EGF receptor regulation of cell motility: EGF induces disassembly of focal adhesions independently of the motility-associated PLCγ signaling pathway

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SUMMARY

A current model of growth factor-induced cell motility invokes integration of diverse biophysical processes required for cell motility, including dynamic formation and disruption of cell/substratum attachments along with extension of membrane protrusions. To define how these biophysical events are actuated by biochemical signaling pathways, we investigate here whether epidermal growth factor (EGF) induces disruption of focal adhesions in fibroblasts. We find that EGF treatment of NR6 fibroblasts presenting full-length WT EGF receptors (EGFR) reduces the fraction of cells presenting focal adhesions from ~60% to ~30% within 10 minutes. The dose dependency of focal adhesion disassembly mirrors that for EGF-enhanced cell motility, being noted at 0.1 nM EGF. EGFR kinase activity is required as cells expressing two kinase-defective EGFR constructs retain their focal adhesions in the presence of EGF. The short-term (30 minutes) disassembly of focal adhesions is reflected in decreased adhesiveness of EGF-treated cells to substratum.

We further examine here known motility-associated pathways to determine whether these contribute to EGF-induced effects. We have previously demonstrated that phospholipase Cγ (PLCγ) activation and mobilization of gelsolin from a plasma membrane-bound state are required for EGFR-mediated cell motility. In contrast, we find here that short-term focal adhesion disassembly is induced by a signaling-restricted truncated EGFR (c'973) which fails to activate PLCγ or mobilize gelsolin. The PLC inhibitor U73122 has no effect on this process, nor is the actin severing capacity of gelsolin required as EGF treatment reduces focal adhesions in gelsolin-devoid fibroblasts, further supporting the contention that focal adhesion disassembly is signaled by a pathway distinct from that involving PLCγ. Because both WT and c'973 EGFR activate the erk MAP kinase pathway, we additionally explore here this signaling pathway, not previously associated with growth factor-induced cell motility. Levels of the MEK inhibitor PD98059 that block EGF-induced mitogenesis and MAP kinase phosphorylation also abrogate EGF-induced focal adhesion disassembly and cell motility.

In summary, we characterize for the first time the ability of EGFR kinase activity to directly stimulate focal adhesion disassembly and cell/substratum detachment, in relation to its ability to stimulate migration. Furthermore, we propose a model of EGF-induced motogenic cell responses in which the PLCγ pathway stimulating cell motility is distinct from the MAP kinase-dependent signaling pathway leading to disassembly and reorganization of cell-substratum adhesion.

Key words: Migration, EGF receptor, Adhesion, Focal contact, Cell-matrix interaction

INTRODUCTION

Cell motility is required for the physiologic processes of wound repair and organogenesis and for the pathologic process of tumor invasion (Clark, 1996; Stossel, 1993). Cell motility requires the coordinated activation of numerous cell processes, which can be grouped into the biophysical phenomena of membrane protrusion, formation of cell-substratum connections at the leading edge, translocation of the cell body and nucleus, and breaking of cell-substratum interactions in the uropod (Lauffenburger and Horwitz, 1996; Stossel, 1993). Signals from the extracellular milieu dictate cell migration. Many growth factors, including the ligands that act through the epidermal growth factor receptor (EGFR), enhance fibroblast cell motility (Manske and Bade, 1994). To better understand the biochemical bases of growth factor-induced cell motility, the signaling pathways which affect each of the required biophysical processes need to be defined.

Cell adhesion to the substratum plays a crucial role in cell migration, not only providing structural anchorage that

supplies needed traction and tracks for cell migration but also functioning as signal transduction transmitters (Dedhar and Hannigan, 1996). Alterations in cell-substratum avidity are critical for cell motility, as changes in adhesiveness can directly affect cell locomotion (Palecek et al., 1997). Focal adhesions, visualized by interference reflection microscopy (IRM), are discrete transmembrane regions in which complexes of cytoskeletal and membrane components are assembled and tightly linked with the underlying substratum via specific receptors such as integrins and proteoglycans (Burridge and Chrzanowska-Wodnicka, 1996). The presence of focal adhesions is a hallmark that distinguishes stationary cells from locomoting ones. Years ago, it was demonstrated that these aggregates of actin filament connection to the membrane were not required for cell motility (Couchman et al., 1982). Rather, in migrating fibroblasts focal adhesions are absent or only transiently identifiable (Dunlevy and Couchman, 1993; Matsumoto et al., 1994), though the more separated 'close contacts' may still be identified (Burridge et al., 1988). Thus, for a cell to move, focal adhesions must be disrupted. Surprisingly, little has been reported on the growth factor-induced signaling pathways leading to focal adhesion disassembly in adherent cells (Dunlevy and Couchman, 1993; Murphy-Ullrich et al., 1996), and whether focal adhesion disassembly, per se, is required for growth factor-induced motility. In particular, earlier reports have failed to note focal adhesion disassembly upon stimulation by EGF (Dunlevy and Couchman, 1993; Herman et al., 1987). It is possible that EGFR-mediated enhanced cell motility results in detachment from substratum by purely biophysical tearing without focal adhesion disassembly, as has been observed during rapid haptotaxis of fibroblasts (Regen and Horwitz, 1992). Therefore, we investigated whether and how cell-substratum connections were affected by EGF treatment, under conditions conducive to fibroblast migration.

The signaling pathways by which motility-inducing events lead to focal adhesion disruption are only now being elucidated. In migrating neutrophils, calcium-activated calcineurin has been implicated in disassembly of established adhesions (Hendey and Maxfield, 1993) to enable recycling of integrins to newly established adhesions at the migratory front (Lawson and Maxfield, 1995). In fibroblasts migrating on defined substratum, in contrast, elements from many of the focal contacts are left behind on the matrix (Palecek et al., 1996; Regen and Horwitz, 1992); this is postulated to require actin-based contraction as a peptide inhibitor of actin-myosin interactions prevents disruption of adhesions (Crowley and Horwitz, 1995). Recently, a pathway has been proposed for integrin-mediated haptotaxis which involves MAP kinase activation of myosin light chain kinase (Klemke et al., 1997); however, inhibition of these enzymatic activities prevented cell translocation but not cell attachment or spreading which results in focal adhesion assembly. Another recent report suggests that integrin-triggered MAP kinase activity reduces integrin avidity in a negative feedback loop (Hughes et al., 1997). While the authors did not examine focal adhesion presence, their results would predict the involvement of this pathway in focal adhesion disruption. However, the mechanisms by which growth factors, as opposed to integrins, negatively modulate focal adhesions and cell adhesiveness acutely have not been explored extensively (Herman et al.,

1986). This distinction is likely important as there are differences between integrin-mediated and EGFR-mediated cell motility; as examples, inhibition of PLC activity, which blocks EGF-induced locomotion, does not affect basal (presumably integrin-mediated) cell motility (Chen et al., 1994a) and membrane protrusion rate appears to be rate-limiting for haptotactic locomotion but not during maximal EGF-induced motility (M. F. Ware et al., unpublished data). To more precisely determine the effect of EGF exposure on cell detachment from substratum, focal adhesions and cell adhesion need to be investigated directly.

EGFR activation promotes cell motility via specific intracellular signaling pathways distinct from those inducing mitogenesis (Chen et al., 1994a). One required pathway involves the activation of PLCγ-1 and the subsequent hydrolysis of phospho-inositide bisphosphate (Chen et al., 1994b); this signaling pathway also is utilized by other motility-inducing growth factors such as PDGF and IGF-1 (Bornfeldt et al., 1994; Kundra et al., 1994). The generation of inositol trisphosphate and diacylglycerol activates protein kinase C and increases cytoplasmic calcium; both of these effectors have been reported to modulate processes required for cell motility (Hinrichsen, 1993; Janmey, 1994; Zimmerman and Keller, 1992). In addition, actin modifying proteins, such as gelsolin, are mobilized from a membrane-association upon hydrolysis of phospho-inositide bisphosphate (Chen et al., 1996), which is hypothesized to effect the cytoskeletal reorganization required for motility. Reports suggest that both the actin reorganization and calcium transients may contribute to either membrane protrusive force or cell-substratum detachment (Hendey and Maxfield, 1993; Herman et al., 1986; Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996; Stossel, 1993). In short, the biophysical consequences of the PLCγ pathway are not known.

In this study, we utilize NR6 mouse fibroblasts expressing signaling-restricted EGFR to investigate whether EGFR activation can alter the interaction between cells and substratum. We report that the loss of focal adhesions was concomitant with EGFR activation in a time-, dose-, and kinase-dependent manner that reflects EGFR-mediated cell motility (Chen et al., 1994a,b). This acute loss of focal adhesions was mirrored by decreased adhesiveness. We determined that the intracellular signaling pathway which modulates EGFR-mediated loss of focal adhesions is distinct from the PLCγ-gelsolin pathway required for cell motility (Chen et al., 1994b, 1996). Rather, initial data suggest that EGF-induced disassembly of focal adhesions are regulated via activation of MAP kinases, akin to integrin-mediated haptotaxis (Klemke et al., 1997). Thus, we have demonstrated that growth factor-induced cell motility requires the coordinate activation of at least two divergent intracellular signaling pathways which lead to distinct biophysical processes required for cell motility.

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 (Chen et al., 1994a,b; Wells et al., 1990). WT EGFR is a

full-length cDNA derived from a placental cDNA library (Welsh et al., 1991). M[721] is a kinase-inactive full-length clone in which methionine replaces lysine in the ATP-binding pocket. c'647, c'973 and c'1000 represent EGFR in which stop codons are introduced just distal to the amino acid number indicated. WT, c'1000 and c'973 EGFR constructs present ligand-activated kinase and signal mitogenesis but only WT and c'1000 promote cell motility and activate PLCγ (Chen et al., 1994b). M[721] and c'647 EGFR lack kinase activity and do not transmit mitogenic or motility signals (Chen et al., 1994a,b).

The constructs were expressed on NR6 cells, 3T3-derivatives which lack endogenous receptors (Chen et al., 1994a,b). This was accomplished by retrovirus-mediated transduction as previously described (Wells and Bishop, 1988). Polyclonal lines were established by selection in G418 (Gibco/BRL). 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 nM to 0.7 nM).

Cell culture

NR6 cells expressing the various EGFR constructs were passaged in MEMα medium supplemented with fetal bovine serum (FBS; 7.5%), penicillin (100 units/ml), streptomycin (200 μg/ml), non-essential amino acids, sodium pyruvate (1 mM), glutamine (2 mM) and G418 (350 μg/ml). Primary dermal and lung fibroblasts were isolated from mice in which the gelsolin gene was disrupted by targeted recombination (Witke et al., 1995). Homozygous and heterozygous disrupted fibroblasts were obtained upon necropsy by standard isolation procedures. The fibroblasts were cultured in Dulbecco's modification of Eagle's medium (4.5 g/l glucose), 7.5% FBS, penicillin (100 units/ml), streptomycin (200 μg/ml), non-essential amino acids, sodium pyruvate (1 mM), glutamine (2 mM) and amphotericin (2.5 μg/ml). Cells were passaged (37°C, 90% humidity, 5% CO_2) at subconfluence by trypsinization (0.25%, 1 mM EDTA). Cells are quiesced in the medium containing 1% dialyzed FBS for 24 hours before experimentation.

Focal adhesion assessment

The presence of focal adhesions was assessed as described previously (Dunlevy and Couchman, 1993; Murphy-Ullrich et al., 1993, 1996). Briefly, cells were seeded on glass coverslips (22 mm × 30 mm) and, after becoming adherent (>24 hours) were switched to medium containing 1% dialyzed FBS for 24 hours. The cells were treated with EGF as described in the text. After experimental treatments, cells on glass coverslips were washed three times with 37°C prewarmed PBS before and again after a 30 minute cell fixation in 3% glutaraldehyde in PBS at 37°C. Coverslips were mounted onto glass slides in PBS. Cells were examined by interference reflection microscopy (IRM) using a Zeiss Axiovert 10 microscope (Murphy-Ullrich et al., 1993, 1996). Cells having at least 3 classic, cicatrix-shaped adhesion plaques (Burridge et al., 1988) were designated as positive; the vast majority of adhesion-positive cells had >20 adhesion plaques, and most adhesion-negative cells had no identifiable plaques. A minimum of 250 cells were counted per coverslip, with each experiment being performed in triplicate. The identities of the slides were coded prior to reading by a second, blinded investigator.

The PLC inhibitor 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) (BIOMOL), were added at 1 μM 15 minutes prior to EGF treatment as previously described (Chen et al., 1994b; Smith et al., 1990). PD98059 (New England Biolabs) (Dudley et al., 1995), diluted in MEMα containing 1% dialyzed FBS so that the final concentration of DMSO was <0.05%, was added at various concentrations 90 minutes prior to EGF treatment; this agent blocks MEK phosphorylation and activation of erk1 and erk2 without affected other MAP kinases.

These conditions closely mimic those under which EGFR-mediated cell motility has been studied (Chen et al., 1994a,b): near confluence and after 24 hours quiescence in 1% dialyzed FBS. This results in a lower percentage of cells expressing focal adhesions; in the presence of 7.5% FBS, the percentage of cells with observable focal adhesions is increased >20% (data not shown). That only half of the cells demonstrate focal adhesions is not unusual under our conditions of low serum (Woods et al., 1993). In short, the initially depressed number of cells displaying focal adhesions is partly due to designing the experiments to be directly comparable to those evaluating EGFR-mediated cell motility.

Fluorescence microscopy

Cells were seeded on glass coverslips at subconfluence, quiesced in 1% dialyzed FBS for 24 hours and then treated with EGF as described in the text. For immunofluorescence of vinculin, cells were fixed for 20 minutes in 3% freshly hydrolyzed paraformaldehyde in PBS at room temperature or in paraformaldehyde with 0.1% Tween-20, at 37°C for 5 minutes. Cells were stained with a 1:150 dilution of a monoclonal antibody to vinculin (clone hVIN-1, V-9131, Sigma) for 30 minutes at room temperature, washed 3 times with PBS, and visualized using FITC-conjugated goat anti-mouse IgG (1:250 dilution). Cells were evaluated using either a Zeiss Axiovert 10 or Olympus BX40 microscope equipped for epifluorescence.

Cell migration assay

EGF-induced migration was assessed by the ability of the cells to move into an acellular area, in vitro wound healing assay, as previously described (Chen et al., 1994a,b). Briefly, cells were plated on plastic and grown to confluence in MEMα with 7.5% FBS (fetal bovine serum). After 24 hours of incubation in medium 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 18 hours and the relative distance traveled by the cells at the acellular front was determined. The EGF-induced migration was calculated as a percentage 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.

Hele-Shaw flow cell adhesion assay

Cell-substratum adhesiveness was quantitated by measuring the level of shear stress required to detach cells (Usami et al., 1993). WT NR6 cells were grown on Amgel-coated non-tissue culture polystyrene slides for 12 hours in MEMα containing 1% dFBS; slides were coated with the human extracellular matrix Amgel (0.5 μg/ml) (Siegal et al., 1993) for 75 minutes at room temperature, after which they were blocked with bovine serum albumin (BSA) (1%) for 60 minutes at room temperature, and finally washed thrice with phosphate buffered saline. The slides were placed in a flow chamber that induces Hele-Shaw flow patterns. The surface shear stress varies linearly with position along the lengthwise midline according to the formula: $\tau_w = (6\,\mu\,Q/h^2\,w_1)(1-z/L)$; where τ_w is the surface shear stress, μ is the fluid viscosity, Q is the flow rate, h is the channel height, w_1 is the channel width at the origin of the flow field, z is the distance from the origin along the midline, and L is the length of the flow field (Palecek et al., 1997). The dimensions of the flow chamber are w_1=1.35mm, L=5.65cm, and h=3.65mm. The flow medium is composed of MEMα with 25 mM Hepes, pH 7.4, and 1% BSA. The medium and cells are maintained at 37°C. Flow is controlled by a pressurized head. The flow chamber is placed on the stage of Zeiss Axiovert 10 microscope; position was precisely controlled by a Ludl 99S008 motorized stage connected to a nuDrive amplifier. Cells in 20 fields, at 2.5 mm intervals, were counted prior to and again after 5 minutes of constant flow.

RESULTS

EGFR activation mediates loss of focal adhesions

Previous reports have shown that migrating fibroblasts lack focal adhesions (Dunlevy and Couchman, 1993; Matsumoto et al., 1994). As EGF exposure induces increased cell motility, we examined whether EGFR signaling results in disassembly of focal adhesions. NR6 cells expressing WT EGFR were treated with various doses of EGF at 37°C for 30 minutes. IRM assessment revealed a decrease of focal adhesion-positive cells in a EGF dose dependent manner (Fig. 1a). The maximum EGF effect occurs at the dose of EGF around 0.1 nM which coincides with the EGF concentration that induces cell motility (Chen et al., 1994a). The disassembly of focal adhesions was rapid, with the maximum decrease occurring by 10 minutes of EGF (25 nM) exposure (Fig. 1b). Similar disassembly of focal adhesions was seen upon EGF stimulation of NR6 cells expressing c′1000 EGFR (data not shown). The disassembly of focal adhesions requires intact EGFR signaling, as EGF exposure of cell expressing two different kinase-inactive receptors, M^{721} and c′647, failed to induce this reduction in focal adhesion-positive cells (Fig. 1c).

The disassembly of focal adhesions as determined by IRM was mirrored by a somewhat slower dissolution of vinculin aggregates as determined by immunofluorescence (Fig. 2). Upon EGF exposure, the vinculin aggregates appear to diffuse and no longer be co-incident with IRM-identifiable focal adhesions before becoming indistinguishable from a low level diffuse background staining. Because the relationship of vinculin aggregation to focal adhesion presence remains controversial, as focal adhesions may form in the absence of vinculin (Volberg et al., 1995) and that vinculin may be redistributed while other focal adhesion components remain aggregated (Tidball and Spencer, 1993), we utilized IRM determinations of focal adhesions throughout this study. Upon EGF stimulation, WT and c′973 EGF-expressing NR6 cells may reduce their cell-substratum interface by up to 30% (Welsh et al., 1991). However, this change in cell spreading in itself would not cause an appreciable change in focal adhesion positive cells for two reasons: (1) the vast majority of cells scored as focal adhesion-positive had in excess of 20 plaques, while the negative cells usually presented no identifiable plaques; and (2) EGF treatment resulted in the dissolution of plaques and vinculin aggregates under the center of the cell not just those towards the periphery.

EGF reduces cell adhesiveness to substratum

Disassembly of focal adhesions would be predicted to result in decreased cell adhesion to substratum. Therefore, we ascertained whether cell adhesion to a human extracellular matrix, Amgel (Siegal et al., 1993), was affected by EGF stimulation. Detachment of cells by shear stress was chosen as a measurement of global cell adhesion to the substratum independent of precise molecular linkage. WT NR6 cells were dissociated with trypsin and allowed to attach to an Amgel coated-glass surface (0.5 µg/ml Amgel) for 12 hours. The cells were then treated with EGF (25 nM) for 30 minutes before being subjected to a shear force for 5 minutes. The shear force which dislodged half the EGF-treated cells ranged from 11,000 to 16,000 µdynes/cm^2 in three separate
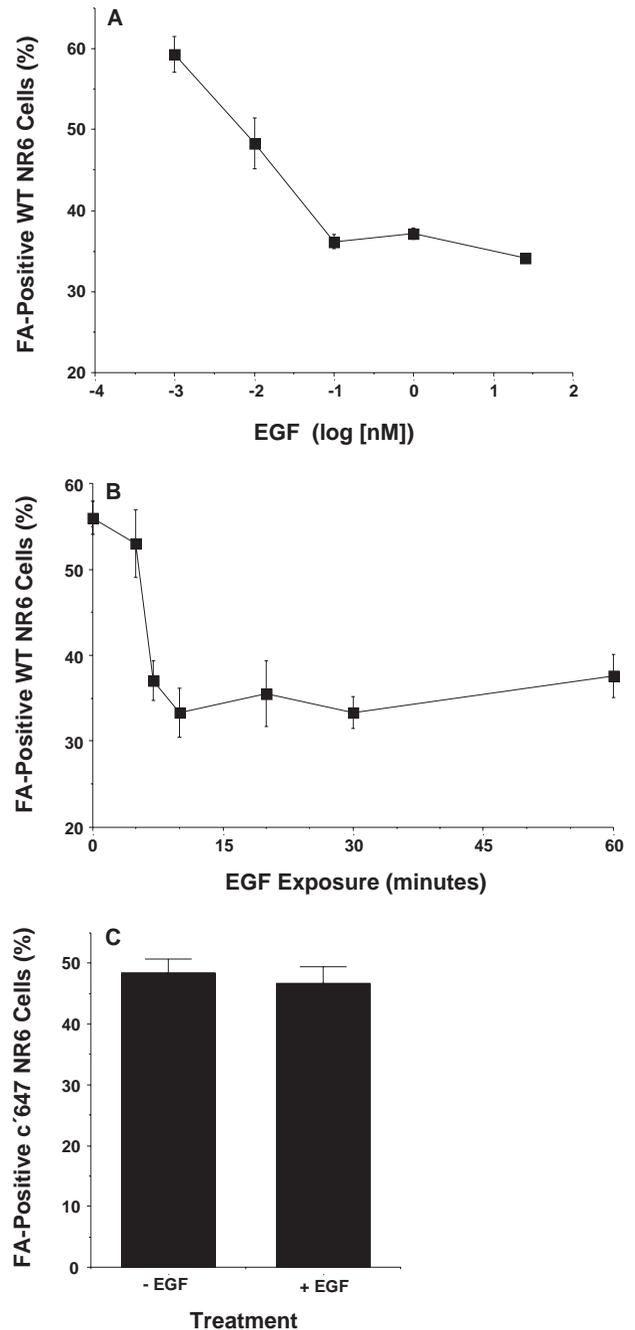
Fig. 1. EGF induces loss of focal adhesions in NR6 cells expressing kinase active EGFR. Cells were plated on coverslips as described in Materials and Methods. WT NR6 cells were exposed to various concentrations of EGF for 30 minutes (A) or for different times to 25 nM EGF (B) prior to fixation and examination by IRM. Cells expressing the cytoplasmic truncated, kinase-deficient EGFR, c′647 NR6, were exposed to 25 nM EGF for 30 minutes prior to examination (C); EGF did not alter focal adhesions at either shorter or longer time periods nor in cells expressing M^{721} EGFR. Results shown are the mean ± s.e.m. of at least 3 experiments each performed in triplicate.

experiments (Fig. 3). The untreated cells were not removed from the substratum at the maximally attained shear of 17,000-19,000 µdynes/cm^2.

IRM

α−vinculin IF

0 min

30 min

60 min
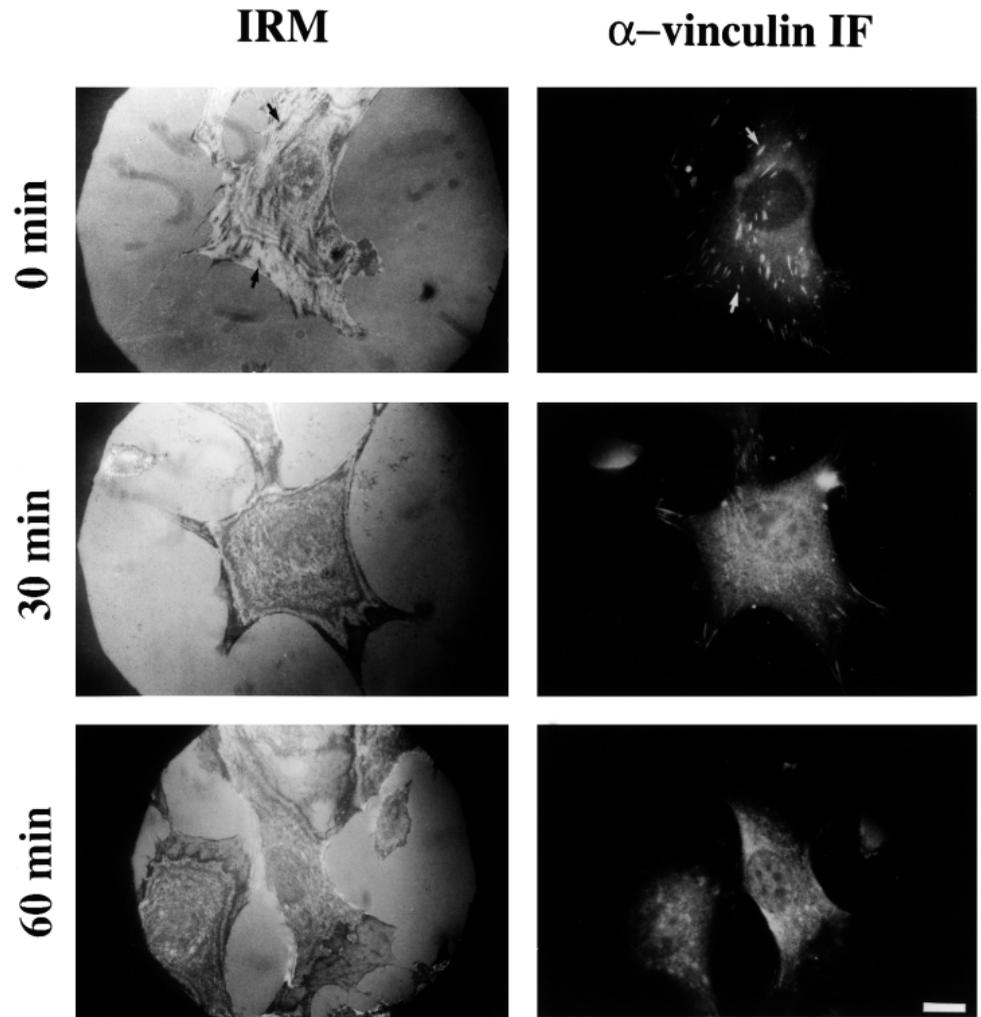
Fig. 2. EGF-induced disassembly of focal adhesions is mirrored by disappearance of vinculin aggregates. WT NR6 cells were exposed to EGF (25 nM) for various times prior to fixation and staining with a monoclonal antibody directed against vinculin and a secondary fluorescein-labelled goat anti-mouse antibody. The same cells were examined sequentially for vinculin aggregates by immunofluorescence microscopy (right panels) and focal adhesions by IRM (left panels). Representative cells are shown. Arrows point to classic cicatrix-shaped co-incident focal adhesions and vinculin aggregates; the large peripheral dark areas noted by IRM at 30 and 60 minutes are attributed to membrane apposition seen during cell retraction (Burridge et al., 1988). Bar, 10 μm.
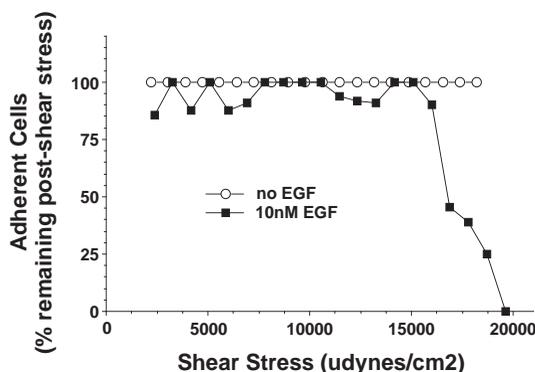
Fig. 3. EGF reduces adhesiveness of WT NR6 cells to substratum. WT NR6 cells were adhered to an Amgel coated surface for 12 hours. The cells were exposed to EGF (25 nM) for 30 minutes prior to shear flow for 5 minutes. The percentage of cells remaining adherent to substratum was determined at intervals along the midline of the flow chamber. Shown is a representative shear flow experiment. In all experiments, the highest attainable shear stresses which were in the range of 18,000 μdynes/cm², could not detach nontreated cells, while the same maximal shear stresses removed all the EGF-treated cells.

PLC activation is not required for focal adhesion disassembly mediated by EGFR

PLCγ has been demonstrated to be required for cell motility induced by numerous growth factors including EGF (Chen et al., 1994b), PDGF (Kundra et al., 1994), and IGF-1 (Bornfeldt et al., 1994). The mechanism by which PLCγ functions to promote cell motility is unknown at present, and thus it is important to determine whether focal adhesion disassembly requires this signaling pathway. EGF does not induce cell motility or PLCγ activity in NR6 cells expressing the c′973 EGFR (Chen et al., 1994a,b). However, in the c′973 NR6 cells EGF (25 nM) exposure leads to a 50% decrease in focal adhesion-positive cells in a pattern similar to that of WT NR6 cells (Fig. 4). These data suggested that PLCγ signaling was not required for growth factor induced focal adhesion disassembly.

It is possible that c′973 EGFR utilize an alternate signaling pathway or induce loss of focal adhesions via different mechanisms than WT EGFR. To confirm this separation of focal adhesion disassembly from the PLCγ signaling pathway, the PLC inhibitor U73122 was used to block PLC activity. Pre-exposure of WT NR6 cells to U73122 or its inactive congener U73343 (either agent at 1 μM for 15 minutes prior to EGF, a treatment which blocks cell motility; Chen et al., 1994b), had
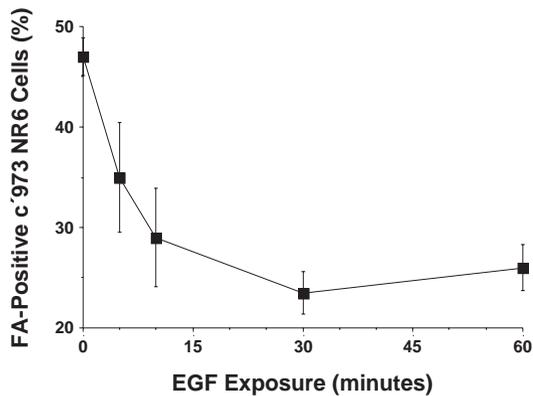
Fig. 4. EGF induces disassembly of focal adhesions in cells expressing the non-motility-associated c′973 EGFR. c′973 NR6 cells were exposed to EGF (25 nM) for various times prior to fixation and examination for focal adhesions. Results shown are the mean ± s.e.m. of at least 3 experiments each performed in triplicate.

no effect on EGF-induced loss of focal adhesion-positive cells (Fig. 5). In parallel control experiments, this concentration of U73122 inhibited EGF-induced cell motility. These experiments confirmed that PLC activation and PLC activities are not required for EGFR mediated decrease of focal adhesion-positive cells.

EGF induces focal adhesion disassembly but not cell motility in gelsolin-devoid fibroblasts

Mobilization of actin modifying proteins, including gelsolin, secondary to PLCγ hydrolysis of PIP2 is critical for EGFR-mediated cell motility (Chen et al., 1996). To determine whether focal adhesion disassembly proceeds in the absence
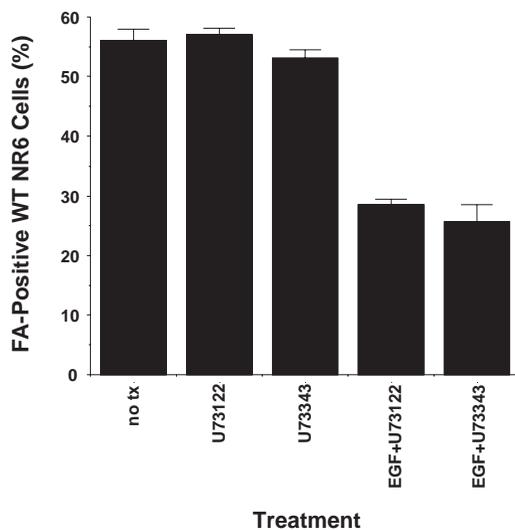
Fig. 5. Inhibition of PLC activity does not abrogate focal adhesion disassembly induced by EGF. WT NR6 cells were treated with the pharmacological inhibitor of phospholipase C, U73122 (1 μM), its inactive congener, U73343 (1 μM), or untreated (no tx) for 15 minutes prior to 30 minute exposure to EGF (25 nM). Results shown are the mean ± s.e.m. of at least 3 experiments each performed in triplicate.
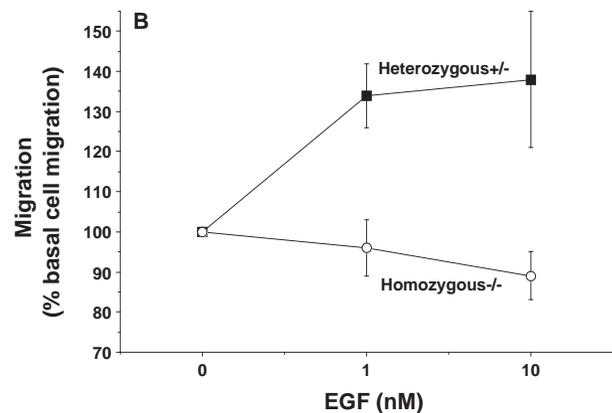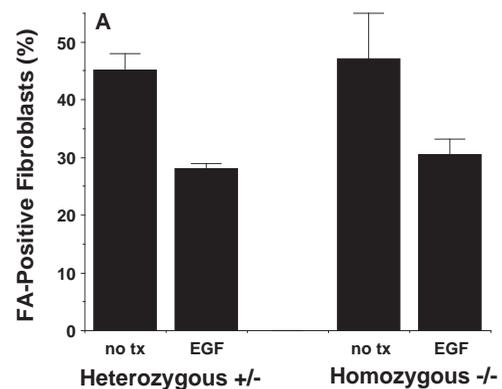
Fig. 6. EGF induces focal adhesion disassembly in gelsolin-devoid fibroblasts. Lung fibroblasts were isolated from gelsolin-devoid mice and heterozygous littermates. The cells were examined for EGF-induced dissolution of focal adhesions by IRM after 30 minutes (A) and cell motility by an in vitro 'wound healing' assay after 18 hours (B). Two independent fibroblast isolates were tested in the absence (no tx) or presence of EGF (25 nM). Results in both panels are the mean ± s.e.m. of 3 experiments performed in duplicate or triplicate.

of gelsolin, gelsolin-devoid fibroblasts were isolated from the lungs and skin of mice in which the gelsolin gene was disrupted (Witke et al., 1995). Gelsolin-devoid homozygous fibroblasts serve as a experiment group, while heterozygous fibroblasts serve as a control group. Our results demonstrate that EGFR induced focal adhesion disassembly in both experiment and control groups (Fig. 6a). It is important to note that only the heterozygous disrupted cells expressing gelsolin and not the homozygous gelsolin-devoid cells responded to EGF by increased cell motility (Fig. 6b). These results demonstrated a clear dissociation between the gelsolin requirement for EGFR-mediated cell motility (Chen et al., 1996) and gelsolin-independence of focal adhesion disassembly.

Inhibition of MAP kinase prevents EGF-induced focal adhesion disassembly

In an initial dissection of the signaling pathway to focal adhesion disassembly, we postulated that MAP kinase may be involved, because MAP kinase is activated by both full-length and truncated EGFR constructs that lack the carboxy terminal autophosphorylation sites, such as c′973 (Chen et al., 1994a; Decker, 1993). Recently, MAP kinase signaling has been

Fig. 8. PD98059 prevents EGF-induced cell motility. WT NR6 (A) or Hs68 human diploid dermal fibroblasts (passage <12) (B) cells were treated with various concentrations of PD98059 for 90 minutes prior to EGF exposure (10 nM). Cell motility was determined in an in vitro 'wound healing' assay after 18 or 24 hours. Results shown are the mean ± s.e.m. of at least 3 experiments each performed in triplicate.

Fig. 7. PD98059 prevents EGF-induced mitogenesis and focal adhesion disassembly. To ascertain the requirement for erk MAP kinases in signaling of focal adhesion dissolution, the effect of an inhibitor of MEK, PD98059, was determined on EGF-induced erk phosphorylation (A), mitogenesis (B) and focal adhesion disassembly (C). WT NR6 cells were treated with various concentrations of PD98059 for 90 minutes prior to EGF (25 nM) stimulation. Mitogenesis was determined by thymidine incorporation 18-24 post-EGF exposure (Chen et al., 1994a). Phosphorylated erks were detected using a specific monoclonal antibody to tyrosyl-phosphorylated p42 and p44 (New England Biolabs); specimens were matched for either cell number or total protein in each experiment. A representative immunoblot is shown in A (repeated four times with WT and twice with c′973 NR6 cells); in B and C the results shown are the mean ± s.e.m. of 3 experiments each performed in duplicate or triplicate.

proposed as required for integrin-mediated haptotaxis (Klemke et al., 1997), further bolstering a role for these enzymes in cell motility. The MEK-specific pharmacological inhibitor PD98059 was added to WT EGFR-expressing NR6 cells prior to EGF exposure. We could note partial inhibition of MAP kinase phosphorylation at concentrations as low as 1 μM (Fig 7a). EGF-induced mitogenesis (as determined by incorporation of thymidine; Chen et al., 1994a,b) was affected at a low dose of 1 μM (Fig. 7b). Acute disassembly of focal adhesions also was prevented in a dose-dependent manner by PD98059 (Fig. 7c). PD98059 prevented approximately two-thirds of the focal adhesion disassembly; this incomplete abrogation may be related to only partial inhibition of erk activation or additional pathways leading to focal adhesion disassembly. These findings suggest that regulated activation of the erk MAP kinases is required for cell motility.

Cell migration of WT NR6 cells, as determined by in vitro wound healing assay (Chen et al., 1994a,b), was inhibited partially at 1 μM PD98059, with near complete abrogation by 20 μM (Fig. 8a). Furthermore, EGF-induced motility of Hs68 human diploid dermal fibroblasts (obtained from American Type Culture Collection, Rockville, MD) was abrogated by PD98059 in a similar dose-dependent manner (Fig. 8b).

DISCUSSION

These studies demonstrate that EGF induces disassembly of established focal adhesions, and that this cell response is actuated by a pathway distinct from the previously described PLCγ-actin modifying protein signaling cascade. Furthermore, this disassembly of focal adhesions is concomitant with acutely decreased cell adhesiveness to the substratum. Lastly, a pharmacological inhibitor, specific for MEK, which prevents focal adhesion disassembly also blocks EGF-induced motility. Thus, we have refined a model of EGFR-mediated cell motility to include at least two divergent, required pathways, one to cytoskeletal reorganization and the other to destabilization of cell-substratum interactions.

This is the first time, to our knowledge, that EGF has been reported to directly induce focal adhesion disassembly. Earlier communications failed to note focal adhesion disassembly upon acute EGF stimulation of rat embryo fibroblasts (Dunlevy and Couchman, 1993) and vascular smooth muscle cells (Herman et al., 1987). The discrepancy with our results may be due to differences in serum factors (as the previous analyses were performed on cells in the presence of high levels of serum or immediately upon removal from high serum), cell type (embryo fibroblasts do not require gelsolin for normal motility in the presence of serum; Witke et al., 1995) or cell interactions with specific substrata. However in the present experiments, EGF exposure resulted in loss of focal adhesions in both the immortal murine NR6 cells and explanted mouse fibroblasts (Figs 1 and 6). The EGFR-mediated decrease in cells possessing focal adhesions was reproducible and was reflected by a concomitant reduction in cell adhesiveness (Fig. 3). Disruption of cell-substratum contacts during cell locomotion may occur through regulated disassembly with recycling of components, as noted in neutrophil movement (Lawson and Maxfield, 1995) or traumatic detachment of focal plaques, as seen during rapid fibroblast haptotactic locomotion (Palecek et al., 1996; Regen and Horwitz, 1992). Our findings suggest that EGFR-mediated motility may utilize, at least in part, regulated adhesion disassembly and recycling of components to break and reform cell-substratum contacts.

Focal adhesion disassembly required EGFR kinase activity, but not the carboxy-terminal autophosphorylation motifs (Figs 1c and 4). This suggested that the biophysical process of detachment from the substratum did not require activation of the motility-associated PLCγ signaling (Bornfeldt et al., 1994; Chen et al., 1994b; Kundra et al., 1994). This was further demonstrated by focal adhesion disassembly in the face of inhibitors of PLC activity (Fig. 5). PLCγ activity is proposed to enhance cell motility through the mobilization of actin modifying proteins from an inactive membrane-associated localization to an active submembrane cytoskeletal locale (Arora and McCulloch, 1996; Chen et al., 1996; Janmey and Stossel, 1987). Gelsolin, an actin filament-severing protein critical for EGFR-mediated motility (Chen et al., 1996, and Fig. 6b), is not required for focal adhesion disassembly (Fig. 6a). Thus, we have identified a pathway for focal adhesion disassembly and modulation of cell-substratum interaction which diverges from the PLCγ pathway at the receptor level.

In an initial attempt to characterize the biochemical signals that modulate cell-substratum interactions, the MAP kinase pathway was probed. The erk MAP kinases are activated to similarly strong levels by full-length WT and truncated c′973 EGFR (Chen et al., 1994a; Decker, 1993), with c′973 presumably initiating the cascade via either SHC phosphorylation or erbB2 (VanEpps-Fung et al., 1996; Wright et al., 1995). A pharmacological inhibitor of MEK, PD98059 (Dudley et al., 1995), partially inhibited the EGFR-mediated loss of focal adhesion-positive cells (Fig. 7). This inhibition was achieved at concentrations which blocked EGF-induced cell motility and proliferation (Fig. 8). Therefore, activation of the erk MAP kinases are postulated to be required for EGFR-mediated cell motility. MAP kinase may also serve as a convergent element in growth factor- and integrin-mediated motility (Hughes et al., 1997; Klemke et al., 1997). However, the biochemical process which leads from MAP kinase to focal adhesion disassembly awaits further dissection. One possibility is that MAP kinase initiates inside-out signaling to alter integrin activation state allowing dissociation from the substratum, as a recent report demonstrated cell rounding after 24 hour activation of the erk MAP kinase pathway (Hughes et al., 1997). As this study did not determine whether erk activation either resulted in focal adhesion disassembly or prevented new adhesions from forming, further investigations are needed to determine whether the rapid focal adhesion disassembly noted herein are accomplished by this mechanism. Another possible pathway involves myosin-based contraction via activation of myosin light chain kinase (Klemke et al., 1997). This was required for cell haptotaxis; however, the specific biophysical aspect actuated by the myosin-based contraction (e.g. cell body translocation or cell-substratum detachment) was not determined. Furthermore, as cell attachment and extent of cell spreading were unaffected by inhibition of erk MAP kinase activation, the role of this signaling pathway in focal adhesion function and turnover is difficult to discern. In initial experiments, the myosin light chain kinase inhibitor ML-7 did not prevent EGFR-mediated focal adhesion disassembly at 30 minutes (data not shown), suggesting that the direct pathway may involve inside-out integrin signaling. Still, it is tempting to speculate that the latter pathway, involving myosin light chain kinase will be relevant at least in part for the rapid focal adhesion disassembly we observed. First, myosin II motors contribute to rear detachment of locomoting fibroblasts (Crowley and Horwitz, 1995; Jay et al., 1995). Second, EGF exposure of c′973 EGFR-expressing NR6 cells results in a rapid centripetal retraction of cytosol with extended cell processes containing phalloidin-staining filaments (Chen et al., 1996; Welsh et al., 1991); this phenotype would be consistent with tension-modulated in addition to avidity-modulated disassembly of contacts. Alternatively, the focal adhesion disassembly noted may result from a combination of decreased integrin avidity coupled with myosin-based retraction of membrane. However, the biochemical basis of this biophysical process will need to be determined by specific interventions of subsequent intermediaries, measuring both focal adhesion disassembly and adhesive strength.

A role for MAP kinase in growth factor-induced cell motility may not seem intuitive. Rather, the erk MAP kinases have been postulated to be involved in mitogenic signaling (Bornfeldt et al., 1995; Cobb et al., 1994; Waskiewicz and Cooper, 1995). In fact, disruption of this signaling pathway with PD98059, blocked EGF-induced thymidine incorporation in the NR6

cells (Fig. 7b), similar to inhibition of mitogenesis induced by other tyrosine kinase receptors (Dudley et al., 1995; Milasincic et al., 1996). However, the specific biophysical process triggered by this cascade, modulation of cell-substratum interactions, likely is key also to the mitogenic process (Bornfeldt et al., 1995; Varner et al., 1995). That the erk MAP kinases provide dual functions, transmission of signals to the nucleus and regulation of cell-matrix connections and signals, is an elegant integration of the multiple processes required for cell proliferation.

A model for cell motility can be refined based on the foregoing considerations. Growth factor-induced cell motility requires coordinate processes: lamellipodial extension, establishment of cell-substratum adhesions at the leading edge, and detachment from the substratum at the tail. The biochemical events which direct these process can only be speculated at present. We have defined at least two distinct intracellular signaling pathways, those utilizing PLCγ and MAP kinase, required for EGFR-mediated cell motility. The PLCγ-actin modifying protein cascade is postulated to provide at least part of the motive force required lamellipodial extension, as inhibition of PLC diminishes the amount of membrane extension (M. F. Ware et al., unpublished data). Activation of phosphatidyl-inositol 3′-kinase, which is required for PDGF- and HGF-induced fibroblast motility (Derman et al., 1996; Kundra et al., 1994; Wennstrom et al., 1994) but not for EGFR-mediated motility of NR6 cells as wortmannin does not inhibit this cell response (data not shown), may provide a similar signal by altering the phospho-inositide binding site availability or affinity for actin modifying proteins (Lu et al., 1996; Singh et al., 1996). Rho and rac may be speculated to be involved in establishing new cell-substratum attachments (Hotchin and Hall, 1995). The current pathway, involving the erk MAP kinases, is suggested to function in detachment of the locomoting cell from the substratum. Within this framework, specific experiments can be designed to link specific biochemical signaling pathways to their resultant biophysical motility responses.

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