing, fetal development, bone remodeling, angiogenesis, and the inflammatory response. Aberrant cell movement, on the other hand, contributes to the pathogenesis of many diseases, such as atherosclerosis and tumor invasion and metastasis. Cell movement is modulated by signals from extracellular environment. However, the intracellular signal transduction pathways that lead to this biological response are not fully delineated.

The epidermal growth factor receptor (EGFR)[1], upon ligand stimulation, elicits augmented cell movement (4, 6, 11). This cellular response is separable from EGF-induced mitogenesis (11). Our previous study demonstrated that EGF-induced cell movement depends on functional tyrosine kinase activity and the presence of at least one autophos-

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1. *Abbreviations used in this paper*: EGFR, EGF receptor; IP, inositol phosphate; MAP, mitogen-activated protein; MBP, myelin basic protein; PKC, protein kinase C; PLC, phospholipase C; SH2, *src* homology 2; WT, wild-type.

phorylatable tyrosine within the carboxy-terminal regulatory region of the EGFR. Phospho-tyrosine motifs interact with *src* homology 2 (SH2) domains in effector molecules activating these second messengers (9, 30); these SH2 domain interactions with phosphotyrosines display varying degrees of specificity according to the amino acids surrounding the tyrosine (18, 26, 29, 36, 42, 43, 55). Multiple EGFR phospho-tyrosine motifs are capable of signaling cell movement, suggesting either a promiscuity in the interactions with SH2 domains or parallel intracellular pathways leading to cell movement (11). Thus, the search for the immediate downstream molecules that mediates the motility response has been pointed toward SH2 domain containing signal transducers.

Phospholipase C γ (PLCγ) is activated by the EGFR tyrosine kinase after physical associating with the receptor via its SH2 domains (27). PLCγ activity and subsequent effector molecules can be linked to the motogenic machinery. PLCγ is capable of associating via its SH3 domain with actin filaments (3). PLCγ hydrolyzes PIP_2 to produce IP_3 and DAG. This hydrolysis releases profilin and gelsolin, actin-sequestering and -severing proteins (2, 19). IP_3 mobilizes intracellular calcium which also has been shown to modulate actin assembly (44). These findings provide a theoretic framework for the participation of PLCγ in the pathway leading to cell motility. Recent studies have demonstrated that PLCγ activity is required for chemotactic transmigration of cells through a collagen gel in response to PDGF-BB (8, 25). However, the cell property elicited by PLCγ activation (i.e., cell movement, enhanced gel proteolysis, etc.) is undefined.

In this study, we utilize the EGFR-devoid NR6 fibroblast cell line (33) to investigate the link between EGF-induced cell movement and PLCγ activity. Various EGFR mutants were expressed in this cell background to avoid signaling from native EGFR (20, 52). The EGFR constructs varied in their ability to elicit a motogenic response upon activation (11). EGF-induction of PLCγ activity and cell movement correlated closely. A pharmacological agent which specifically inhibits PLC activity abrogated induced cell movement, but had no effect on EGFR-mediated mitogenesis. Molecular inhibition of PLCγ activity by antisense oligonucleotides or a dominant-negative fragment (23) also diminished the motility response. Lastly, in an attempt to map further downstream intermediaries, activation of mitogen-activated protein (MAP) kinase was found not to be sufficient to induce cell movement.

Materials and Methods

Generation of NR6 Cells Expressing EGFR Constructs

The construction of the EGFR and stable expression in NR6 cells were by standard methods, and have been described previously (11, 52). Briefly, wild-type (WT) EGFR is a full-length cDNA (46) derived from a placental cDNA library (53). M^{721} is the kinase-inactive counterpart in which methionine replaces lysine in the ATP-binding pocket (13). *c*973, *c*991, and *c*1000 represent EGFR in which stop codons are introduced just distal to the amino acid number indicated. *c*$1000F^{992}$ was created from *c*1000 by replacing the sole remaining autophosphorylation site at Y^{992} with a phenylalanine (F^{992}) (10). The fusion mutants *c*$958f^{988-996}$ and *c*$958f^{1146-1154}$ link a minimal kinase-active EGFR (50) to the phosphotyrosine motifs around Y^{992} and Y^{1148}, respectively. *c*1186F3 represents a full-length EGFR in which the three major autophosphorylation sites (Y^{1173}, Y^{1148}, and Y^{1068}) (17) have been negated by phenylalanine replacement. The EGFR are shown schematically in Table I.

The constructs were expressed on NR6 cells, 3T3-derivatives which lack endogenous receptors (33). This was accomplished by retroviral-mediated transduction as previously described (51). Polyclonal lines were established by selection in G418 (GIBCO BRL, Gaithersburg, MD). The infectant cell lines presented high, but physiologic levels of receptors (50,000–250,000 EGF-binding sites per cell) with similar dissociation constants (K_d were 0.2–0.7 nM); different EGFR levels within this range do not affect ligand-induced cell motility (11). All of the EGFR possessed kinase activity, except for M^{721}; the cells which presented the kinase-active EGFR all demonstrated a mitogenic response to EGF.

Cell Motility Assay

EGF-induced migration was assessed by the ability of the cells to move into an acellular area as previously described (11, 14). Briefly, NR6 cells were plated on plastic and grown to confluence in MEMα with 7.5% FBS. After 24 h of incubation in media with 1% dialyzed FBS, an area was denuded by a rubber policeman at the center of the plate. The cells were then treated with or without 25 nM EGF and incubated at 37°C. Photographs were taken at 0 and 24 h and the relative distance traveled by the cells at the acellular front was determined. The EGF-induced migration was calculated as a percent of basal motility observed in the non-EGF-treated cells tested in parallel at each time point. Mitomycin-C (0.5 μg/ml) was present throughout the motility assays to avoid interference from the mitogenic response.

PLC Activity Assays

PLC activity was monitored by a functional assay in which the production of inositol phosphate species (IP) was measured (38, 39). NR6 cells expressing mutant EGF receptors were grown to ~90% confluency, under the same conditions used in motility assays. Cells were labeled in serum free media containing 5 μCi/ml [^{3}H]myo-inositol for 12–14 h, after which the cells were washed twice with PBS to remove unincorporated label. LiCl (10 mM) was introduced to inhibit IP hydrolysis by inositol phosphatases. After 15 min of LiCl incubation, saturation level of EGF (25 nM) was added and the incubation continued for another 30 min. The reaction was terminated by removing the media and adding boiling water to the cells. Cell lysates were collected and boiled for 5 min. The lysates were briefly centrifuged to remove particulate material, and the soluble cytosolic fraction retained for analysis.

Inositols and inositol phosphates were separated on Dowex (AGI-X8 100-200 mesh) anion-exchange mini column. Inositol was eluted with water and IP with 100 mM ammonium formate and 100 mM formic acid. The IP content was quantitated by scintillation counting and normalized against the protein content in the lysate determined by Bradford Protein Assay (Bio Rad Labs., Hercules, CA). The EGF-elicited PLC activity was expressed as percent of non-EGF-treated basal IP production to account for variations in labeling.

A complete profile of the individual inositol phosphate species was generated by HPLC separation to confirm the anion-exchange chromatography (38). Split samples from the cell lysate were injected into a Partisil 10 SAX anion-exchange analytical column (0.45 × 25 cm). Inositol was eluted with water for 10 min. Inositol phosphate species were eluted in the order of IP, IP_2, and IP_3 by a 5-min linear gradient of 0–10% 1 M ammonium formate, pH 3.8, followed by 20-min of isocratic elution with 10% 1 M ammonium formate. Elution then continued with 25-min linear gradient of 10–100% of 1 M ammonium formate followed by a 15-min isocratic 100% 1 M ammonium formate. The flow rate was set at 1.2 ml/min and 1-min fractions were collected.

PLC activity responsiveness after prolonged EGF treatment was determined after 12 h of continuous exposure of cells to EGF. After 12 h of incubation, 10 mM LiCl was added. After an additional 30-min incubation, cells were lysed and lysates were analyzed as described above.

Thymidine Incorporation Assay

EGF-induced mitogenesis was assessed by the incorporation of [^{3}H]thymidine in the target cells. Cells were plated on plastic and grown to confluence in MEMα with 7.5% FBS. The cells were then switched to media containing 1% dialyzed FBS for 24 h. The cells were subsequently treated with or without EGF (25 nM) and incubated at 37°C for 16 h. [^{3}H]thymidine (5 μCi/ml) was added and incubation continued for another 10 h. The cells were then washed with ice-cold PBS twice and incubated in 5% trichloroacetic acid at 4°C for 30 min. After two washes with PBS the cells were lysed in 0.2 N NaOH and the incorporated [^{3}H]thymidine counted by scintillation counter.

Pharmacological PLC Inhibitors

The pharmacological agents, U73122 (1-(6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) and its inactive congener, U73343 (1-(6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidine-dione), were added to the cells to inhibit PLC activity (7, 41). Both U73122 and U73343 (BIOMOL Res. Labs., Inc., Plymouth Meeting, PA) were dissolved in chloroform and dried under a nitrogen stream. The dried film was then dissolved in medium containing serum proteins to complex the drugs for intracellular delivery.

These compounds were introduced at 1 μM into the media in the PLC activity assays at the same time LiCl was added. In the cell migration and [^{3}H]thymidine incorporation assays, these compounds (1 μM) were introduced 15 min before the addition of EGF. EGF-induced PLC activity and cell migration responses were measured in parallel with the control non-EGF-treated cells.

Cloning and Expression of Dominant-negative PLCγ-1 in NR6 Cells

A dominant-negative PLCγ-1 gene fragment (designated as PLCz) which encodes the Z region SH2 and SH3 domains (amino acids 517–901) of this enzyme (23) was cloned from a human placenta cDNA library by standard PCR techniques. The oligonucleotide primers used were: 5′ sense I^{517}: CCTCTAGAATGATCTACTACTCTGAGGAGACC and 3′ anti-sense $stop^{902}$: GGCAAGCTTCTACATGCTGATGGAGAAGACGAAG. These primers have XbaI and HindIII sites to allow subsequent cloning into the pXf vector for expression. Subcloning placed the PLCz fragment under the control of a SV40 early promoter present in the pXf vector (12). The pXf vector contains a DHFR gene transcribed from a second SV40 early promoter.

The pXf/PLCz expression vector was transfected into selected NR6 infectant cell lines (c′1000, c′1086F3, and c′991) using the lipofectin reagent (GIBCO BRL). Cells were selected in 400 mM methotrexate. Stable expression of PLCz in these cell lines was demonstrated by western blotting with mixed monoclonal anti-PLCγ-1 antibodies (UBI; 05-163). The PLCz expressing cell lines were tested for PLC activity, cell movement, and thymidine incorporation as described above.

Anti-sense PLCγ-1 Oligonucleotides

Anti-sense PLCγ oligonucleotides were employed to down-regulate PLCγ expression. Oligonucleotide A1 (GGGGGTCCCGACGCCCGCCAT) was a 21-mer anti-sense to the sequence encoding the first seven amino acids in the rat PLCγ sequence. A1-thio was the same sequence with the first and the last base containing a thio-linkage to prevent degradation. A2 (GCTGTACATGAGGCTGCGGTA) was a 21-mer anti-sense to the sequence encoding the amino acids 217–223 which was absolutely conserved across murine, rat and human species. S1 (CGGAGGAAGAAGATTGCCCTG), a 21-mer sense oligonucleotide, encoding amino acids 945–951 in the human PLCγ, was used as a control to demonstrate sequence specificity of any effects. The oligonucleotides were dissolved in medium and introduced at 20 μM during the final 8 h of the metabolic labeling in the PLC activity assays. In the cell migration assays, oligonucleotides (20 μM) were introduced 8 h before the addition of EGF and remained present throughout the entire assay period. The effects of these oligonucleotides on EGF-induced PLC activity and cell migration responses were assessed in parallel with the control oligonucleotides and nontreated cells.

Phospholipid extraction was used to determine the basal incorporation of [^{3}H]myo-inositol in the presence of antisense oligonucleotides. After metabolic labeling with or without oligonucleotides as described above, cells were lysed in 1 ml of cold methanol. Chloroform (0.5 ml) containing 0.63 mg/ml of butylated hydroxytoluene was added to the lysate. 188 μl of 1:2 chloroform/methanol were added, followed by 563 μl of water. The lower chloroform layer containing phospholipids was collected. A 200-μl aliquot was air-dried and incorporated label measured by scintillation counting. The effects of antisense oligonucleotides were assessed in parallel with the nontreated control cells.

MAP Kinase Assay

MAP kinase activity was measured as the rate of phosphorylation of the MAP kinase-specific substrate myelin basic protein (MBP) by cell extract as previously described (1). Briefly, cells were plated on plastic and grown to confluence. After 5 min of treatment with EGF (25 nM), cells were rinsed twice with ice-cold PBS and once with cold extraction buffer (50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 0.1 mM Na_3VO_4, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 μg/ml pepstatin A, and 1 mM benzamidine). Cells were scraped into extraction buffer, homogenized for 20 s and centrifuged at 14,000 *g* for 5 min at 4°C. Supernatants were collected as the cytosolic extracts. The cytosolic extracts were then mixed at room temperature with the assay buffer so the final concentrations of assay components were 50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 0.1 mM Na_3VO_4, 1 mM dithiothreitol, 10 μM calmidazolium, 2 μM PKI peptide, 10 mM $MgCl_2$, 0.33 mg/ml MBP, 330 nM ATP, and 15 nM [^{32}P]γATP. Reactions were terminated after 0, 2, 4, 6, and 10 min by spotting aliquots of the assay mixture onto PE81 phosphocellulose filter paper squares which were washed three times with 175 mM phosphoric acid, once with ethanol, once with ether, air dried, and counted. Initial studies revealed that the 10-min time period was in the linear range; MAP kinase activity was determined as the increase of MBP phosphorylation over time. The EGF-induced MAP kinase activity was determined by comparing the MAP kinase activity of EGF-treated cells to non-EGF-treated cells; the EGF-induced MAP kinase activity was calculated and expressed as the percent of activity seen in non-EGF-treated cells.

The effects of U73122 on EGF-induced MAP kinase activity was assessed. 1 μM of U73122 was introduced to the cells 15 min before the addition of EGF and the EGF-induced MAP kinase activity was evaluated as above.

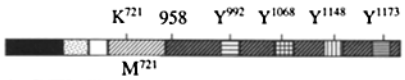
Results

EGF Induces PLC Activity Only in Cell Lines which Demonstrate EGF-enhanced Movement

Previously, we demonstrated that EGF augments cell movement only in NR6 cells that express EGFR constructs which contain at least one autophosphorylatable tyrosine (11). Cell movement was determined by the ability of cells to move into a denuded area on a culture dish. To standardize the assays, the EGF-induced movement after 24 h of EGF exposure was calculated as a percent of basal motility observed in non-EGF-treated cells tested in parallel. NR6 cells expressing EGFR constructs which undergo autophosphorylation (WT, c′1000, $c'958f^{988-996}$, $c'958f^{1146-1154}$) demonstrated enhanced movement in the presence of saturating concentrations of EGF (25 nM) (Table I); this is in agreement with our earlier report (11). An additional mutant c′1186F3, a full-length receptor with the three major autophosphorylatable tyrosines (Y^{1173}, Y^{1148} and Y^{1068} [17]) replaced by phenylalanines, was tested for EGF-induced motility. As would be predicted (11, 43), this construct underwent autophosphorylation upon EGF stimulation, presumably at the remaining two minor phospho-tyrosine motifs (centered on Y^{992} and Y^{1086} [43, 50]). The NR6 cells expressing this EGFR construct also demonstrated augmented cell movement in the presence of EGF. The parental NR6 cells, devoid of endogenous receptors, and NR6 cells expressing non-autophosphorylated receptors (c′973, c′991, $c'1000F^{992}$, and M^{721}) all failed to demonstrate increased movement in the presence of EGF (Table I) (11).

These findings suggested that activation of the immediate downstream effector molecule in the signaling pathway leading to enhanced cell movement involved SH2 domain interactions. EGFR must be autophosphorylated to be able to activate PLCγ (35, 48), a signaling molecule which has been implicated in altering cell motility (8, 19, 25). Therefore, we tested the various NR6 cell lines for EGF-induced PLC activity (Table I). PLC activity was assessed by the generation of [^{3}H]inositol phosphates in cells metabolically labeled with [^{3}H]myo-inositol. PLCγ upon activation hydrolyzes PIP_2 to generate IP_3 and DAG; IP_3 is rapidly converted to IP by either direct stepwise dephosphorylation or following

Table I. EGF-induced PLCγ Activity and Cell Migration in NR6 Cells Expressing EGFR Constructs

Construct	Schematic of EEGF receptor	Phosphotyrosyl*	Migration‡	PLC activity§
Parental	No EGF Receptor	NA	101 ± 4	97 ± 2
WT		+++	225 ± 41	337 ± 74
M^{721}		–	99 ± 11	91 ± 5
c'1000		+	170 ± 11	335 ± 55
c'1000F^{992}		–	121 ± 14	121 ± 4
c'991		–	105 ± 18	110 ± 10
c'973		–	114 ± 4	101 ± 11
c1186F3		+	207 ± 11	383 ± 109
c'9581$f^{988-996}$		+	177 ± 25	240 ± 75
c'958$f^{1146-1154}$		+	172 ± 23	259 ± 81

Extracellular ligand-binding domain

Transmembrane domain

Tyrosine kinase domain

Carboxy-terminal regulatory domain

Domain around autophosphorylatable tyrosine Y^{992}

Domain around autophosphorylatable tyrosine Y^{1064}

Domain around autophosphorylatable tyrosine Y^{1148}

Domain around autophosphorylatable tyrosine Y^{1173}

M^{721}, lysine replaced by methionine in the ATP-binding pocket of the tyrosine kinase domain.
F, phenylalanine replacing the autophosphorylated tyrosines at the indicated sites.
* EGF-induced, as determined by anti-phosphotyrosyl antibody Western blot of immunoprecipitated EGFR.
‡ EGF-induced migration after 24 h of exposure to 25 nM EGF; expressed as percentage of non-EGF-treated cells, mean ± SEM, n = 3 to 7.
§ EGF-induced PLCγ activity measured over a 30-min treatment with 25 nM EGF; expressed as percentage of non-EGF-treated cell, mean ± SEM, n = 3 to 7.

conversion to IP_4 and subsequent dephosphorylation. Accumulation of IP in the presence of lithium, an inhibitor of inositol monophosphatase, is a good measure of phosphoinositide signaling (5, 39). Production of IPs was determined and quantified by anion exchange chromatography followed by scintillation counting. To account for experimental variations in metabolic labeling and extractions, IPs production was calculated as cpm per 100 μg protein; the production induced by EGF was expressed as percent of IPs produced by non-EGF-treated cells tested in parallel. We observed a concordance between EGF induced cell movement and augmented PLC activity (Table I). The NR6 cells expressing autophosphorylatable EGFR all demonstrated ligand stimulation of PLC activity while the cell lines presenting non-autophosphorylated EGFR did not.

HPLC separation of the inositol species confirmed that IP_3 was generated from the labeled inositol pool (Fig. 1). As lithium exerts its most potent inhibition towards inositol monophosphatase, inositol monophosphate was the major species which accumulated after EGF treatment of responsive cell lines. The low level of IP_3 observed is consistent with a brief <2–5 min burst of IP_3 formation (I(1, 4, 5)P_3 for the initial minute converting to I(1, 3, 4)P_3 as the major species after that), followed by a prolonged lower level of IP_3 generation (data not shown and reference 21).

PLC Activation by EGF Is Demonstrable Even After 12 Hours of Exposure

NR6 cell movement in response to EGF is observed only after 6–12 h of exposure (11). However, PLC activity was monitored during the initial 30 min of EGF stimulation to avoid interference from inositol turnover and recycling and depletion of [^{3}H]-labeled inositol. Furthermore, as EGF exposure stimulates phospho-inositide metabolism (Table I, Fig. 1), EGF treatment may be expected to exacerbate the depletion of labeled inositol. However, it was critical to determine if IP_3 production was increased throughout the migration assays.

EGF-augmented PLC activity was measured after 12 h of EGF stimulation (Fig. 2). After metabolic labeling of NR6 cells expressing WT EGFR, the cells were washed, incubated in media without [^{3}H]myoinositol, and either treated or not with saturation concentrations of EGF (25 nM). After a further 12-h incubation, LiCl (10 mM) was added to prevent IP hydrolysis, and production of IP determined. Cells which were not exposed to EGF during the 12 h after labeling incubation, demonstrated significant induction of PLC activity in response to addition of EGF; the absolute amount of label recovered in the IP fraction was reduced 15–20% in cells stimulated 12 h after labeling com-

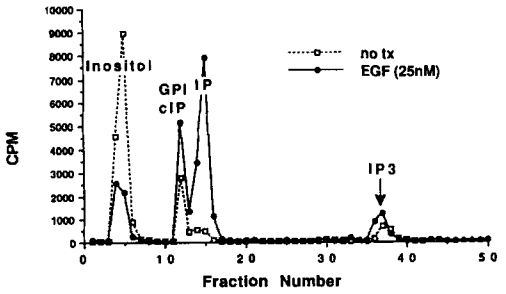


Figure 1. EGF-induced inositol phosphate production profile in NR6 cells expressing WT EGF receptor. Cells were labeled 12–14 h with [^{3}H]myo-inositol and then treated with or without EGF (25 nM) for 30 min in the presence of LiCl (10 mM). Cytosolic extracts were obtained and fractionated by HPLC. Fractions were collected and the [^{3}H]inositol phosphate contents quantified by scintillation counting. Relative amounts of individual inositol phosphate contents are reported as the actual cpm from scintillation counting. Peaks were identified by known standards run in parallel. On Dowex mini-column chromatography the species collected are GPI, cIP and IP. EGF treated (*EGF*) inositol phosphate production profile is depicted as solid line and no EGF treatment (*no tx*) as dashed line. *GPI*, glycerol phosphoinositol; *cIP*, cyclic inositol phosphate; *IP*, inositol monophosphate; *IP3*, inositol trisphosphate. This figure is of a representative analysis.

pared to cells stimulated immediately after labeling with [^{3}H]myoinositol. The WT-expressing NR6 cells which were exposed to EGF throughout the 12-h incubation produced [^{3}H]IP at nearly twice the rate of unstimulated cells. HPLC analyses revealed that IP_3 was being generated at a higher rate in the EGF-exposed cells than in the non-treated cells (Fig. 2 *b*); the relative EGF-induction and species profile of IP_3 accumulation was similar to that seen with the 30-min EGF exposure.

Pharmacologic Agents Which Inhibit PLC Activity Also Decrease EGF-induced Cell Motility

The correlation between EGF-induced PLC activity and cell motility suggested that PLCγ is the immediate downstream effector in the EGFR-mediated motogenic pathway. However, this correlation may represent an epiphenomenon due to promiscuity of phospho-tyrosine/SH2 domain interactions (11, 35, 42). A causal relationship between PLCγ and cell motility would be demonstrated if inhibition of PLC reduced cell movement.

Pharmacological agents can inhibit specific enzymes in complex biochemical pathways. U73122 has been shown to inhibit specifically PLC while its inactive analogue U73343 does not affect this enzymatic activity (7, 41). EGF-induced PLC activity and cell motility were determined in the presence of these agents (Fig. 3). In three cell lines expressing responsive EGFR constructs (WT, c1186F3, and c1000), U73122 inhibited EGF-induction of both PLC activity and cell movement; the inactive analogue, U73343, had little affect on these responses. We noted only partial inhibition of both PLC and cell motility at the doses used (1 and 2 μM); higher concentrations which fully blocked EGF-induction of PLC activity (>5 μM) resulted in cell toxicity over the 12–24 h required for the motility assays (data not shown). The effect of U73122 on basal cell movement and IP production was negligible (<15% decrease) in these cell lines.

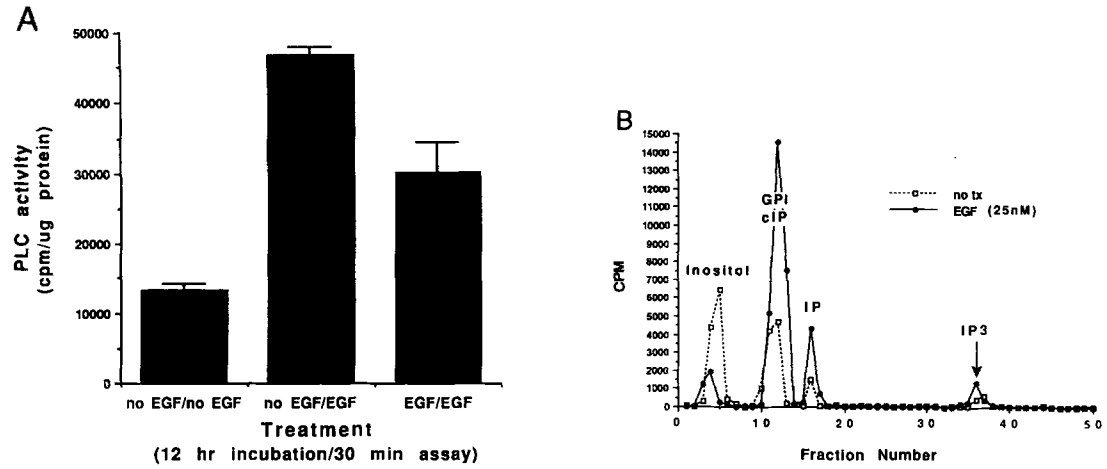


Figure 2. EGF-induced PLC activity in NR6 cells expressing WT EGF receptors. PLC activity was measured as the production of inositol phosphates by anion-exchange chromatography (*A*) or HPLC separation (*B*). Cells were labeled 12–14 h with [^{3}H]myoinositol and then incubated in media without label for 12 h in the absence or presence of EGF (25 nM). Inositol phosphate production was measured for 30 min; LiCl (10 mM) was present only for 15 min before and during the 30-min measurement period. Relative amounts of inositol phosphates productions are reported as the actual cpm per μg protein in cytosol extract in *A* or cpm in *B*. (*A*) No EGF/no EGF, basal inositol phosphate production during the 30-min incubation; no EGF/EGF, acute (30 min) EGF-induced inositol phosphate production after 12 h after removal of label; EGF/EGF, inositol phosphate produced over 30-min assay period after 12 h of continuous exposure to 25 nM EGF. Mean ± SEM, $n = 3$, each experiment in triplicate; $P < 0.01$ between no EGF/no EGF and EGF/EGF. (*B*) The analysis shown was performed as described in Fig. 1. EGF treated (*EGF*) inositol phosphate production profile is depicted as solid line and no EGF treatment (*no tx*) as dashed line. *GPI*, glycerol phosphoinositol; *cIP*, cyclic inositol phosphate; *IP*, inositol monophosphate; *IP3*, inositol trisphosphate. This figure is of a representative analysis.

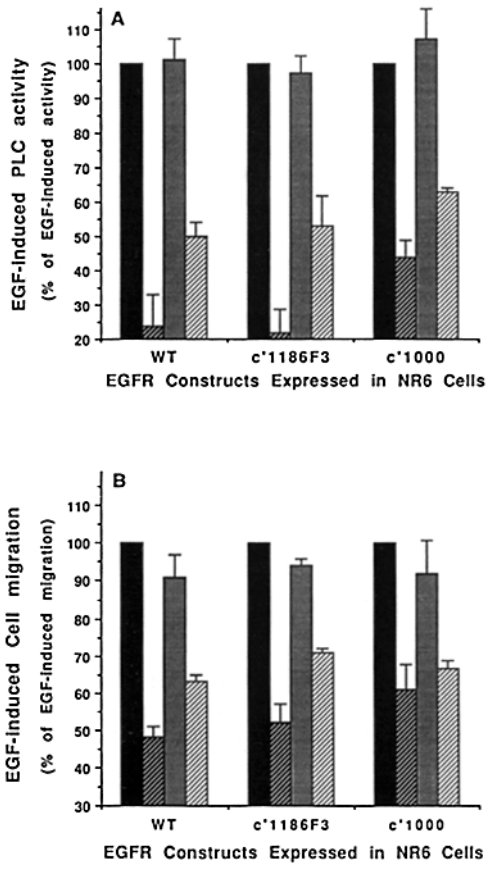


Figure 3. Effects of the pharmacologic agents U73122 and U73343 on EGF-induced (*A*) PLC activity and (*B*) cell movement in three NR6 cell lines. NR6 cells lines expressing WT, c'1186F3, and c'1000 EGFR all demonstrated EGF-induced PLC activity and cell motility responses. Cells were treated with EGF (25 nM) in the presence or absence of U73122 or U73343 (1 μM) during the assay periods (30 min for PLC activity, 24 h for cell migration). The effects of U73122 and U73343 on the EGF-induced responses were calculated as percent of the EGF-induced responses observed in the absence of U73122 and U73343. ■, EGF-induced PLC activity or cell migration in the absence of drug treatment; ▨, basal PLC activity or cell migration as percent of EGF-induced responses; ▩, EGF-induced response in the presence of the inactive congener U73343; ▨, EGF-induced response in the presence of U73122. Values are mean ± SD; n = 3–6.

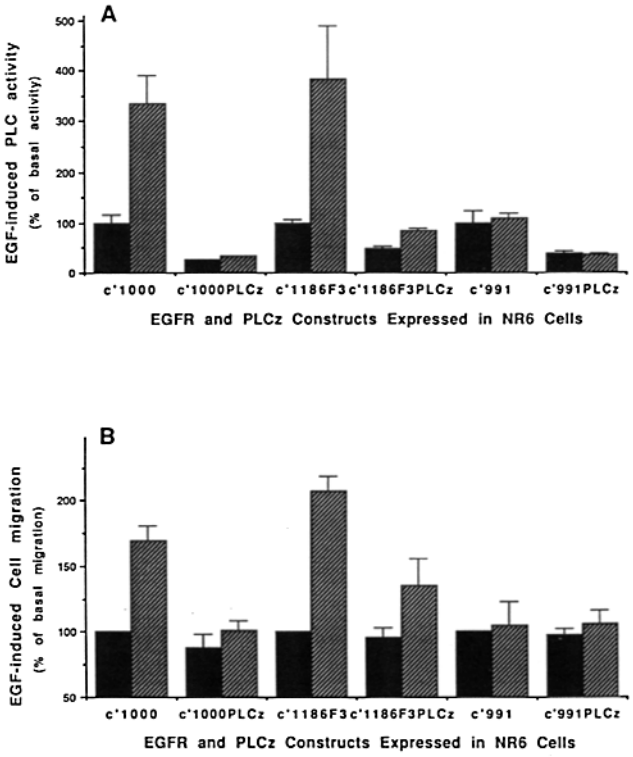


Figure 4. Effects of over-expressing dominant negative PLCz on EGF-induced (*A*) PLC activity and (*B*) cell movement in three NR6 cell lines. Solid bars depict PLC activity or cell migration in the absence of EGF treatment; hatched bars are EGF-induced responses. Values are mean ± SD; $n > 3$.

A Dominant-negative PLCγ-1 Fragment or Antisense Oligonucleotides Diminish EGF-induction of Both PLC Activity and Cell Motility

Specific interruption of EGFR activation of PLCγ is required to demonstrate a causal relation between induced PLC activity and cell motility. U73122 is not specific for the tyrosine kinase activated isoform of PLC, PLCγ, and may also inhibit other phospholipases which are triggered by the EGFR (32). Therefore, we attempted to either block the activation of PLCγ by interfering with its binding to EGFR or to down-regulate the level of PLCγ in the cell.

The Z region of human PLCγ-1, covering the SH2-SH2-SH3 domains, was isolated by reverse transcriptase/PCR and cloned into a eukaryotic expression vector. PLCz was expressed in three selected infectant NR6 lines (c'1000, c'1186F3, and c'991), as determined by immunoblotting with a mixture of monoclonal antibodies (data not shown). PLC activity and cell movement were tested in these infectant sublines (Fig. 4). The presence of PLCz disrupted both EGF-induced responses in parallel. The infectant PLCz sublines demonstrated lower basal PLC activity than their respective infectant lines, but basal cell movement was unchanged. EGF-induction of these responses was blocked or severely diminished in the PLCz sublines.

Anti-sense oligonucleotides down-regulated PLCγ activity in the WT EGFR infectant cells (Fig. 5). The sense oligonucleotide control had no affect on EGF-induced cell responses. Two distinct anti-sense oligonucleotides were tested for the ability to decrease the EGF-induced PLC and motility responses. Accumulations of IPs were decreased by 35–60% in these cells, and augmented cell movement by 56–82%; basal activities were relatively unaffected by these treatments. The slightly greater inhibition of cell movement compared to IP generation may be due to extended exposure to anti-sense down-regulation in the cell migration assay; the oligonucleotides were present for 8 h before addition of EGF and remained in the media throughout the 24-h motility assay. As anti-sense down-regulation does not affect previously synthesized proteins, the extended exposure in the migration assay may result in a greater decrease in PLC activity during the measurement of this response.

Induced Cell Motility Correlates with PLC Activity

EGF-induction of PLC activity was compared to induction of cell movement (Fig. 6). NR6 cell lines which expressed

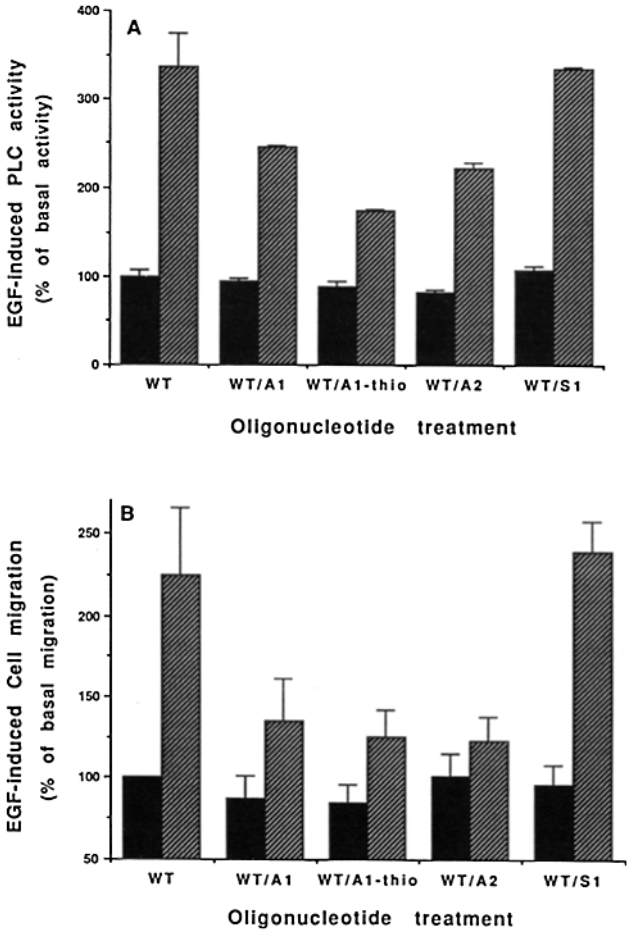


Figure 5. Effects of anti-sense PLCγ oligonucleotides on EGF-induced (*A*) PLC activity and (*B*) cell movement in NR6 cells. NR6 cell lines expressing WT EGFR were treated with EGF (25 nM) in the presence or absence anti-sense PLCγ oligonucleotide (20 mM) during the assay periods (8 h preincubation for PLC activity, 8 h before incubation plus subsequent 24-h assay period for cell migration). Oligonucleotide A1 was a 21-mer anti-sense to the sequence encoding the first seven amino acids in the rat PLCγ sequence. A1-thio was the same sequence with the first and the last base containing sulfur groups to prevent degradation. A2 was a 21-mer anti-sense to the sequence encoding the amino acids 217-223. A sense sequence S1, a 21-mer encoding amino acids 945–951, that was used as a control to demonstrate the inhibitory effects on PLC activity was sequence specific. The effects of these oligonucleotides on the EGF-induced responses were expressed as percent of the EGF-induced responses observed in the absence of EGF and oligonucleotide treatment. Solid bars depict PLC activity or cell migration in the absence of EGF treatment; hatched bars are EGF-induced responses. Values are mean ± SD; $n > 3$.

autophosphorylatable (Fig. 6, *Y*) and non-autophosphorylatable (Fig. 6, *O*) EGFR represented the doubly responsive and doubly nonresponsive lines, respectively. U73122 treatment (Fig. 6, *U*) partially inhibited both parameters in the three responsive cell lines tested (Fig. 3). Diminution of PLC activity correlated with reduced cell motility in the infectant sublines expressing PLCz (Fig. 6, *Z*) and the WT EGFR line treated with the oligonucleotides (Fig. 6, *A*). EGF-induced PLC activity and cell motility was expressed as percent of basal (non-EGF-treated). The two responses

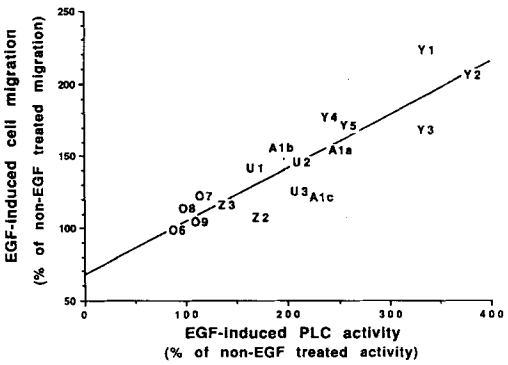


Figure 6. Correlation of EGF-induced PLC activity with cell migration responses. EGF-induced PLC activity and cell migration was calculated as percent of non-EGF-treated responses for each NR6 cell lines tested. *O*, cells lines expressing EGFR constructs that do not respond to EGF in migration and PLC activity; *Y*, cell lines expressing EGFR constructs that undergo autophosphorylation upon EGF stimulation and demonstrate augmented PLC activity and cell migration responses; *U*, responses demonstrated by the three EGF-responsive cell lines (Fig. 3) in the presence of U73122 (1 μM); *Z*, responses demonstrated by the two EGF-responsive cell lines expressing dominant negative PLCz. Numbers denote cell lines expressing various mutant EGFR constructs (*1*, *WT*; *2*, c'1186F3; *3*, c'1000; *4*, c'957f$^{988-996}$; *5*, c'958f$^{1146-1154}$; *6*, M^{721}; *7*, c'1000F^{992}; *8*, c'973; and *9*, c'991). Ala, Alb, Alc, and Als represent WT EGFR expressing cells treated with anti-sense PLCγ oligonucleotides A1, A1-thio, A2, and S1, respectively. Values are the mean of the cumulative responses for each cell line tested; $n = 3$–12 for individual responses in each cell line tested. R, 0.81; $P < 0.001$.

correlated strongly (r, 0.81; $P < 0.001$), exhibiting a positive linear relationship.

EGF-induced Mitogenesis Is Independent of the Inhibition of EGF-induced PLC Activity

We have shown previously that EGF-induced motogenesis is separate from the mitogenic response (11): all EGFR con-

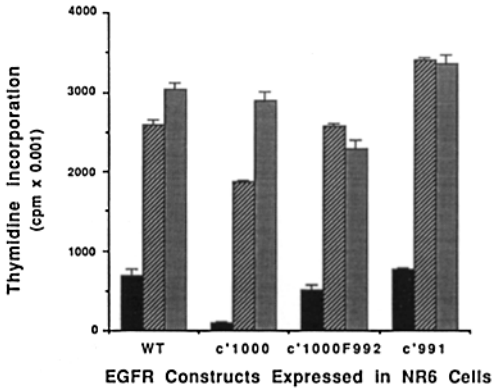


Figure 7. Effect of U73122 on EGF-induced [^{3}H]thymidine incorporation in infectant NR6 cell lines. EGF-induced [^{3}H]thymidine incorporation was determined for each cell line tested in the presence or absence of U73122 (1 μM). Equivalent numbers of cells (~100,000 cells) were tested. The incorporated [^{3}H]thymidine is expressed as actual scintillation counts. ■, basal incorporation with no treatment; ▨, EGF-stimulated incorporation (25 nM EGF); ▩, stimulated incorporation in the presence of U73122. Shown are mean ± SD for three determinations.

structs which exhibited exo-kinase activity elicited mitogenesis, low dose D-actinomycin blocked motility but did not affect EGF-induced mitogenesis, and mitomycin-C treatment abrogated proliferation but spared the ligand-induced motility. The point of divergence of these two EGFR-mediated signaling pathways is unknown. That augmented PLC activity was observed only in the cells expressing autophosphorylatable EGFR (Table I) suggested that the separation of the pathways may occur at the immediate postreceptor level. To further establish the divergence of signals, we assessed EGF-induced mitogenesis in the presence of U73122 (Fig. 7) and in infectant sublines expressing PLCz (Fig. 8). EGF-induced mitogenesis was determined by incorporation of [^{3}H]thymidine. At concentrations of U73122 (1 μM) that inhibit EGF-induced PLC activity and cell motility, thymidine incorporation was not diminished regardless of the autophosphorylation status of the expressed EGFR. In the c'1000PLCz and c'1186F3PLCz sublines, [^{3}H]thymidine incorporation, if anything, was increased, not decreased. Thus, disruption of EGFR-mediated PLCγ activity and blockage of induced cell movement does not negatively affect the mitogenic response.

Activation of MAP Kinase Is Not Sufficient to Elicit EGF-induced Cell Motility

Activation of the MAP kinase cascade has been implicated in linking receptor tyrosine kinases to numerous biological responses including mitogenesis and differentiation (15, 28). EGFR-mediated signals activate MAP kinase; this activation, however, can be accomplished by EGFR in which the five mapped autophosphorylation sites have been replaced by phenylalanines (16). We have shown that EGF-induced motility response requires phospho-tyrosine motifs in the receptor (11). These findings suggested that activation of MAP kinase pathway is not sufficient to elicit cell motility.

To demonstrate that the MAP kinase activation is not sufficient for signaling cell movement, we determined the EGF-induced MAP kinase activity in NR6 cells expressing both motogenic (WT and c'1000) and nonmotogenic (c'1000F^{992} and c'973) EGFR constructs. EGF-stimulated phosphorylation of MBP was similar in cells expressing WT and c'973 EGFR constructs (Figs. 9 and 10). Augmented MAP kinase activity was mirrored by EGF-induced tyrosyl phosphorylation of p42 MAP kinase. These findings confirm that the ability to trigger MAP kinase by an EGFR lacking the carboxy-terminal region is intrinsic to the receptor. Thus, activation of MAP kinase pathway correlates with EGF-induced mitogenesis, but this activation is not sufficient to elicit enhanced motogenesis.

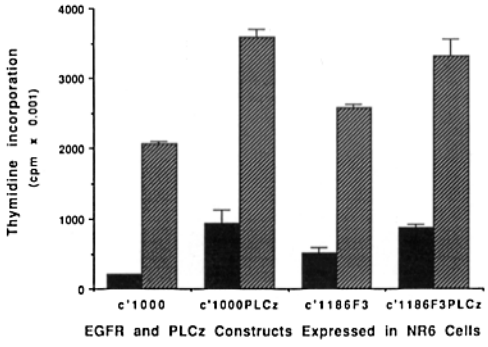


Figure 8. Effect of expressing dominant negative PLCz on EGF-induced [^{3}H]thymidine incorporation in infectant NR6 cell lines. EGF-induced [^{3}H]thymidine incorporation in three mutant EGFR cell lines with or without the expression of PLCz. Equivalent numbers of cells (~100,000 cells) in each cell line were tested. The incorporated [^{3}H]thymidine is expressed as actual scintillation counts. Solid bars depict basal incorporation with no treatment; hatched bars are EGF-stimulated incorporation (25 nM EGF). Shown are mean ± SD for six determinations.

Discussion

We demonstrated previously that EGF-induced cell motility requires the presence of a phospho-tyrosine motif in the intracellular regulatory region of the EGFR (11). This suggests that the immediate downstream effector molecule in the motogenic pathway was activated by SH2 domain interactions. Numerous SH2-containing effector molecules interact with, and are activated by EGFR (9, 37, 40, 45, 49, 55). At least three of these pathways can be linked to cell motility. Activation of small GTP-binding proteins of the *rho* subfamily leads to formation of focal adhesions (34), which is con-

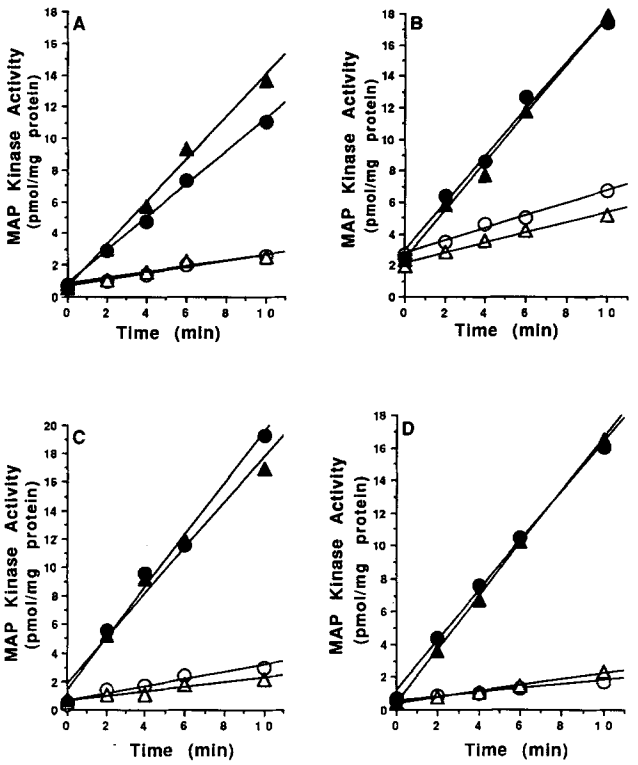


Figure 9. EGF-induced MAP kinase activity in infectant NR6 cell lines expressing mutant EGF receptors. MAP kinase activity in (*A*) WT, (*B*) c'973, (*C*) c'1000, and (*D*) c'1000F^{992} was measured as phosphorylation of the MAP kinase specific substrate MBP by cytosolic extract and expressed as picomoles ATP incorporated/mg protein in cell extract. The effect of U73122 (1 μM) was also tested in parallel. ○, basal activity observed in non-EGF-treated cells; ●, activity observed with 25 nM EGF treatment; △, activity observed in cells treated with U73122; ▲, EGF-induced activity observed in cells treated with U73122. *A–D* are of representative analyses.

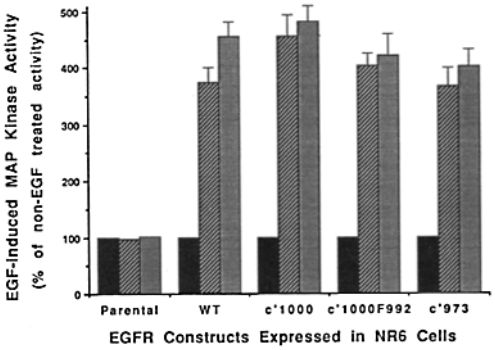


Figure 10. EGF-induced MAP kinase activity. Stimulation of MAP kinase activity was demonstrated by the increase in the rate of MBP phosphorylation. This is expressed as percent of the MAP kinase activity observed in non-EGF-treated cells. The effect of U73122 (1 μM) treatment on EGF-induced MAP kinase activity also is expressed as percent of basal MAP kinase activity. ■, basal activity observed in non-EGF-treated cells; ▨, activity observed with 25 nM, activity observed with 25 nM EGF treatment; ▩, EGF-induced MAP kinase activity in the presence of U73122. Values are mean ± SD; n = 2-4 for each cell line tested.

sistent with the conclusion that activation of GAP by the PDGFβ receptor inhibits cell movement (25). Recent studies have demonstrated that phosphatidylinositol 3′ kinase activation is required for chemotaxis signaled via the PDGFβ receptor (25, 54). A third signaling pathway which involves PLCγ may promote cell motility. PLC hydrolysis of PIP_2 releases actin-severing and -sequestering proteins which lead to the dissolution of stress fibers and focal adhesions, enabling a cell to move (2, 19, 44). In addition, activation of PLCγ has been shown to be required for (25, 54) or associated with (8) PDGFβ receptor-mediated chemotaxis. The specific intermediary effector molecules in the motogenic pathway have not been investigated in other receptors with intrinsic tyrosine kinase activity.

We investigated the necessity of PLCγ activation in EGFR-mediated motogenesis. We examined a series of cell lines expressing genetically engineered EGFR for EGF-induced PLC activity. Activation of PLCγ by EGFR was determined by a functional assay because EGFR may phosphorylate PLCγ without activating it (unpublished observations), presumably by phosphorylation of non-activating tyrosines (24), and PLCγ may be activated non-enzymatically by EGFR (22). Enhanced inositol phosphate production was observed only in the cell lines which demonstrate EGFR-mediated cell movement (Table I). EGF enhanced PLCγ activity in all cell lines expressing autophosphorylated EGFR, further demonstrating the promiscuity of SH2 domain interactions with EGFR phospho-tyrosine motifs. EGF-induced cell movement was noted only in cells which also were responsive by PLC activity. This correlation between the biochemical and biologic responses suggested that PLCγ was the immediate downstream effector.

Specific inhibition of PLCγ was required to demonstrate a causal relationship between this enzyme and cell motility. A specific inhibitor of phospholipase C activity, U73122, was employed to determine whether inhibition of PLCγ would also block induced cell movement. Concurrent exposure of the cells to EGF and U73122 diminished both PLC activity and cell movement. The drug had little effect on the basal rates of either parameter. This agent inhibits all PLC isoforms, and may have some activity towards other phospholipases (32). To diminish the activation of PLCγ by EGFR, a dominant-negative PLC fragment, consisting of the Z region (23), was expressing in select infectant lines. EGF-induction of both PLC activity and cell motility was decreased in these cells. As this fragment is postulated to exert its inhibitory effect by binding to receptor phospho-tyrosine motifs, it is possible that other, non-PLCγ SH2-mediated interactions also are interrupted. The U73122 data points to a phospholipase and the PLCz data indicate a SH2 domain containing molecule as being required for induced cell motility; PLCγ is the only candidate which fulfills both parameters. However, to definitively identify PLCγ as a required intermediary, we down-regulated this enzyme activity by anti-sense oligonucleotides. Cells expressing the WT EGFR were exposed to anti-sense oligonucleotides directed towards two distinct regions of PLCγ. This treatment partially abrogated both PLC activity and motility responses in parallel (Fig. 6). Thus, PLCγ is required for EGFR-mediated movement. These experiments place PLCγ directly downstream of the EGFR.

These findings do not imply that other pathways are not necessary for the full motility response. Kundra and colleagues (25) present evidence that both PLCγ and PI-3′ kinase are required for PDGF-BB-induced chemotaxis through a collagen matrix. However, chemotaxis in such an assay results from a number of cell phenotypes, of which cell movement is just one. Therefore, it is not certain that cell movement in itself requires other immediate effector molecules to be activated by the receptor. In addition, certain other signaling pathways may modulate the motility response. Activation of GAP (25) or protein kinase C (PKC) (unpublished observations) limit cell migration; presumably through modulation of net cell adhesiveness. To determine which other signaling pathways, if any, are required to induce cell movement will need similar analyses demonstrating both positive and negative correlations.

EGFR-mediated cell motility is separable from mitogenesis (4, 11), but the level at which the signaling pathways diverge is undefined. PLCγ activation is not required for EGF-induced mitogenesis as EGFR constructs which do not activate PLCγ can elicit the mitogenic response (16, 48, 52). We now show that inhibition of PLCγ activity by U73122 or PLCz does not lead to a reduction in the mitogenic response. In fact, in the PLCγ-responsive lines, we note a slight but consistent increase in thymidine incorporation in the presence of U73122 (Fig. 7) and in the cells expressing PLCz (Fig. 8). This may be due to abrogation of feedback inhibition of EGFR signaling by PKC (53); a negative regulatory loop in which PLCγ or PLD (unpublished observations) generates diacylglycerol which then activates PKC. EGFR constructs which are resistant to PKC inhibition (53) are being expressed on NR6 cells to test this postulate. Though these findings do not eliminate the possibility of redundant parallel pathways in signaling mitogenesis as seen with the PDGF receptor (47), they strongly support a divergence of the mitogenic and motogenic signaling pathways by differential activation by the EGFR itself.

We are attempting to define further downstream effector molecules in the motogenic pathway. Signaling through EGFR and related tyrosine kinases activates the MAP ki-

nase. This molecule occupies a central station in transducing signals from the extracellular milieu to the nucleus. In addition, MAP kinase elicits numerous cellular responses without involving transcription (28, 31). We sought to determine if activation of MAP kinase was sufficient to elicit cell motility. In the infectant NR6 cell line which expresses the nonmotogenic c'1000F^{992} and c'973 EGFR, EGF exposure increased MAP kinase activity (assessed by MBP phosphorylation) equivalently to the increase seen in WT EGFR-expressing cells (Fig. 9). This finding is in agreement with published results using another non-autophosphorylated EGFR in 3T3 cells (16). We can not determine if MAP kinase is necessary for EGFR-mediated cell motility, either in a parallel pathway or as being permissive for cell movement, due to the lack of nontoxic inhibitors and suitably signaling-restricted EGFR constructs. However, activation of MAP kinase is not sufficient, in itself, to elicit the motogenic response. It is likely that MAP kinase is not involved directly in the EGFR-mediated motogenic signaling pathway, but rather in the mitogenic or other signaling pathways.

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