A Role for Gelsolin in Actuating Epidermal Growth Factor Receptor–mediated Cell Motility

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Abstract. Phospholipase C-γ (PLCγ) is required for EGF-induced motility (Chen, P., H. Xie, M.C. Sekar, K.B. Gupta, and A. Wells. *J. Cell Biol.* 1994. 127:847–857); however, the molecular basis of how PLCγ modulates the actin filament network underlying cell motility remains undetermined. We propose that one connection to the actin cytoskeleton is direct hydrolysis of PIP$_2$ with subsequent mobilization of membrane-associated actin modifying proteins. We used signaling-restricted EGFR mutants expressed in receptor-devoid NR6 fibroblast cells to investigate whether EGFR activation of PLC causes gelsolin mobilization from the cell membrane in vivo and whether this translocation facilitates cell movement.

Gelsolin anti-sense oligonucleotide (20 μM) treatment of NR6 cells expressing the motogenic full-length (WT) and truncated c′1000 EGFR decreased endogenous gelsolin by 30–60%; this resulted in preferential reduction of EGF (25 nM)-induced cell movement by >50% with little effect on the basal motility. As 14 h of EGF stimulation of cells did not increase total cell gelsolin content, we determined whether EGF induced redistribution of gelsolin from the membrane fraction. EGF treatment decreased the gelsolin mass associated with the membrane fraction in motogenic WT and c′1000 EGFR NR6 cells but not in cells expressing the fully mitogenic, but nonmotogenic c′973 EGFR. Blocking PLC activity with the pharmacologic agent U73122 (1 μM) diminished both this mobilization of gelsolin and EGF-induced motility, suggesting that gelsolin mobilization is downstream of PLC. Concomitantly observed was reorganization of submembranous actin filaments correlating directly with PLC activation and gelsolin mobilization. In vivo expression of a peptide that is reported to compete in vitro with gelsolin in binding to PIP$_2$ dramatically increased basal cell motility in NR6 cells expressing either motogenic (WT and c′1000) or nonmotogenic (c′973) EGFR; EGF did not further augment cell motility and gelsolin mobilization. Cells expressing this peptide demonstrated actin reorganization similar to that observed in EGF-treated control cells; the peptide-induced changes were unaffected by U73122. These data suggest that much of the EGF-induced motility and cytoskeletal alterations can be reproduced by displacement of select actin-modifying proteins from a PIP2-bound state. This provides a signaling mechanism for translating cell surface receptor-mediated biochemical reactions to the cell movement machinery.

A CTIVATION of the epidermal growth factor receptor (EGFR) and other receptors with intrinsic tyrosine kinase activity (RPTK) enhances cell motility of many cell types contributing to organ development and wound repair under normal conditions and to tumor invasiveness in pathologic signaling states. Cells achieve movement by extending peripheral lamellipodia which requires actin reorganization at the submembranous localization. However, the translation of receptor-activated biochemical processes into the restructuring of the actin cytoskeleton network remains undetermined.

Phospholipase C-γ (PLCγ)[1] activation is required for EGF- (10) and PDGF-induced (30) motility and has been implicated in IGF-1–induced cell motility (7). The molecular basis of how PLCγ modulates the actin filament network underlying cell motility (52) is not well-understood. PLC activity may promote cell motility secondary to a generation of second messengers or by altering the membrane–cytoskeleton associations. Inositol trisphosphate (IP3) and diacylglycerol (DAG) are produced upon PIP2 hydrolysis. IP3 excites intracellular calcium release channels and causes a transient increase in intracellular calcium

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1. *Abbreviations used in this paper:* DAG, diacylglycerol; EGFR, epidermal growth factor receptor; PLC-γ, phospholipase C-γ; PPI, phosphoinositide.

thereby activating calcium-dependent processes (4). Although it has been controversial as to whether these intracellular calcium transients promote or inhibit actin assembly (3, 15, 33, 51), it is widely accepted that the calcium transients can modulate cell movement by activating proteins involved in cytoskeletal dynamics (22, 26). However, calcium transients have been demonstrated not to be required for cell motility under all conditions (19, 24, 46). DAG is a potent activator of members of the ser-thr kinase PKC family and exerts most of its effects through these kinases. Activation of PKC causes phosphorylation and activation of numerous regulators of the actin-cytoskeleton (1, 20, 39, 48). The effects on cell motility, however, are pleiotropic and seem to be tissue-, cell-, and isotype specific (2, 12, 62). Independent of PKC activation, DAG itself has also been shown to enhance actin filament assembly by increasing de novo formation of action nucleation sites (47). Alternatively, activated PLCγ initiates rapid turnover of polyphosphoinositides which can affect the distribution and activities of actin-modulating proteins such as profilin, CapG, and gelsolin (18, 28, 29, 32, 53, 64).

A number of proteins that modulate actin cytoskeleton organization and connection to the membrane can bind to PIP2. Gelsolin can sequester and nucleate monomeric G-actin as well as cap and sever polymerized F-actin; these functions provide a central role for gelsolin in controlling cell movement (11, 52, 54, 61). Gelsolin has been demonstrated to bind to PIP2 (29), the substrate for PLCγ. The phosphoinositide (PPI)-binding motifs in these proteins are well-conserved (50, 63, 64). Upon PPI binding, the F-actin severing capacity of gelsolin family members is inhibited (28, 29, 56, 64) presumably due to the interference with the two actin-binding sites in close proximity (28). In addition, peptides derived from these PIP2-binding motifs can compete gelsolin off PIP2-containing lipid micelles in vitro (28). Binding of PIP2 to profilin, another actin-modifying protein, has been shown to inhibit hydrolysis of PIP2 by PLCγ-1. However, EGFR-activated PLCγ-1 can overcome this inhibition, hydrolyze PIP2, and release profilin (18). The released profilin and gelsolin from PIP2-containing lipid micelles regain their actin-modulating capacity (17, 18, 64). These biochemical events suggest a role for gelsolin and other PIP2-binding molecules in vivo to connect the RPTK-activated phospholipid cycle to actin remodeling. The implication of gelsolin's role in modulating cell motility comes from experiments that regulate in vivo expression of gelsolin. Cells lacking gelsolin demonstrate reduced motility while overexpression of gelsolin increases cell movement (11, 61).

In this study, we provide evidence that at least part of EGF receptor–mediated motility is actuated by gelsolin. Down-regulation of gelsolin in receptor-expressing NR6 fibroblasts abrogates EGF-induced motility. We used signaling-restricted EGF receptors to decipher the signaling mechanisms that translate receptor signaling into actin-cytoskeleton reorganization required for cell movement. Only those EGF receptors that elicit cell motility caused a PLCγ-dependent dissociation of gelsolin from the plasma membrane. Submembranous actin-cytoskeleton changes were observed in parallel with this dissociation event. Finally, cells expressing a peptide that encodes the PIP2-binding domain of gelsolin demonstrated cytoskeletal changes similar to those observed in the presence of EGF. The expression of this peptide also increased cell motility, replacing the EGF-induced motility response.

Materials And Methods

Expression of Signaling-restricted EGFR in NR6 Cells

Design and generation of the EGFR constructs and stable expression in NR6 cells were by standard methods, and have been described previously (9, 58). WT EGFR is a full-length cDNA (55) derived from a placental cDNA library (59). c'973 and c'1000 represent EGFR in which stop codons are introduced just distal to the amino acid number indicated. All three EGFR constructs present ligand-activated kinase and signal mitogenesis but only WT and c'1000 promote cell motility (9).

The constructs were expressed on NR6 cells, 3T3 derivatives that lack endogenous receptors (41). This was accomplished by retrovirus-mediated transduction as previously described (57). 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 0.2–0.7nM).

Cell Migration Assay

EGF-induced migration was assessed by the ability of the cells to move into an acellular area as previously described (9, 10). 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.

In the anti-sense oligonucleotide experiments, cells were treated for 14 h with or without a 21-mer oligonucleotide complementary to the transcription start site (5'thio-CTCCACCACCATAGTGCTGG-thio-G3') of the mouse gelsolin coding sequence (13). A scrambled 21-mer oligonucleotide (5'thio-CGGAGGAAGAAGATTGCCCTG-thio-3') controlled for specificity of gelsolin down-regulation. Oligonucleotides (20 μM) were dissolved in culture medium and introduced 8 h before the addition of EGF (25 nM) and remained present through the entire assay period.

In PLC inhibition assays, the pharmacological agents, U73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H- pyrrole-2,5-dione) and its inactive congener, U73343 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2 ,5-pyrrolidine-dione), were added to the cells to inhibit PLC activity (10). U73122 is a relatively specific inhibitor for most PLC species (5, 10, 49) but does not inhibit PLA isoforms or PLD (our unpublished data). These compounds were introduced at 1 μM into the media 15 min before the addition of EGF.

Western Blot Analyses

In anti-sense oligonucleotide experiments, 3 × 10⁵ cells were treated for 14 h with or without the 21-mer oligonucleotide complementary to the transcription start site of the mouse gelsolin coding sequence (see above). Total cell lysates were loaded in each lane. After PAGE and transfer to a PVDF membrane, two different polyclonal antisera (RaGEL-P, East Acres and the generous gift of D. Kwiatkowski, Harvard University [61]) specific for gelsolin detected the cellular 87-kD form of gelsolin. This was visualized with an AP- or HRP-conjugated secondary antibody followed by development with colorimetric (Promega Corp., Madison, WI) or ECL (Amersham Corp., Arlington Heights, IL) methods.

To determine whether EGF induces gelsolin levels, 3 × 10⁵ cells were treated for 14 h with or without EGF (25 nM) before probing for gelsolin as above.

Plasma Membrane Isolation

Plasma membrane fractions were isolated using previously described methods (21). Cells were swollen with a hypotonic buffer and mechani-

cally disrupted. Plasma membranes were obtained by differential density centrifugation. PMSF (1 mM), leupeptin (200 μM), bestatin (20 μM), aprotinin (5 μg/ml), pepstatin A (2 μM), and methylamine chloride (30 nM) were present in all solutions throughout the isolation procedures to prevent protein degradation and the entire procedure (~5 h) was performed on ice or at 4°C. The isolated plasma membrane fraction were equalized for cell number before loading on SDS-acrylamide gel and protein content in each fraction were determined by Bradford method. In addition, actin content in each membrane fraction was determined by immunoblotting with anti-actin antibodies (A2066; Sigma Chemical Co., St. Louis, MO). The amount of gelsolin present in the membrane was determined by immunoblotting described above.

Actin Filament Staining

Cells were plated on coverslips and quiesced in 1% dialyzed FBS for 24 h. After treatment, cells were fixed for 20 min with 3% paraformaldehyde in PBS followed by two washes with PBS. Cells were stained for 30 min with 2.0 U/100 μl rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR). After two washes with PBS, coverslips were mounted on slides and cells were examined microscopically and photographed using a (Axiovert 10; Carl Zeiss, Inc., Thornwood, NY) microscope equipped for phase and epifluorescence. In inhibition studies, U73122 was added 15 min before addition of EGF.

Expression of the Peptide Encoding PIP2-binding Sequence of Gelsolin

cDNA encoding the amino acid sequence corresponding to PIP2-binding region of murine gelsolin (ATGCAGAGACTCTTCCAGGTCAAAGG-GCGGCGTGTG) was cloned into the pXf vector for expression (10). A second cDNA fragment (ATGGCCCTCTTCTGGGTCGACGGGCTC-CGTGTG) encoding a peptide of same amino acid length was used as control. Subcloning placed the cDNAs fragment under the control of a SV-40 early promoter present in the pXf vector. A DHFR gene transcribed from a second SV-40 early promoter serves as the selectable marker. These expression vectors were transfected using lipofectin reagent (GIBCO BRL) and stable transfectants selected in 400 mM methotrexate.

Results

### Gelsolin Is Required for EGFR-mediated Cell Motility

Cell motility is positively correlated to cellular gelsolin content (11, 61). To determine if EGFR-mediated motility is dependent on gelsolin, we used EGFR-devoid fibroblast cells (NR6) expressing signaling-restricted EGFR (9, 10). A full-length (WT) EGFR and a carboxy-terminal truncated EGFR which contains a single autophosphorylation motif (c'1000) can activate PLCγ and signal cell motility, while a nonautophosphorylated truncated EGFR (c'973) cannot (10). The infectant NR6 cells were exposed to anti-sense oligonucleotides complementary to the translation start site of the murine gelsolin coding sequence. The anti-sense treatment down-regulated endogenous gelsolin levels 30–60% in WT, c'1000, and c'973 EGFR-expressing cells as determined by immunoblotting with quantification by densitometry (Fig. 1 A). A corresponding decrease in EGF-induced cell motility was observed in the treated WT and c'1000 EGFR NR6 cells (Fig. 1 B). Basal cell motility was only marginally affected. A scrambled oligonucleotide served as a control in half the experiments, and did not affect endogenous gelsolin expression or cell motility response. The anti-sense treatment had little effect on cell motility in the nonmotogenic c'973 EGFR-expressing cells. These observations suggest a requirement for gelsolin in EGFR-mediated cell movement.

To determine if EGF enhances cell motility by increasing gelsolin levels, we examined total cell gelsolin content after EGF treatment. We did not note higher levels in NR6 cells expressing either the motogenic WT and c'1000 EGFR or the nonmotogenic c'973 EGFR after 14 h treatment with EGF (Fig. 2). Therefore, it was unlikely that EGF promotes cell motility by increasing cellular gelsolin levels; rather, EGF may induce cell motility by altering gelsolin subcellular distribution or activity.

Gelsolin Dissociates from Plasma Membrane upon PLCγ Activation

In vitro, the activities of various actin modifying proteins are diminished when bound to PIP2 (17, 18, 28, 64). EGFR-phosphorylated PLCγ can cleave PIP2, release these gelsolin and profilin from PIP2-containing lipid micelles, and restore gelsolin F-actin–severing activity (17, 18, 64). To determine whether EGF causes a similar alteration in the membrane-associated of gelsolin in vivo, we
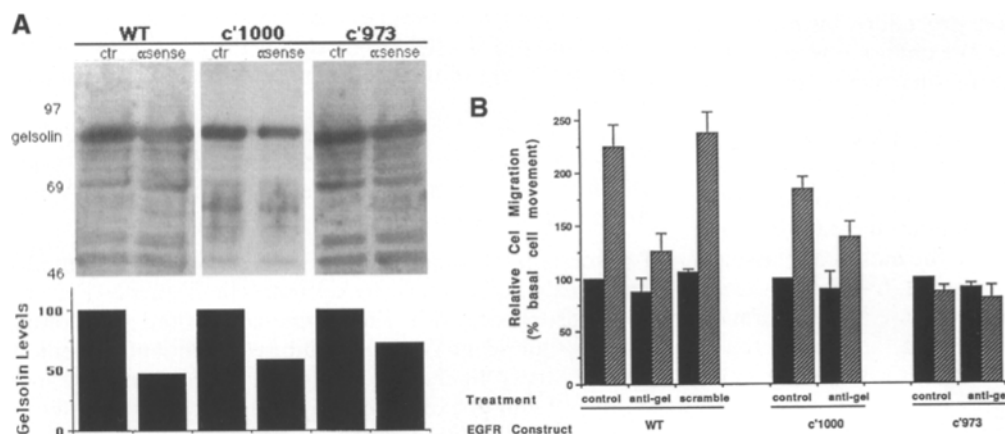
Figure 1. Effects on endogenous gelsolin levels and inhibits EGF-induced cell movement of anti-sense oligonucleotide treatment. (*A*) Composite immunoblot showing endogenous expression of gelsolin in signaling-restricted EGFR-expressing NR6 cells. Shown are representative experiments performed four times with each cell line. Densitometric readings are shown below the immunoblot (calculated as percent of untreated member of each pair). (*B*) In vitro cell movement in EGFR–expressing cells expressed as percent of basal cell movement. Individual experimental points were performed in triplicate; values are mean ± SEM; *n* = 2–4. *P* < 0.03 for EGF-induced motility between control and gelsolin anti-sense oligonucleotide treatment in WT- and c'1000 EGFR–expressing cells. *P* > 0.3 between control and gelsolin anti-sense oligonucleotide treatment in c'973 EGFR expressing–cells. ■, -; ▨, EGF.
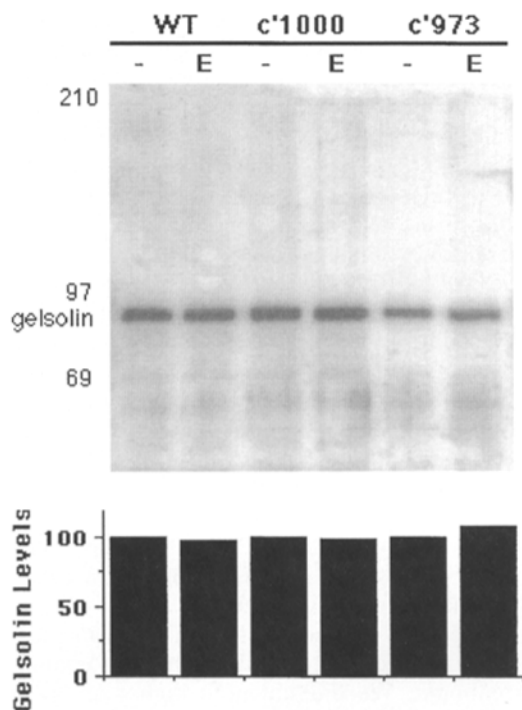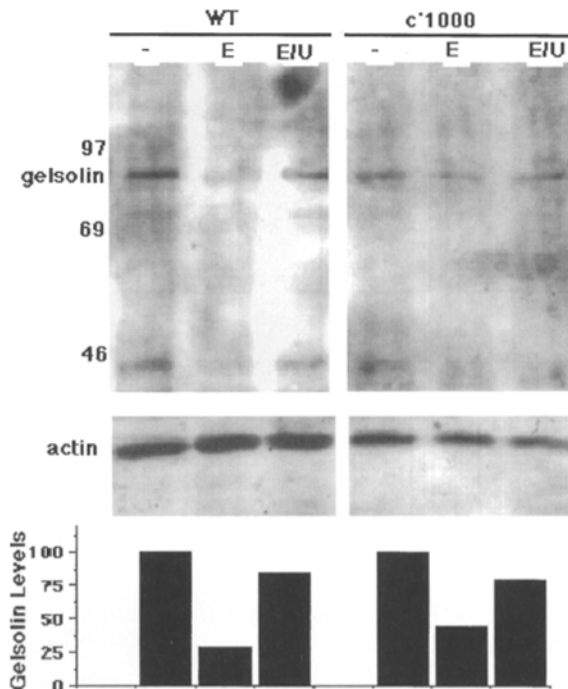
Figure 2. Endogenous gelsolin expression after 14 h treatment with EGF. Shown is one of four experiments with similar results. Densitometric readings for this immunoblot are shown below (calculated as percent of untreated member of each pair).

Figure 3. Effect of EGF and PLC inhibitor U73122 on plasma gelsolin content in cells expressing motility-responsive, PLCγ-activating EGFR. Immunoblot demonstrating membrane-associated gelsolin in WT- and c'1000 EGFR–expressing NR6 cells in the presence (*E*) or absence (-) of EGF or EGF and U73122 (*E/U*). Representative immunoblots are shown; n = 2–4 for each cell line; amounts loaded on gel were equalized for cell number; the differences in protein content of the membrane fraction among various treatments were within 10%. Middle panel shows the immunoblot for actin present in each membrane fraction. Densitometric readings for gelsolin content are shown in the bottom panel (calculated as percent of untreated within each cell line).

isolated plasma membranes from the NR6 sublines. Plasma membranes were isolated by differential centrifugation methods described above. The absence of cytoplasmic contamination of the plasma membrane preparations was demonstrated by lack of both LDH activity and immunoreactivity with anti-pan erk antibodies in parallel membrane fractions (data not shown). Amounts of membrane loaded on the gel were equalized for cell number. Protein content in each fraction was measured by the Bradford method; differences among various treatments were within 10% (data not shown). In addition, we determined the actin content in the membrane fraction to control for variation in membrane isolation procedure by immunoblotting with anti-actin antibody. We did not observe a difference in actin content in the plasma membrane upon EGF treatment (Figs. 3 and 4).

In the presence of EGF, we observed a significant decrease in the gelsolin content of the plasma membrane fraction of cells expressing the motility-responsive WT and c'1000 EGFR (Fig. 3); a concomitant increase in cytosolic gelsolin was not noted because the membrane-associated gelsolin constitutes only a small fraction of total gelsolin (data not shown). This decrease in membrane-associated gelsolin was not observed in the non-motogenic, non-PLCγ activating c'973 EGFR expressing cells (Fig. 4).

If this dissociation of gelsolin from the membrane fraction was due to PLCγ activation, disruption of this signaling pathway should prevent the dissociation. The cells were pretreated with U73122 (15 min), a selective PLC inhibitor (10), before addition of EGF. U73122 inhibited the

EGF-induced decrease in plasma membrane gelsolin content (Fig. 3) and decreased, in parallel, the EGF-induced cell movement in the motility-responsive WT and c'1000 lines (Fig. 5). U73343, an inactive congener for U73122, also was tested in parallel as a control; the results were identical to those observed in cells with no treatment. These findings strongly suggest that PIP2 hydrolysis is important for gelsolin association with the plasma membrane.

EGF-induced Gelsolin Mobilization and Cell Motility Is Accompanied by Reorganization of the Actin Cytoskeleton

To ascertain the functional consequences of EGFR-mediated signals on the actin cytoskeleton, actin filaments (F-actin) were visualized with rhodamine-conjugated phalloidin (Fig. 6). Quiescent WT cells exhibited abundant elongated F-actin stress fibers extending to the cell membrane. Within 10 min of exposure to EGF, the actin cytoskeleton demonstrated reorganization. In the protruded lamellipodia, stress fibers did not extend to the membrane; rather, short actin filaments had formed into submembranous arcs (Fig. 6). These changes persisted throughout 12 h of EGF treatment, corresponding to the time frame over
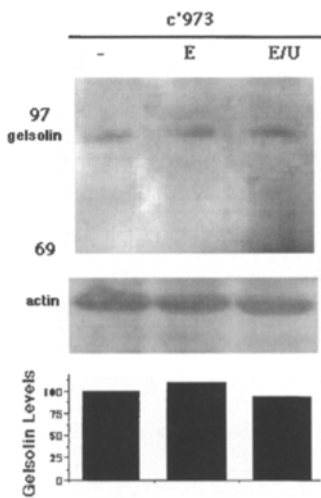
Figure 4. Effect of EGF and PLC inhibitor U73122 on plasma gelsolin content in cells expressing motility non-responsive, non–PLCγ-activating c'973 EGFR. The experimental design and presentation is as per Fig. 3. A representative immunoblot is shown; $n = 4$.

which we measure cell motility (9). Parallel to these submembranous actin arcs were membrane ruffles as observed by phase contrast microscopy (Fig. 7). Cells expressing the nonmotogenic c'973 EGFR demonstrated neither the subterminal foreshortened stress fibers nor the submembranous actin arcs (Fig. 6). The cytoskeletal structure in the c'973 cells reflected EGF-induced lamellipodia retraction previously reported (59).

The cytoskeletal reorganization in WT-expressing cells was dependent on PLCγ activation. U73122 prevented the appearance of EGF-induced lamellipodia protrusions with the submembranous actin arcs (Figs. 6 and 7). However, these cells demonstrated the contracted phenotype. Together, these data demonstrate a causal relationship between EGF-induced PLCγ activation with release of gelsolin from the plasma membrane and remodeling of the actin cytoskeleton in the submembrane region of cell protrusions.
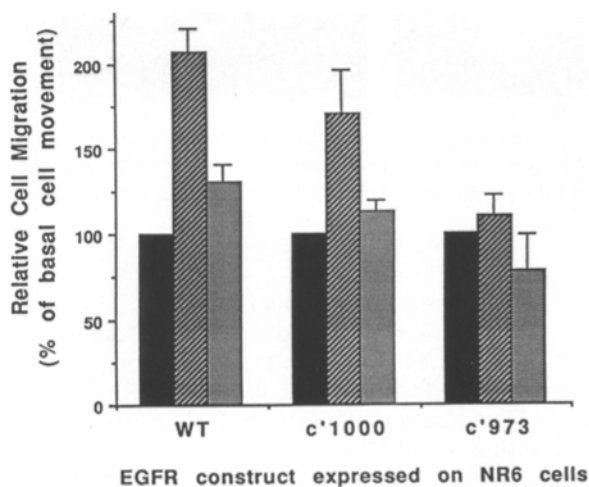
Figure 5. Effect of EGF and PLC inhibitor U73122 on cell motility response in cells expressing EGFR constructs. Individual experimental points were performed in triplicate; values are mean ± SEM; $n = 2$–4. $P < 0.03$ for EGF-induced motility between U73122 treated and nontreated or U73343-treated cells expressing WT and c'1000 EGFR; $P > 0.3$ in cells expressing c'973 EGFR. ■, -, ▨, E, ■, U/E.

Expression of a Peptide Encoding a PIP2-binding Sequence Mimics EGF-induced Gelsolin Dissociation from the Plasma Membrane and the Induced Cell Motility Response

The preceding experiments suggested that PIP2 hydrolysis with release of gelsolin from a membrane association mediates at least part of EGFR-activated motility. However, the observed gelsolin dissociation and cytoskeletal reorganization may be secondary to induced cell motility. A causal relationship between gelsolin translocation and cell motility would be established if direct mobilization of gelsolin resulted in augmented cell motility. Gelsolin binds to PIP2 via motifs in its NH2-terminal domains which are loosely conserved among many actin-modifying proteins (Table I) (28, 64). To test the prediction that release of gelsolin and other actin-modifying proteins from the membrane enhances motility, we expressed such a peptide in the NR6 cells. We transfected a cDNA construct which encodes MRLFQVKGRRV (corresponding to amino acids 135–146 in murine gelsolin [13]) into WT, c'1000, and c'973 EGFR-expressing NR6 cells. A second construct serves as control peptide; MALFWVDGLRV was encoded because it has been demonstrated that both the primary and secondary structure of the peptides are important in PIP2 binding in addition to the electromagnetic interaction among charged molecules (28).

In the WT- and c'1000 EGFR–expressing cells, expression of the competitive peptide augmented the basal cell motility (Fig. 8 *A*). In the c'973 EGFR–expressing cells, expression of the competitive peptide also augmented basal cell movement to a level comparable to that observed in WT or c'1000 cells; the percent increase, however, was not as prominent since c'973 cells present higher basal motility (9, 10). If EGF enhances cell motility via release of actin-modifying proteins from the membrane then the competitive peptide would be predicted to blunt the EGF response. EGF exposure did not further enhance cell motility in WT and c'1000 cells expressing the competitive peptide.

The level of gelsolin which associated with membrane fractions was significantly reduced in WT EGFR cells expressing the PIP2 peptide compared with cells expressing the control peptide (Fig. 8 *B*). EGF exposure decreased the plasma membrane gelsolin content only in cells expressing the control peptide. Thus, the expression of this PIP2 peptide mimicked EGF-induced gelsolin mobilization and cell motility responses.

Expression of the Peptide Encoding PIP2-binding Sequence Mimics EGF-induced Cytoskeletal Reorganization

The cytoskeletal structure of cells expressing the PIP2 peptide was examined. Cells expressing the PIP2 peptide demonstrated protruding lamellipodia lacking stress fibers and the formation of submembranous, short, thin filaments even in the absence of EGF stimulation (Fig. 9). This morphology mimicked what was induced by EGF in control cells (Figs. 6 and 7). EGF did not further accentuate the cytoskeletal phenotype. U73122 did not alter the actin reorganization, suggesting that the mobilization of gelsolin was downstream of PLC activities. Cells express-
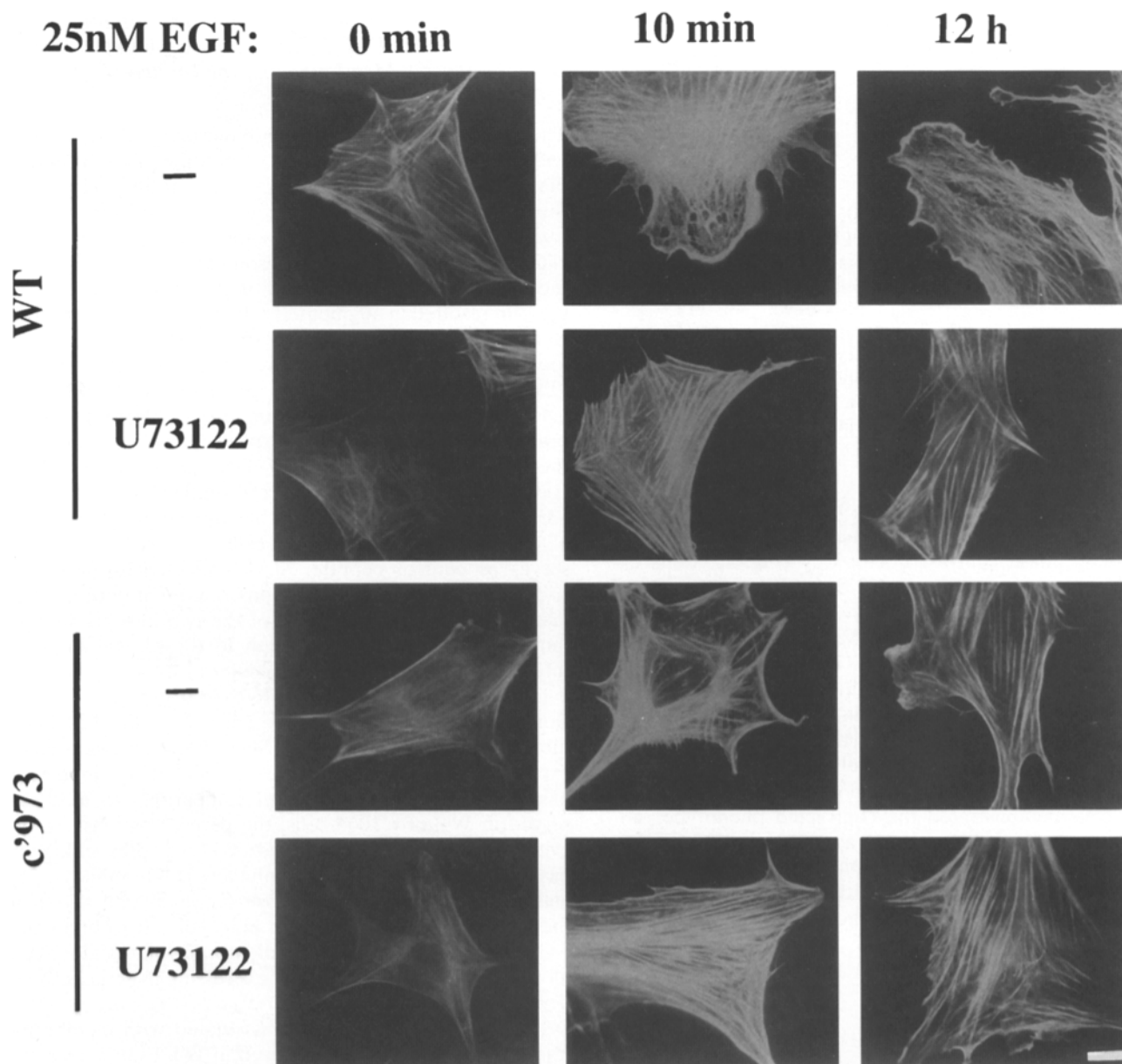
Figure 6. Effect of EGF and PLC inhibitor U73122 on actin cytoskeleton reorganization demonstrated by rhodamine-phalloidin stain-ing WT- and c'973 EGFR–expressing NR6 cells were treated with EGF (25 nM) in the absence or presence of U73122 (1 μM) and ana-lyzed by rhodamine-phalloidin staining after various times. Cells shown represent the predominant morphology for each treatment, $n \geqslant 3$. Bar, 10 μm.

ing control peptide responded identically to nontrans-fected cells in cell motility, gelsolin mobilization and cy-toskeletal changes (Fig. 8, *A* and *B* and Fig. 9).

Discussion

Our data strongly suggest that EGFR-mediated cell motility is actuated, at least in part, by mobilization of membrane-associated gelsolin. We have demonstrated that dissocia-tion of gelsolin from the membrane parallels EGF-induced motility and actin reorganization, which is seen only upon activation of motogenic EGFR. Signaling-restricted EGFR were used to sort through the pleiotropic cell responses. c'1000 EGFR, which contain a single phosphotyrosine mo-

tif, signal this cell event, while nonmotogenic c'973 EGFR, which are fully mitogenic and induce lamellipodia retrac-tion (59), promote neither gelsolin dissociation nor sub-membrane cytoskeletal reorganization. EGFR-mediated gelsolin mobilization is dependent on and downstream of PLCγ activation. In addition, direct dissociation of gelso-lin, and presumably other PIP2-binding actin modifying proteins, mimics the EGFR-mediated cell motility and ac-tin reorganization responses. Thus, we can envision a mo-togenic signaling pathway which leads from receptors with intrinsic tyrosine kinase activity, via activation of PLCγ, to direct cytoskeletal reorganization required for cell motility.

The intracellular level of gelsolin has been correlated with cell motility (11, 61). Here we observed a parallel re-
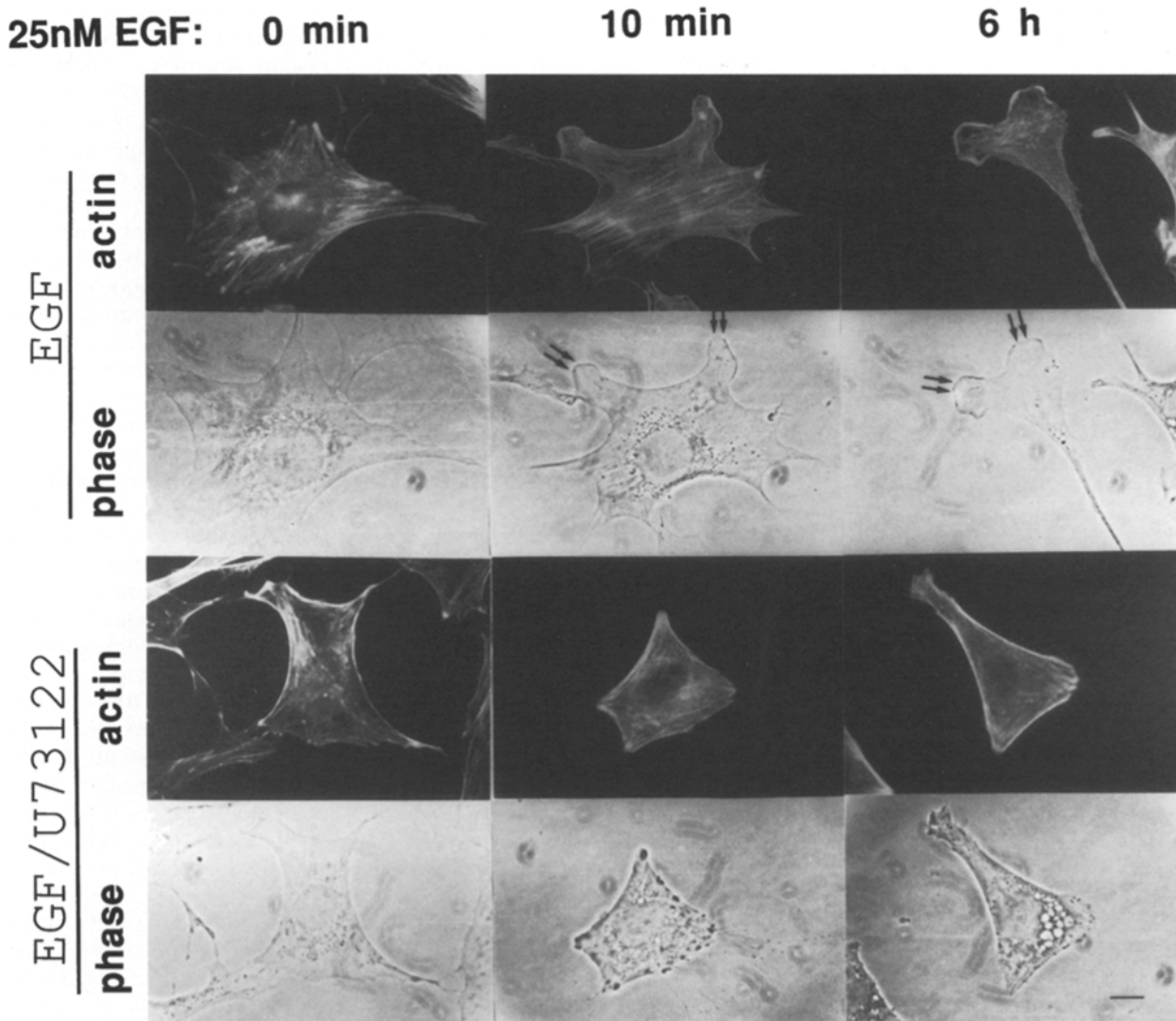
Figure 7. Actin cytoskeleton reorganization demonstrated by rhodamine-phalloidin staining and corresponding membrane ruffles by phase contrast in the presence of EGF and U73122. WT EGFR-expressing NR6 cells were treated with EGF (25 nM) in the absence or presence of U73122 (1 μM) and analyzed by rhodamine-phalloidin staining after various times. Cells shown represent the predominant morphology for each treatment, $n \geqslant 3$. Double arrows designate membrane Wff6s. Bar, 10 μm.

duction in EGF-induced motility and endogenous gelsolin level upon gelsolin anti-sense oligonucleotide treatment. It is possible, though unlikely, that the coincident decrease in cellular gelsolin content and EGF-induced cell motility was due to nonspecific effects of hybrid DNA-RNA molecules generated by anti-sense treatment. The decrease in gelsolin content was specific for the gelsolin-complementary oligonucleotide, as gelsolin content was still decreased when compared with a control oligonucleotides complementary to phospholipase Cγ-1 (data not shown). The outsized decrease in motility for a 30–60% diminution in gelsolin is consistent with a model in which the majority of gelsolin is not PIP2 bound but rather in a cytosolic localization. If the on-rate of gelsolin for PIP2 is substantially less than for actin (as has been shown for profilin [18, 34]), then a decrease in total endogenous gelsolin is likely to be reflected as a preferential loss of PIP-associated (and perhaps motility-associated) gelsolin. Furthermore, fibroblast motility, though decreased, is still evident in gelsolin-devoid

fibroblasts (61). Therefore, it is likely that EGFR-mediated motility is more sensitive to gelsolin levels than basal cell movement. Evidence for such a model awaits an experimentally tractable system in which cellular gelsolin levels can be modulated.

It is likely that other gelsolin-independent mechanisms also are necessary for eliciting the motogenic response since non-stimulated cells in our model system and gelsolin-deficient fibroblasts (61) demonstrate basal cell movement. These mechanisms may involve both additional signaling pathways and altered activation of other actin modifying proteins. The dissociation of gelsolin from the membrane fraction observed upon PLCγ activation may represent a general regulatory mechanism modulating functioning of other PIP2-binding, actin-modifying proteins.

It was surprising that the PIP2-binding peptide induced cell motility in the absence of EGFR signaling as motility is an asymmetric cell response. Nonhomogenous subcellular distribution and differential activation of the cell motil-

Table I. PIP2-binding Domains of Actin-modifying Proteins and Competitive Peptides

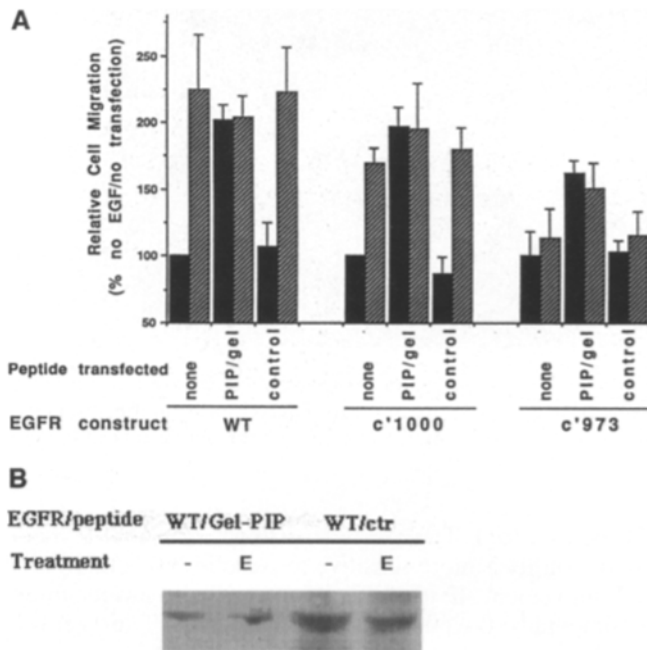| Protein	Amino acid	Sequence
Gelsolin	135–142	K S G L K Y K K
Gelsolin	161–169	K L F Q V K G R R
CapG	139–147	K L Y Q V K G K K
Villin	137–145	R L L H V K G K R
Cofilin	13–22	K V F N D M K V R K
Profilin	123–136	K C Y E M A S H L R R
PIP2 peptide		M R L F Q V K G R R V*
Control peptide		M A L F W V D G L R V*

Sequences are taken from reference 64.
*stop codon.

ity machinery may also be required to initiate cell motility. While gelsolin is distributed throughout the cell (14), profilin is preferentially localized to dynamic lamellipodia in migrating cells (8). Though both gelsolin and profilin have the capacity to promote and inhibit actin polymerization, under conditions mimicking the in vivo environment, gelsolin is reported to preferentially sever filamentous actin while profilin nucleates actin monomers, the event required for actin polymerization (16, 23). Together, mobilization of multiple PIP2-binding proteins, including CapG

A

B

Figure 8. Effect of PIP2-binding domain peptide on (*A*) EGF-induced cell motility and (*B*) membrane association of gelsolin. PIP/gel denotes cells transfected with cDNA encoding PIP2 binding sequence (Table I). Control denotes the cDNA encoding the control peptide. Each treatment was performed in triplicate; values are mean ± SEM; *n* = 3. The immunoblot shown here is a representative of three experiments. The membrane isolation of two independent transfected WT-expressing NR6 cell lines were performed in the same experiment. *P* < 0.01 for basal cell motility between cells with no transfection and cells transfected with PIP/gel in all three cell lines expressing EGFR constructs. *P* > 0.3 for cell motility between cells with no transfection and cells transfected with cDNA encoding control peptide in all three cell lines expressing EGFR constructs. *P* > 0.3 between EGF-treated and nontreated cells in all three PIP/gel-expressing cell lines.

(53) may produce the asymmetric actin-severing and -nucleating activities in the cell, leading to increased cell motility in the direction of F-actin generation. Therefore, while gelsolin is required for EGF-induced motility (Fig. 1), the motility-inducing and cytoskeletal effects of the PIP2-binding peptides are likely mediated by multiple members of this functional superfamily of actin-binding proteins.

In addition, there are published data supporting roles for downstream activation of PKC and cytoplasmic calcium transients in modulating cell motility. These messengers are both increased by EGF-induced activation of PLCγ. Calcium concentration significantly affects actin polymerization and gelsolin's actin-severing activity (27, 31), and, thus may play an important role in maintaining the homeostasis of the actin cytoskeleton and the aforementioned basal motility. PKC has been demonstrated to participate in focal adhesion formation and disruption as well as cytoskeletal rearrangement (62). All of these structures are essential components for cell motility (52).
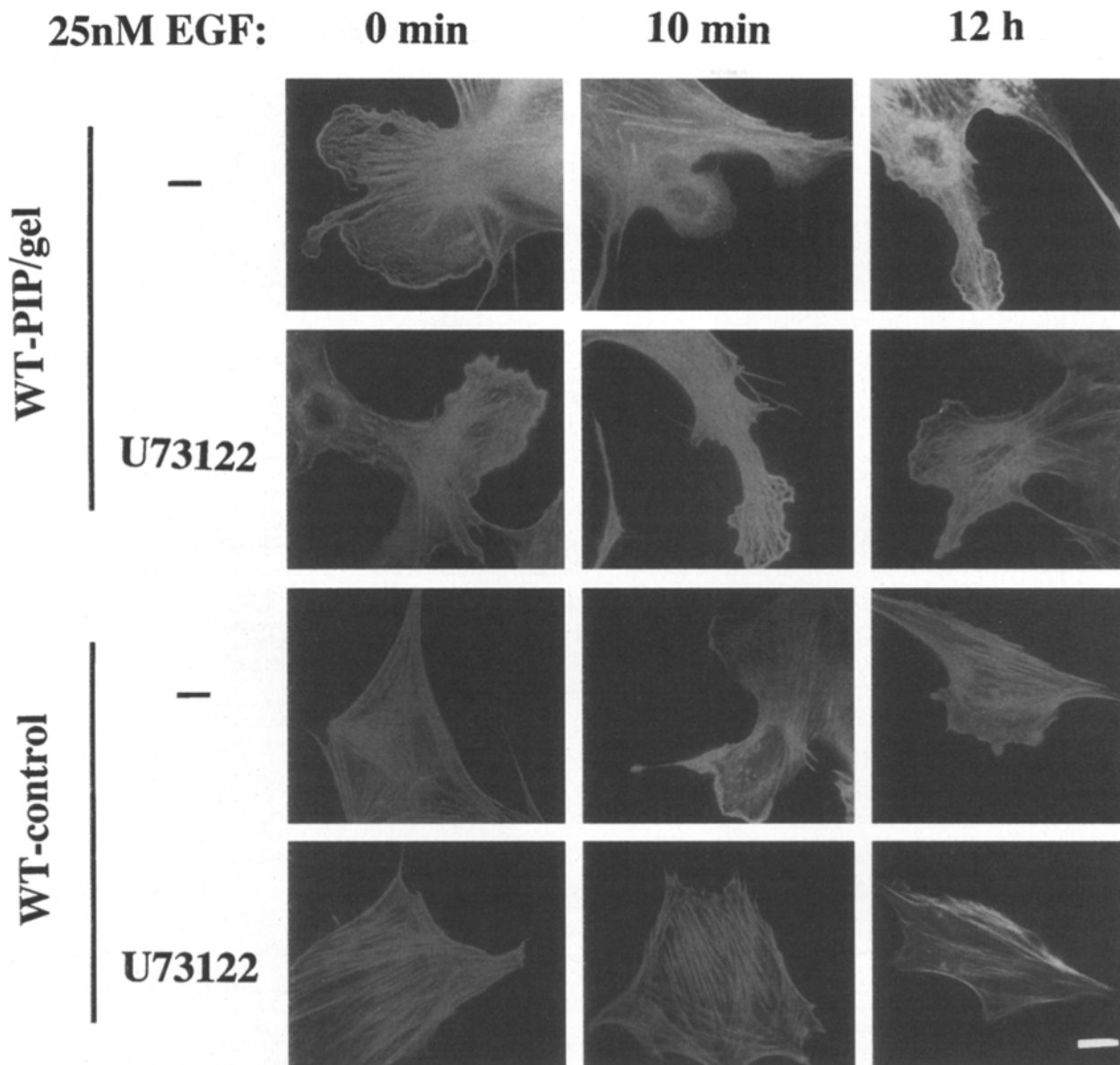
Other signaling pathways activated by receptors with intrinsic tyrosine kinase activity likely also participate in triggering cell motility. PDGF-β receptor–mediated cell motility requires receptor binding and subsequent activation of PI-3 kinase, another phosphoinositol-regulatory enzyme (7, 30). Small G proteins, such as rac and rho transmit signals for membrane ruffling and cytoskeletal reorganization effected by this class of receptors (40, 43, 44, 45). These pathways alter phospholipid content, profile, and metabolism. Thus, the polyphosphoinositide cycle may serve as a central point of convergence. It is unsurprising that altering activities of any one arm, PLCγ, PI3-kinase, or small G-proteins, will tip the balance and induce or inhibit cell motility.

Cell motility is a dynamic process involving cycles of actin cytoskeleton disruption, polymerization, and reorganization (52). Herein, we demonstrated for the first time the in vivo mobilization and potential activation of gelsolin upon EGF stimulation. It is reasonable to speculate that other actin modifying proteins which bind to PIP2 also are mobilized upon PLCγ activation. In vitro profilin, gelsolin, and other actin modifying proteins bind reversibly to PIP2 with this binding limiting their activities (18, 28, 35, 36, 38). PLCγ hydrolysis of PIP2 releases these factors from PIP2-containing micelles in vitro and restores their actin-modulating capacity (17, 18, 64). It is easy to conceive that the ability to modulate the actin modifying activities of this superfamily of proteins is critical to regulate cell motility. Extended hydrolysis of PIP2, on the other hand, has been associated with diminished motility (6). These observations are consistent with previous findings linking PPI turnover with cell motility (10, 32, 37). Thus, regulated redistribution of the small amount of plasma membrane-associated gelsolin and other proteins would contribute significantly to EGF-induced cell movement. Repeatedly activated PLCγ in these cells (10) mobilizes membrane-associated gelsolin family members, thus allowing them to interact and modulate the actin cytoskeleton at the sites of cytoskeleton interaction with the cell membrane (47). The localized, subtle alterations in the ratio of plasma membrane-associated gelsolin to "free", presumably active gelsolin, would be coupled with the rapid and efficient downregulation of activated EGFR (42, 60) and PLC (25).

Figure 9. Effect of PIP2-binding domain peptide on actin cytoskeleton reorganization. WT EGFR-expressing NR6 cells expressing the PIP2-binding or control peptide were analyzed by rhodamine-phalloidin staining. Cells shown are representative of the majority of cells for each treatment, $n \geqslant 3$. Bar, 10 μm.

This model provides a ready mechanism underlying chemotactic cell motility toward higher ligand concentrations.

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