# Cell Movement Elicited by Epidermal Growth Factor Receptor Requires Kinase and Autophosphorylation but Is Separable from Mitogenesis

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***Abstract.*** The EGF receptor (EGFR) upon activation signals increased cell movement. However, the domains within the receptor, and the pathway which trigger movement are undefined. We expressed EGFR mutants at physiologic levels in receptor-devoid NR6 cells to investigate this biologic response. The receptors possessed kinase activity and underwent autophosphorylation as predicted by primary amino acid sequence. EGF-induced cell motility was assessed *in vitro* by excess migration into an acellular area and colony scatter in the presence of saturating concentrations of EGF.

Wild-type (WT)-EGFR signaled increased motility. However, replacing the conserved lysine$^{721}$ with methionine resulted in a kinase-inactive receptor which did not elicit movement. Removal of the entire terminus by truncation (c′973) also abrogated ligand-induced motility. Thus, we concentrated on the carboxy-terminal domains. EGF-induced movement was seen with a less-truncated mutant (c′1000) that contained a single autophosphorylated tyrosine (tyrosine$^{992}$). Other mutants, c′991 and c′1000F$^{992}$, in which this tyrosine was removed did not signal motility. Fusion mutants which presented other autophosphorylated tyrosine domains also exhibited EGF-induced movement. These findings suggested that the presence of both an autophosphorylated tyrosine signaling domain and the kinase activity are necessary for this biologic response.

All kinase-positive mutants signaled cell proliferation but only those that contained autophosphorylatable tyrosines induced movement. The motility responses mediated by these EGFR were identical in the presence or absence of mitomycin-C, at a dose (0.5 μg/ml) which completely inhibited cell proliferation. On the other side, D-actinomycin (50 ng/ml) blocked EGF-induced motility but did not affect thymidine incorporation. Thus, EGF-induced mitogenesis and cell motility are mediated through different pathways.

ACTIVATION of the epidermal growth factor receptor (EGFR)[1] signals many cellular biological responses ranging from mitogenesis to induction of differentiation (6, 9, 31, 49). The mitogenic activity of EGFR and its transduction as the oncogene v-*erb*B initially evoked interest in its roles in cell proliferation and tumor growth. Increased signaling by either overexpression of wild-type EGFR (17) or expression of down-regulation-defective receptors (32, 51) results in cell transformation and tumor formation. Numerous studies have associated increased levels of EGFR and amplification of its gene with progression of certain *de novo* human neoplasias (19, 27, 36, 37, 55). As tumor progression requires cell phenotypes other than simply dysregulated proliferation, such as invasiveness and motility, it has been speculated that EGFR-stimulated responses other than mitogenesis may be involved. EGFR activation has been shown to elicit cell migration and increased random movement (1, 4, 53). Ligand-stimulation leads to production of extracellular matrix degrading proteases and extracellular matrix constituents (33, 34, 46, 54). The EGFR's role in matrix remodeling and cell migration has widespread implications in organogenesis and tumor invasion and metastasis.

Recent investigations of receptors with intrinsic tyrosine kinase activity have uncovered amino acid motifs crucial for specific signaling pathways and interactions with secondary effector molecules. Much of the non-kinase intracellular region has been deleted in the EGFR with retention of the mitogenic response (16, 51). The EGFR's long carboxyl-terminus has been shown to regulate kinase activity (3, 13, 48), and to include at least five tyrosines which can be autophosphorylated upon ligand stimulation. These phosphotyrosines can associate with the *src* homology-2 (SH2) domains found in numerous second messengers (e.g., PLC-γ, GAP, PI-3 kinase) (8, 28, 30, 42), and through these interactions initiate signaling.

At least two of these signaling pathways can be linked to cell movement. PLC-γ, which associates preferentially with

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1. *Abbreviations used in this paper*: EGFR, epidermal growth factor receptor; WT, wild-type.

$Y^{992}$ on EGFR (10, 42), has been implicated in the pathway responsible for the reorganization of the cytoskeleton. PLC$\gamma$ hydrolyzes $PIP_2$ which leads to the release of profilin, a cytoplasmic actin-binding protein (22). Alternatively, EGFR activation leads to membrane ruffling and reorganization of focal adhesions through activation of members of the *rho* subfamily of GTP-binding proteins (40, 41). These events may be responsible for mediating EGFR effects on cell motility. However, it has not been shown that such interactions are required to elicit this response. EGFR lacking autophosphorylated tyrosines are capable of activating protein kinase C (52) and MAP kinase (15).

In this study, we expressed genetically engineered EGFR mutants on the murine fibroblast cell line NR6, which lacks the endogenous EGF receptors. We investigated the ability of various EGFR to elicit cell motility. These truncation and point mutants isolated a single autophosphorylated tyrosine, $Y^{992}$. Further truncation/fusion mutants which isolated other autophosphorylation sites were examined. Ligand-induced receptor autophosphorylation and tyrosine kinase activity were determined to be required for the biological response of cellular motility. Furthermore, this ligand-induced augmentation of cell motility was separable from mitogenesis.

## Materials and Methods

### Construction of EGFR Mutants

EGFR mutants were constructed by standard methods. The point mutant $M^{721}$ was constructed by replacing $K^{721}$ with a methionine codon (12) by site-directed mutagenesis (26). The mutants c'973, c'991, and c'1000 also were generated by site-directed mutagenesis in which stop codons were encoded after the amino acid number indicated. The point mutant c'$1000F^{992}$ was created from c'1000 by replacing $Y^{992}$ with a phenylalanine codon ($F^{992}$). The fusion mutants c'$958f^{988-996}$, c'$958f^{1146-1154}$, and c'$958f^{1170-1178}$ linked c'958 with fusion segments containing the domains around $Y^{992}$, $Y^{1148}$, and $Y^{1173}$, respectively. This was accomplished by introducing a Sal I site (encodes -V-D-) distal to amino acid 958 by site-directed mutagenesis and recreating the specified sequences by oligonucleotide linkers. Clones were verified by sequencing. Deduced amino acid sequences of the constructs are shown in Fig. 1.

### Stable Expression of EGFR in NR6 Cells

The EGFR were expressed in NR6 cells, 3T3-derivatives which lack endogenous receptors (39). This was accomplished by retrovirus-mediated transduction as previously described (50, 51). The constructs were cloned into the *gag* position of a Moloney MLV-derived vector with an SV40 early promoter-driven neomycin-phosphotransferase gene in the *env* position, as the selectable marker. Plasmid DNA was introduced into an ecotropic packaging cell line, $\psi$ (29), using the lipofectin reagent (GIBCO/BRL, Gaithersburg, MD). Packaged viruses were then used to infect the NR6 mouse fibroblast cells in the presence of 4 $\mu$g/ml of polybrene in growth media (MEM$\alpha$ supplemented with 7.5% FBS). 6 h later, these media were replaced with polybrene-free growth media. 36 h after infection, cells were selected in media containing 350 $\mu$g/ml G418 (GIBCO/BRL). Polyclonal lines consisting of more than 20 colonies were established and maintained in G418 containing media (52). At least two separate infectant lines were generated and subsequently assayed for each construct. In all cases the independent lines presented near-identical EGFR characteristics and biologic responses, and are not distinguished in the results.

### Detection and Characterization of EGFR

Presence of EGFR on the surface of infectant NR6 lines was determined by binding parameters and the ability of EGF to stimulate tyrosine kinase activity and cell proliferation. Scatchard analyses determined the apparent binding sites and affinity for EGF. Cells were grown to confluence in 6-well culture plates (~250,000 cells/well). After incubating 30 min in serum-free media, cells were incubated with 0.1 nM $^{125}$I-labeled EGF and unlabeled EGF (0–100 nM) in binding buffer (DME with 25 mM Hepes, pH7.4, and 0.2% BSA) at 4°C for 2 h. Unbound $^{125}$I-EGF was collected from the supernatant and two washes with binding buffer. The cells were lysed and the bound $^{125}$I-EGF was collected. Free and bound $^{125}$I-EGF were counted and $B_{max}$ and $K_d$ for each cell line were calculated from scatchard plots after subtracting background counts (radioactivity bound in presence of 10–100 nM EGF). All constructs presented monophasic binding graphs.

| EGFR construct | Deduced amino acid sequence[a] |
|---|---|
| WT | ..LVIQGDERMH LPSPTDSNFY RALMDEEDMD DVVDADEYLI PQQGFFSSPS.. |
| c'1000 | ..LVIQGDERMH LPSPTDSNFY RALMDEEDMD DVVDADEYLI PQQGFF* |
| c'$1000F^{992}$ | ..LVIQGDERMH LPSPTDSNFY RALMDEEDMD DVVDADEFLI PQQGFF* |
| c'991 | ..LVIQGDERMH LPSPTDSNFY RALMDEEDMD DVVDADE* |
| c'973 | ..LVIQGDERMH LPSPTDSNF* |
| c'$958f^{988-996}$ | ..LVIQ VD DADEYLIPQ* |
| c'$958f^{1146-1154}$ | ..LVIQ VD PDYQQDFFP* |
| c'$958f^{1170-1178}$ | ..LVIQ VD NAEYLRVAP* |

*Figure 1.* Amino acid sequence of EGFR constructs. The sequences are deduced from sequencing of the cDNA clones. Sequences are shown from $L^{995}$. * denotes a stop codon.

Kinase activity was determined by exposure of 90% confluent cells (~200,000 cells) to EGF (25 nM) for 5 min at 37°C. Cells were lysed directly in SDS-PAGE (polyacrylamide gel electrophoresis) sample buffer under reducing conditions, and proteins size-separated through a 7.5% gel. After transfer to a PVDF membrane, tyrosyl-containing proteins were detected by an antibody which recognizes phosphotyrosine residues (PT03; Oncogene Sciences). Visualization was by a second antibody conjugated to alkaline phosphatase followed by color development (ProtoBlot system; Promega).

Autophosphorylation was determined by immune-precipitation of EGFR after EGF exposure. The cells were lysed in lysis buffer (10% glycerol, 1% Triton X-100, 100 mM NaCl, 20 mM Hepes pH7.4, and 1 mM Na Vanadate) and EGFR were immune-precipitated with an anti-EGFR monoclonal antibody against the extracellular domain (GR01; Oncogene Sciences) followed by protein G-Sepharose 4B. The precipitates were washed under stringent conditions (lysis buffer containing 500 mM NaCl and 0.1% SDS). The immune-precipitates were fractionated through a 7.5% polyacrylamide gel followed by transfer to a PVDF membrane. Anti-phosphotyrosine antibody (PT03; Oncogene Sciences) detected phosphotyrosyl-containing EGFR. The blots were subsequently reprobed with an anti-EGFR antibody (LA22; UBI) to visualize non-phosphorylated EGFR.

EGFR-stimulated proliferation was determined by automated counting of cells after 4 d exposure to saturating concentrations of EGF (10 nM) (51). Briefly, 20,000 cells were plated in a 35-mm dish, let adhere in MEM$\alpha$ with 7.5% serum, and then switched to MEM$\alpha$ supplemented with 1% dialyzed FBS (dFBS) for 48 h before testing. EGF was added and the cells counted 4 d later. Numbers of cells were compared with and without added ligand.

### Cell Migration Assays

EGFR-mediated motility was determined by two methods. In the first, cells were tested for the ability to move into a denuded area on a culture dish (5, 14). NR6 cells were plated on plastic and grown to confluence in MEM$\alpha$ with 7.5% FBS. The cells were then switched to 1% dFBS medium for 24 h. A rubber policeman introduced an acellular area at the center of the plate. The cells were then treated with or without EGF (25 nM) in parallel and incubated at 37°C. Photographs were taken at 0, 12, and 24 h. Cell migration was determined by measuring the distance cells moved into the acellular area over time. This was done by measuring the distance between the two boundaries of cells on the photographs of same magnification. The measurements were taken by two individuals independently on a blinded basis to eliminate subjectivity; difference between the two sets of measurements were less than 5%. Ligand-induced migration was compared to the non-EGF treated (basal) migration and expressed as % of basal migration in each mutant EGFR expressing cell line at each time point. Mitogenesis-independent cell migration was determined in the presence of mitomycin-C (0.5 $\mu$g/ml).

Colony scattering, as a second indicator of induced random cell movement, was adopted from studies of HSF/HGF (5, 21). NR6 cell lines ex-

pressing different EGFR constructs were plated sparsely and maintained in MEMα supplemented with 7.5% FBS. Upon compact colony formation (~200–400 cells per colony), the cells were switched to 1% dFBS medium for 24 h. The cells were then treated with or without EGF (25 nM) and incubated at 37°C. Photographs were taken on preselected colonies at 0, 12, and 24 h. The areas occupied by the same colony at these time points were measured from photographs. The measurements again were taken by two individuals on a blinded basis. Colony scatter was defined as the increase in colony area over time. EGF-induced colony scattering was expressed as % additional increase in colony area as compared to the non-EGF treated (basal) colony scattering. A second series of experiments controlled for ligand-induced mitogenesis by incorporation in the media of mitomycin-C (0.5 μg/ml).

### *[³H]Thymidine Incorporation Assay*

Pharmacological effects of mitomycin-C and D-actinomycin on the EGF-induced mitogenic response were assessed by the ability of cells to incorporate [³H]thymidine. Cells were plated on plastic and grown to confluence in MEMα with 7.5% FBS. The cells were then switched to 1% dFBS medium for 24 h. The cells were subsequently treated with or without EGF (25 nM) and incubated at 37°C for 16 h. [³H]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% TCA at 4°C for 30 min. After two washes with PBS the cells were lysed in 0.2N NaOH and the incorporated [³H]thymidine counted by scintillation counter. The effects of drug treatments were evaluated by [³H]thymidine incorporation assays in the presence or absence of mitomycin-C (0.5 μg/ml) or D-actinomycin (5 μg/ml or 50 ng/ml).

## *Results*

### *Kinase Activity of the Receptor Is Required for EGF-induced Motility*

Wild-type (WT) EGFR were expressed in the NR6 cells (51). A point mutation within the full-length EGFR replaced the ATP-complexing lysine at amino acid 721 with methionine ($M^{721}$) (25). $M^{721}$ did not demonstrate auto- or exo-kinase activity (Table I) (12). These cells were examined for motility in response to EGF (Fig. 2).

Cell movement was determined by two methods. In the first, an area of a monolayer culture was denuded and the relative distance traveled by the cells at the acellular front was determined (14, 45). The absolute migration of each cell line was determined either in the presence or absence of EGF (Fig. 2 *A*). To standardize our assays and account for slight day-to-day variation in the measurement of migration, we calculated the EGF-induced migration as a percent of basal motility observed in the non-EGF exposed cells tested in parallel at each time point. WT-EGFR NR6 cells demonstrated enhanced migration in the presence of saturating concentrations of EGF (125% increase) while $M^{721}$-EGFR expressing cells responded similarly to parental NR6 cells (Fig. 2 *B*).

Increased cell movement also would be reflected in colony scattering (21), which can be quantified by the area described by the outlying cells of the colony. EGF-induced colony scattering was determined by increase in area occupied by individual colonies in the presence of EGF and expressed as % additional increase in area as compared to non-EGF treated colonies tested in parallel at each time point (Fig. 2 *C*). Again, WT- but not $M^{721}$-EGFR expressing cells presented enhanced motility in the presence of saturating level of ligand. This suggested that the kinase activity of the receptor is required in the pathway that leads to the biological response of motility.

### *Tyrosine Autophosphorylation of EGFR Constructs*

Expression of EGFR mutants on transformed B82 cells (10, 11) revealed that the tyrosine residue at position 992 is the only one autophosphorylated proximal to the putative calpain hinge at 1037 (20). Four EGFR (WT, $c'1000$, $c'1000F^{992}$, and $C^{'991}$) which isolated this autophosphorylation site were expressed on NR6 cells. The polyclonal infectant lines demonstrated homogeneous receptor distribution by immunostaining (data not shown) and presented EGFR with normal physiologic binding characteristics (Table I) (7). All four EGFR exhibited EGF-stimulated exo-kinase activity as determined by an increase in intensity and number of cell proteins recognized by anti-phosphotyrosine antibodies (data not shown). Only the WT and $c'1000$ EGFR underwent ligand-induced autophosphorylation. Replacement of tyrosine by phenylalanine at position 992 eliminated autophosphorylation in the $c'1000$-truncated EGFR, though this, and the $c'991$-truncated EGFR retained a tyrosine residue at position 974 (Fig. 1).

Recent findings suggest that individual autophosphorylation domains in the EGFR may substitute for each other in biologic functions and protein–protein interactions (42, 44).

*Table I. Intrinsic Properties of EGFR Constructs Expressed in NR6 Cells*

| Construct | Apparent $B_{max}$* | Apparent $K_d$‡ | NR6 growth‡ | Phosphotyrosyl§ |
|---|---|---|---|---|
| Parental | nd‖ | na‖ | 100 | na |
| WT | 100 | 0.4 nM | 214 ± 36 | +++ |
| $M^{721}$ | 50 | 0.3 nM | 107 ± 5 | – |
| $c'1000$ | 180 | 0.7 nM | 308 ± 78 | + |
| $c'1000F^{992}$ | 140 | 0.4 nM | 352 ± 41 | – |
| $c'991$ | 240 | 0.4 nM | 278 ± 79 | – |
| $c'973$ | 80 | 0.3 nM | 266 ± 43 | – |
| $c'958f^{988-996}$ | 120 | 0.2 nM | 255 ± 11 | + |
| $c'958f^{1146-1154}$ | 260 | 0.4 nM | 260 ± 64 | + |
| $c'958f^{1170-1178}$ | 35 | 0.1 nM | 307 ± 58 | + |

* Properties of the major binding site as determined by "best-fit" calculation after subtracting non-specific binding (sites with dissociation constants, ≤0.1 nM could not be detected); $B_{max}$ is $\times 10^{-3}$.
‡ Induced growth (as determined by cell counting) after 96 h in 10 nM EGF vs no EGF, expressed as % of no EGF treatment; $n$ = 4–15.
§ EGF-induced, as determined by anti-phosphotyrosyl antibody Western blot of immunoprecipitated EGFR.
‖ nd, none detected; na, not applicable.

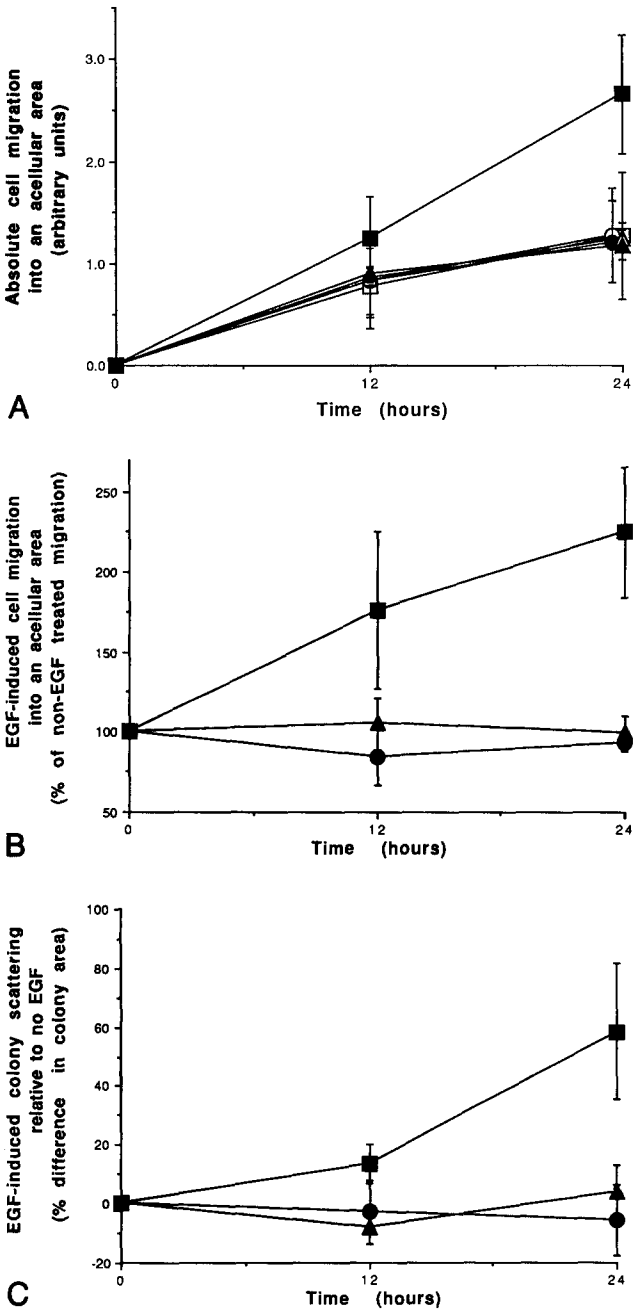


*Figure 2.* EGF-induced cell motility in NR6 cells expressing kinase active (WT) and inactive ($M^{721}$) constructs. (*A*) Migration into an acellular area was measured at different time points from photographs similar to the ones shown in Fig. 3. Absolute migration was determined by measuring the distance between the two boundaries of cells on the photograph. Cells were exposed to EGF or diluent (non-EGF treated) and tested in parallel for each EGFR construct expressing cell line. (*B*) EGF-induced cell migration into the denuded area was calculated as the percentage of the basal (non-EGF treated) migration tested in parallel at each time point. (*C*) Colony scatter was determined similarly. EGF-induced colony scattering was assessed as the difference in percentage increase over non-EGF-treated scattering at each time point. All assays were performed in the presence of mitomycin-C (0.5 μg/ml). Values are mean ± SEM; $n > 4$ for each cell line tested. $p < 0.01$ at 24 h between WT +EGF and WT basal (no EGF treatment) or other cell lines; $p > 0.5$ for all other paired comparisons at 24 h. -■- - WT-expressing NR6 cells, -▲- - $M^{721}$-expressing cells, and -●- - parental NR6 cells. Open symbols in *A* denote cells tested in the absence of EGF, filled symbols are cells in the presence of EGF.

Therefore, fusion mutants were generated which linked the autophosphorylation domains surrounding the tyrosines at 992, 1148, and 1173 to a minimal kinase-positive truncation mutant (11) ($c'958f^{988-996}$, $c'958f^{1146-1154}$, and $c'958f^{1170-1178}$, respectively). These receptors demonstrated the predicted intrinsic properties, such as ligand-induced auto- and exo-kinase activities (Table I). A non-autophosphorylatable truncation mutant (c'973) (11, 51) served as a control receptor being of approximately equal length (973 amino acids vs 969 for the truncation/fusion EGFR).

### *Autophosphorylation at $Y^{992}$ Increases Ligand-induced Cell Movement*

The presence of a tyrosine at position 992 enables the c'1000 EGFR to activate PLC-γ (47), a secondary effector molecule which has been linked to cytoskeletal reorganization (22). It was of interest to determine if EGFR phosphorylated at this tyrosine increased cell movement. In cell migration into an acellular area, significantly increased EGF-induced migration (70 and 125%, respectively, over the untreated) were observed with cells expressing the autophosphorylatable constructs, c'1000 and WT (Figs. 3 and 4 *A*). No ligand-induced augmentation was seen with cells presenting c'991 or $c'1000F^{992}$ EGFR. The NR6 cells expressing the $Y^{992}$ containing constructs, c'1000 and WT, also demonstrated 45 and 60% increases, respectively, in EGF-induced colony scattering as compared to the non-EGF-treated cells (Fig. 4 *B*), while the autophosphorylation-negative mutant cell lines failed to show any EGF effect. These data suggested that EGF-induced cell motility depended on the presence of an autophosphorylatable tyrosine.

### *Cell Movement Is Induced by Other Autophosphorylation Positive Constructs*

The $Y^{992}$ autophosphorylation domain was fused to a minimal kinase-positive truncation mutant ($c'958f^{988-996}$). In parallel, two other truncation/fusion EGFR were constructed representing two of the major in vivo autophosphorylation sites ($c'958f^{1146-1154}$ and $c'958f^{1170-1178}$) (18). These EGFR were used to determine if different autophosphorylation domains could substitute for each other in triggering the motility biologic response. These autophosphorylatable constructs demonstrated 70–130% increases in cell migration into an acellular area and 40–80% increases in colony scattering (Fig. 5). These responses were indistinguishable from those exhibited by cells expressing WT or c'1000 EGFR. The mitogenic, non-autophosphorylated control EGFR, c'973, failed to elicit augmented cell motility; these results were indistinguishable from those derived from the c'991, $c'1000F^{992}$, and parental cell lines. With all the EGFR, induced cell movement segregated with the ability of the receptor to be autophosphorylated.

High basal motility rates may either mask augmentation by EGF or already represent maximal movement. For the NR6 lines which failed to demonstrate EGF-induced enhanced motility, we examined the basal rates of cell movement to determine if these cells possessed anomalously high basal motility. Basal colony scattering and cell migration rates did not differ between the positive and negative EGFR mutants (Table II). Furthermore, the EGF-augmented rates of cells presenting the autophosphorylatable constructs ex-

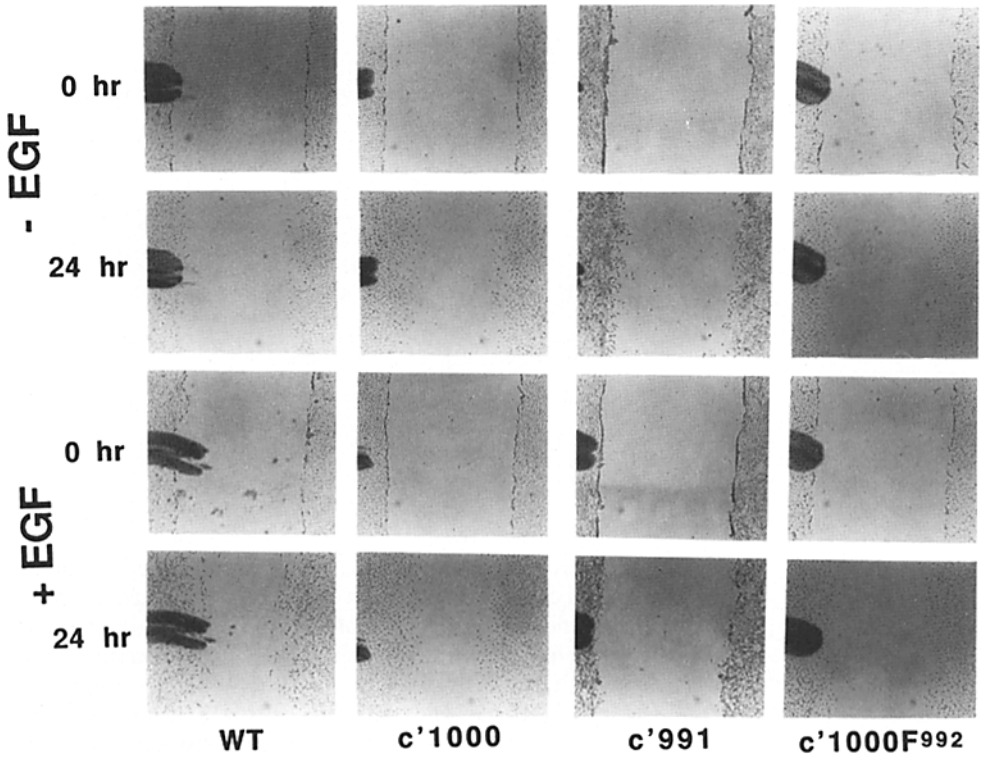


*Figure 3.* EGF-induced migration into an acellular area. Confluent monolayers of NR6 cells were denuded in the center. Cells were treated with or without 25 nM EGF for 24 h, and photographs were taken at different time points. The dark lines on the left side of each photograph marked the plate to ensure that the same spot was photographed and measured. This figure is a representative single experiment with all four cell lines tested simultaneously.

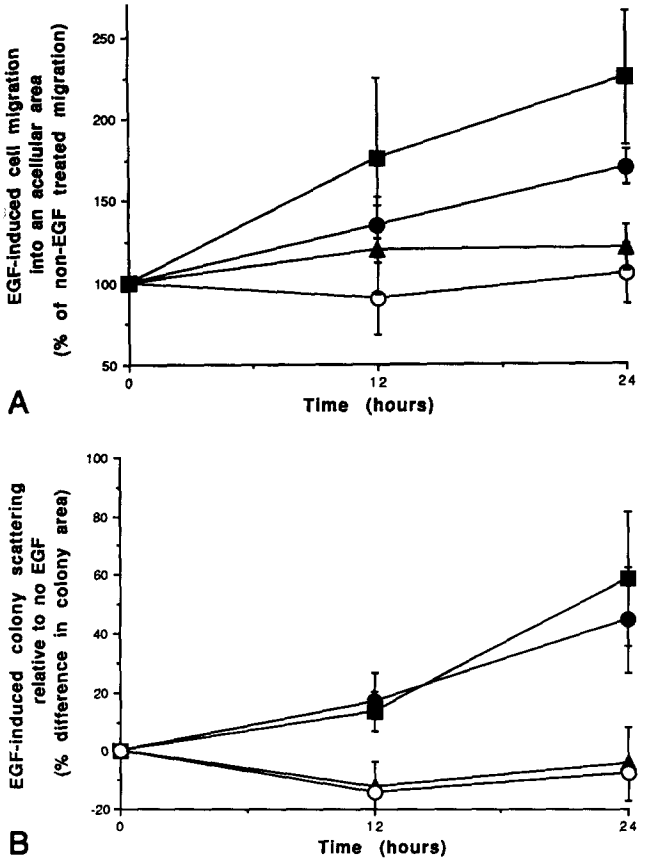


*Figure 4.* EGF-induced motility in NR6 cells expressing EGFR constructs which isolate $Y^{992}$. (*A*) Migration into an acellular area and (*B*) colony scatter were determined as described in Materials and Methods. All assays were performed in the presence of mitomycin-C (0.5 μg/ml). Values are mean ± SEM; $n > 6$ for each cell line tested. -■- WT-expressing NR6 cells, -●- c'1000, -○- c'991, and -▲- $c'1000F^{992}$. $p < 0.01$ between either WT or c'1000, NR6 cells and the non-autophosphorylatable constructs.

ceeded those of the negative EGFR. These findings suggested that the observed increased movement must be ascribed to specific signaling by the EGFR.

Differences in receptor numbers and intrinsic characteristics may affect the extent of the biological responses noted. The ligand-binding affinities of the EGFR fell closely together, being within the narrow range of experimental error (Table I). Numbers of receptors per cell (apparent $B_{max}$) did not correlate with the extent of either inducible or basal cell motility. Furthermore, two different infectant WT lines were compared. These expressed different numbers of EGFR (100,000 and 180,000 EGF-binding sites per cell). These cell lines behaved identically in the cell migration assay (176 ± 49 vs 161 ± 63 at 12 h, and 225 ± 41 vs 229 ± 22 at 24 h). This suggests that the ligand-induced motility is independent of receptor number, at least over the range expressed (Table I). Alternatively, diminished ligand-induced EGFR internalization may enable some mutants to signal longer; this was not the case as all the EGFR displayed similar internalization rates ($k_e$ 0.03–0.05 $sec^{-1}$ at 0.1 nM EGF) except for WT (0.10) and c'973 (0.009). Thus, the ability to signal cell motility is considered to be intrinsic to the various EGFR.

### *Ligand-induced Cell Motility Is Independent of and Separable from Ligand-induced Mitogenesis*

It was unlikely that the observed increases in cell motility, noted by 12 h of EGF exposure, were secondary to EGF's

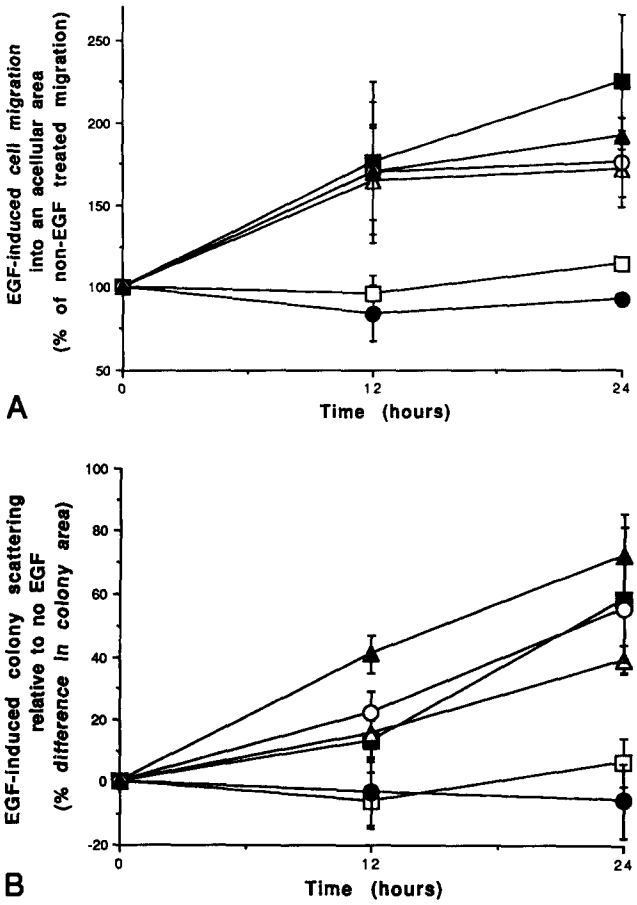


*Figure 5.* EGF-induced cell migration (*A*) and colony scattering (*B*) in NR6 cells expressing autophosphorylatable constructs. Relative rates of migration and increase in colony size were determined as described in Materials and Methods. All assays were performed in the presence of mitomycin-C (0.5 μg/ml). Values are mean ± SEM; $n > 4$ for each cell line tested. -■- WT-expressing NR6 cells, -▲- c'958f$^{1170-1178}$, -△- c'958f$^{1146-1154}$, -○- c'958f$^{988-996}$, -□- c'973, and -●- parental NR6 cells. $p < 0.01$ between any of the NR6 cells lines expressing the autophosphorylatable constructs and parental or c'973-expressing cells.

mitogenic actions. First, all kinase-positive EGFR stimulated mitogenesis (Table I) but not migration. Second, the first wave of EGF-elicited DNA synthesis occurs only after 17–21 h in these cells and cell division after 24 h (data not shown). However, low background growth rates may be accelerated. To ensure that the observed increases in ligand-induced cell motility was not due to EGF's mitogenic effect, ligand-induced mitogenesis of dispersed cells was monitored in parallel with the cell motility assays to determine the correlation of the two events. We compared cell proliferation of the EGF-treated and -untreated cells, and the cell numbers after 24 h were within 20% for all constructs (not significant). Actual enumeration of cells in each colony of the colony scattering assays revealed a <10% discrepancy over the 24 h experimental period.

Motility assays were performed in the presence of 0.5 μg/ml of mitomycin-C to further ensure that EGF-induced mitogenesis did not interfere with our observations in cell motility. At this level of mitomycin-C, the low basal proliferation rate was further reduced and EGF exposure had no effect on cell number. Cells expressing the various EGFR constructs migrated identically in the presence or absence of mitomycin-C (Table III). Thus, the EGF-induced movement was not secondary to the well-described mitogenic actions of this receptor system.

Cell motility and mitogenesis are separable biological events. Actinomycin D has been shown to preferentially block cell migration at concentrations which did not affect mitogenesis (2, 23). To further differentiate the EGF-induced cell migration response from mitogenesis, we treated NR6 cells with two dosages (50 ng/ml and 5 μg/ml) of D-actinomycin. We assessed their effects on EGF-induced [$^3$H]thymidine incorporation as well as the EGF-induced motility. EGF-induced [$^3$H]thymidine incorporation was not

*Table III. Cell Motility in the Presence or Absence of 0.5 μg/ml Mitomycin-C Treatment*

| | Colony scatter* | | Cell migration‡ | |
|---|---|---|---|---|
| Mitomycin-C: | −§ | +‖ | −¶ | +** |
| Construct | | | | |
| WT | 65 ± 26 | 59 ± 23 | 211 | 225 ± 41 |
| c'991 | −16 ± 1 | −8 ± 37 | 89 | 105 ± 18 |
| c'973 | 7 ± 9 | 7 ± 8 | 118 | 114 ± 4 |
| c'1000 | 31 ± 5 | 45 ± 18 | nd‡‡ | |
| c'1000F$^{992}$ | 8 ± 5 | −4 ± 13 | nd | |

* Expressed as percentage increase over non-EGF treated colony scatter.
‡ Expressed as percentage of non-EGF treated migration.
§ $n = 3$.
‖ $n = 4$–6.
¶ $n = 1$.
** $n = 4$–12.
‡‡ nd, not done.

*Table II. Basal and EGF-induced Cell Motility*

| | Cell migration* | | | | Colony scatter‡ | | |
|---|---|---|---|---|---|---|---|
| | Basal distance | | EGF-induced | | Basal rate | | EGF-induced |
| Autophosphorylation | | | | | | | |
| Positive constructs§ | 1.6 ± 0.3 | ** | 2.9 ± 0.4 | ** | 129 ± 8 | ** | 176 ± 8 |
| Negative constructs‖ | 1.9 ± 0.3 | ¶ | 2.0 ± 0.4 | | 155 ± 7 | ¶ | 148 ± 14 |

* Absolute cell migration after 24 h, in arbitrary units.
‡ Percent of original colony area at time 0.
§ Mean ± SEM of mean values of NR6 cells expressing WT, c'1000, c'958f$^{1170-1178}$, c'958f$^{1146-1154}$, and c'958f$^{988-996}$.
‖ Mean ± SEM of mean values of parental NR6 cells and those expressing c'1000F$^{992}$, c'991, and c'973.
¶ $p > 0.3$, ** $p \leq 0.01$.

*Table IV. Effect of D-actinomycin on EGF-induced Cell Motility*

| | Cell migration* | | |
|---|---|---|---|
| | no D-actinomycin‡ | | 50 ng/ml D-actinomycin§ |
| Construct | | | |
| WT | 225 ± 41 | ‖ | 117 ± 13 |
| c'1000 | 170 ± 11 | ‖ | 103 ± 19 |
| c'1000F$^{992}$ | 121 ± 14 | ¶ | 109 ± 13 |
| c'991 | 105 ± 18 | ¶ | 103 ± 33 |

* EGF-induced migration after 24 h of 25 nM EGF treatment; expressed as percentage of non-EGF-treated cells, mean ± SEM.
‡ $n$ = 4–12.
§ $n$ = 3.
‖ $p < 0.01$; ¶ $p > 0.3$; there were no statistically significant differences between the various lines in the presence of D-actinomycin.

blocked by low dose (50 ng/ml) D-actinomycin. In the presence or absence of the drug at this low dose, EGF elicited the same sevenfold increase in [$^3$H]thymidine incorporation over non-EGF-treated cells ($n$ = 3 cell lines: 7.5× in the presence of D-actinomycin, 6.3× in its absence), with no difference in the basal incorporation between the drug-treated and non-treated cells. However, high dose (5 μg/ml) D-actinomycin inhibited the basal and EGF-induced incorporations. Treated cells showed only 1% incorporation of the non-drug-treated cells and did not respond to EGF exposure ($n$ = 3 cell lines).

D-actinomycin, at either dose, blocked the EGF-induced cell migration and inhibited the basal level of movement (Table IV). Mitomycin-C spared this biological response. These findings indicate that the EGF-induced mitogenesis and cell motility have different sensitivity to DNA polymerase and RNA polymerase blockade and may require different levels of transcription.

## Discussion

EGF induces cell migration in certain receptor-bearing cells (1, 4, 53). However, the nature of this signal is undefined. Recent advances suggest SH2-mediated associations may initiate signaling pathways leading to migration (22, 40, 41, 47). However, other signals and biologic responses do not require intrinsic EGFR-mediated SH2 interactions (15, 52). To dissect the roles of autophosphorylation sites in the carboxy terminus of EGFR in mediating the cellular biological response of cell motility, we have constructed mutants containing various autophosphorylation sites. These mutants demonstrate the expected auto- and exo-kinase properties, even when the particular domains were relocated from distal sites and juxtaposed onto the end of the kinase domain. This suggests that the ability of a particular sequence in the EGFR to be autophosphorylated is dependent on the immediate context of the tyrosine residue. The biological responses that are modulated by autophosphorylation events also may be somewhat promiscuous. Thus, individual phosphotyrosines may substitute for each other in signaling interactions, as has been suggested for the situation involving EGFR Y$^{992}$ and PLC-γ (42, 44).

Herein, we demonstrated that EGF induces cell movement only through EGFR-containing phosphorylatable tyrosine residues. Initially this was noted with EGFR which isolated Y$^{992}$ by truncation of the receptor (c'1000). This receptor is fully capable of mobilizing intracellular calcium (10) and activating PLC-γ (47). c'991 and c'1000F$^{992}$ EGFR, both of which contain a non-phosphorylated tyrosine (Y$^{974}$), did not stimulate cell movement. To determine whether this cellular response is specific for the Y$^{992}$ domain, fusion mutants were constructed to present other single autophosphorylation sites (c'958f$^{1146-1154}$ and c'958f$^{1170-1178}$) (18). These EGFR also augmented cell motility in a ligand-dependent manner. The results further support the contention that phosphotyrosine-mediated interactions may be somewhat promiscuous, allowing similar SH2-binding motifs to substitute for each other (38, 42–44). In addition, the position of these autophosphorylatable tyrosines in relation to the kinase domain may not be critical in mediating the biological response of cell motility since the c'1000 mutant and the fusion mutants stimulated cell movement. Designation of immediate effector molecules which lead to the motility response may require point-mutational analyses in the background of the wild-type receptor.

These data do not demonstrate that the tyrosines must be phosphorylated for motility to be signaled. Replacement of a tyrosine by phenylalanine (e.g., Y$^{992}$ > F$^{992}$) not only prevents phosphorylation, but may interrupt tyrosine-mediated interactions. Investigations have shown weak, but significant, interactions between SH2 domains and unphosphorylated tyrosines (35). However, the presence of these SH2 tyrosine motifs in a kinase-defective EGFR (M$^{721}$) was not sufficient to mediate EGF-induced movement. As the EGFR requires kinase activity to trigger downstream biologic responses (12, 24), it will be difficult to settle this point until the next link in this pathway is identified.

Increased motility was independent of cell proliferation. All the constructs exhibited exo-kinase and elicited mitogenesis in an EGF-dose dependent manner (Table I). NR6 cells expressing c'973 EGFR are more sensitive to ligand-induced proliferation, cell transformation, and tumor promotion (32, 51). However, these cells, and others which presented non-phosphorylatable EGFR, did not demonstrate EGF-enhanced cell movement. In the cell lines which responded to EGF, movement was noted before the onset of DNA synthesis and cell division. Addition of mitomycin-C in concentrations which prevented entry into S-phase, did not affect EGF-induced motility. Contrarily, low dose D-actinomycin inhibited EGF-induced cell motility but spared the ability to incorporate thymidine. In sum, certain EGFR constructs signaled mitogenesis without promoting movement, and an inhibitor of RNA synthesis blocked motility at concentrations which did not effect mitogenesis; and mitogenesis was prevented without affecting migration. Thus, EGF stimulation of cell migration was separable from the mitogenic signal.

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