Biophysical Integration of Effects of Epidermal Growth Factor and Fibronectin on Fibroblast Migration

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ABSTRACT Cell migration is regulated simultaneously by growth factors and extracellular matrix molecules. Although information is continually increasing regarding the relevant signaling pathways, there exists little understanding concerning how these pathways integrate to produce the biophysical processes that govern locomotion. Herein, we report the effects of epidermal growth factor (EGF) and fibronectin (Fn) on multiple facets of fibroblast motility: locomotion speed, membrane extension and retraction activity, and adhesion. A surprising finding is that EGF can either decrease or increase locomotion speed depending on the surface Fn concentration, despite EGF diminishing global cell adhesion at all Fn concentrations. At the same time, the effect of EGF on membrane activity varies from negative to positive to no-effect as Fn concentration and adhesion range from low to high. Taking these effects together, we find that EGF and Fn regulate fibroblast migration speed through integration of the processes of membrane extension, attachment, and detachment, with each of these processes being rate-limiting for locomotion in sequential regimes of increasing adhesivity. Thus, distinct biophysical processes are shown to integrate for overall cell migration responses to growth factor and extracellular matrix stimuli.

INTRODUCTION

Migration is a central cell function in wound healing, tumor metastasis, the immune response, angiogenesis, and development. Cell movement requires coordination of underlying biophysical processes including membrane extension and retraction, formation of new attachments at the cell front, generation of contractile force, and detachment of old attachments at the cell rear (Sheetz, 1994; Lauffenburger and Horwitz, 1996). It remains as yet largely unexplored as to how these processes are coordinated and regulated as an integrated system. A mathematical model has been constructed incorporating the key biophysical processes listed above, with some details of how they might depend on molecular-level properties (Lauffenburger, 1989; DiMilla et al., 1991). Experimental studies, both in vitro (DiMilla et al., 1993; Duband et al., 1991; Wu et al., 1994; Palecek et al., 1996; Huttenlocher et al., 1996; Condic and Letourneau, 1997) and in vivo (Ho et al., 1997), have been found to be consistent with predictions of this model concerning the effects of parameters characterizing interactions of adhesion receptors and extracellular matrix ligands, but a simultaneous examination of the multiple biophysical processes is required to understand the integration of the external signals.

Migration of many cell types depends on the nature of the extracellular matrix (ECM) substratum. The integrins, which are heterodimeric transmembrane proteins, bind to ECM molecules as well as to cytoskeletal elements and certain intracellular signaling molecules within the cell (Ginsberg et al., 1992; Hynes, 1992), providing signals from the ECM in addition to structural linkages (Clark and Brugge, 1995; Juliano and Haskill, 1993; Schwartz et al., 1995). Integrin-mediated signals regulate a variety of cellular functions such as differentiation, proliferation, and migration (Craig and Johnson, 1996; Rabinovitz and Mercurio, 1997; Sastry and Horwitz, 1993). ECM binding leads to the activation of second messengers that are also activated via the binding of growth factors to tyrosine kinase receptors (Kolanus and Seed, 1997; Miyamoto et al., 1996). Recent work has shown that synergy occurs between growth factor- and ECM-mediated events in the regulation of some of these signaling pathways and cell functions (e.g., Woodard et al., 1998; Ware et al., 1998; Mainiero et al., 1996; Plopper et al., 1995; Schubert, 1992). While these studies have concentrated on identification of signaling molecules within the cell which may be activated both by growth factors binding to their receptors and ECM molecules binding to integrins, little is known as to how these signals integrate at the biophysical level in regulating cell locomotion.

Epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF), among other growth factors, induce both mitogenic and motogenic responses in cell types such as fibroblasts, epithelial cells, and keratinocytes (Bornfeldt et al., 1994; Chen et al., 1994a,b; Kundra et al., 1994; Wennstrom et al., 1994). Increased levels of the epidermal growth factor receptor (EGFR), a receptor for EGF and related ligands, are noted in cancers in correlation with tumor progression to the invasive and metastatic state (Aaronson, 1991; Goustin et al., 1986; Heino, 1996; Liebermann et al., 1984). This increased EGFR expression has been suggested to increase EGFR-mediated cell motility and proliferation required for tumor

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progression (Turner et al., 1996; Wells et al., 1990). In addition, EGF-dependent increases in cell proliferation and motility have also been shown to result in accelerated wound healing (Shultz et al., 1991). Thus, EGFR-mediated cell motility has important pathological and physiological ramifications.

Growth factor and ECM effects on migration may operate by influencing cell-substratum adhesiveness, membrane activity, and/or contractile force generation (Lauffenburger and Horwitz, 1996). Growth factors such as EGF and PDGF enhance filopodia formation via cdc42 (Kozma et al., 1995; Nobes and Hall, 1995). They also stimulate short-term lamellipodial extension and membrane ruffling, which requires activation of rac (Ridley and Hall, 1992). In addition to affecting adhesiveness (Palecek et al., 1997), integrin binding to ECM may influence membrane activity and contractile force generation through signaling intermediates such as mitogen-activated protein kinase (MAPK) (Klemke et al., 1997; Lin et al., 1997). These types of studies provide insight into the components involved in regulation of motility by growth factors and ECM. An essential next step is the investigation of how this regulation at the molecular level is coordinated through effects on the biophysical processes that integrate to yield locomotion.

We initially address this integration and coordination of regulation by examining how the effects of EGF and the ECM protein fibronectin (Fn) on fibroblast locomotion speed operate through combined changes in membrane activity and cell/substratum adhesion. We use NR6 murine fibroblast cells, a 3T3-derived cell line, transfected with wild-type human EGFR (WT NR6 cells) as a well-characterized immortalized but nontransformed fibroblastic cell line, with EGF and Fn as well-characterized representatives of growth factor and ECM stimuli. We find that the effect of EGF on cell speed depends on the surface Fn concentration, with EGF able to either enhance or diminish locomotion. EGF reduces the strength of the cell-substratum adhesion at all Fn concentrations, implying that adhesion effects alone do not fully account for the cell speed changes. EGF also affects membrane extension activity: which is again dependent on the surface Fn concentration but in a manner which is not identical to its effects on cell speed. EGF diminishes, then enhances, and finally has no effect on membrane extension activity with increasing Fn concentrations. For maximal migration, cells require both high membrane activity and optimal, intermediate cell-substratum adhesion permitting not only attachment at the cell front but also detachment at the cell rear. EGF and Fn together influence each of these processes, so that their net overall effect arises from the integration of their individual effects on each of the biophysical processes.

MATERIALS AND METHODS

Cell culture

The generation of WT NR6 cells, a 3T3-derived murine fibroblastoid cell line lacking endogenous EGF receptor (EGFR), transfected with wild-type human EGFR, has been described previously (Chen et al., 1994a; Pruss and Herschman, 1977). WT NR6 cells were cultured in MEM-α media supplemented with fetal bovine serum (FBS; 7.5%), penicillin (100 U/ml), streptomycin (200 μg/ml), nonessential amino acids (1 mM), sodium pyruvate (1 mM), glutamine (2 mM), and G418 (350 μg/ml). Cells were passaged at subconfluence by trypsinization (0.25%, 1 mM EDTA) and incubated at 37°C, 90% humidity, and 5% CO_2. Assay medium used while performing the migration, adhesion, and membrane extension activity assays contained MEM-α with HEPES (25 mM), 1 gm/l BSA, 1% dialyzed FBS, penicillin (100 U/ml), streptomycin (200 μg/ml), nonessential amino acids (1 mM), sodium pyruvate (1 mM), glutamine (2 mM), and G418 (350 μg/ml). FACS analysis with the appropriate antibodies demonstrated the expression of the Fn receptors $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins on these cells (data not shown).

Surface preparation and substratum coating

Glass coverslips were acid-washed in 20% HNO_3 for 1 h, rinsed with deionized water for 1 h, and silanized by exposure to hexamethydisilazane vapor (Sigma Chemical Co., St. Louis, MO) for 30 min at 200°C. Coverslips (18-mm diameter) were used for migration and membrane extension activity assays, and 12-mm diameter coverslips were used for the adhesion assays. The coverslips were attached to the bottom of culture dishes using an optically clear adhesive (Norland Chemicals, New Brunswick, NJ). Surfaces were then coated with Fn (Sigma Chemical Co., St. Louis, MO). Thirty-five-mm dishes for migration and membrane extension activity assay were coated with 2 ml of appropriate concentration of Fn in PBS and incubated at room temperature for 2 h. Nonspecific protein adhesion was blocked by subsequent incubation in 1% BSA for 1 h. Dishes were washed three times with PBS and stored at 4°C. This protocol was also used to coat 24-well plates for the adhesion assay while maintaining a constant surface area to volume ratio.

Migration assay

Cell migration speed was measured using time-lapse videomicroscopy of single cells; 30,000 cells were plated onto 35-mm dishes in 2.5 ml serum-free medium. At 12 h post-seeding the medium was changed to 2.5 ml assay medium with or without 25 nM EGF and incubated at 37°C in humidified air for 8 h. At this cell density, soluble ligand concentration is relatively unchanged over a 24-h period (Reddy et al., 1996). Mineral oil (3 ml) was added to the dish to prevent evaporation, and the dish was then placed in a heated stage insert for a Ludl 99S008 motorized stage on a Zeiss Axiovert 35 microscope. Cell boundaries and centroids were identified using image processing software developed by Engineering Technology Center (Mystic, CT) running under a LabVIEW (National Instruments, Austin TX) and Concept Vi (Mystic, CT) environment. Five to ten cells per field in 10 different fields were scanned every 15 min for up to 20 h. The x and y coordinates of the cell centroids were recorded every 15 min. Single cell speed is calculated by determining the total path length as measured by the total centroid displacement divided by the tracking time. The reported cell speed $\pm$SEM for each condition is an average over 70–100 cells. For purposes of testing transient effects, cell speeds were calculated every 15 min by quantifying the centroid displacement every 15 min over a 20-h period starting immediately after addition of EGF, or in the control case, immediately after the media was changed to the assay media; the cells had been incubated in serum-free conditions for 12 h before addition of EGF. As will be presented below in the Results section, cell speed increased toward a plateau for 6–8 h following addition of EGF. Hence all subsequent migration measurements were carried out following an 8-h incubation period.

Adhesion assay

The adhesion assay was performed as previously described (Chu et al., 1994). Briefly, 24-well plates were plated with 20,000 cells per well in

serum-free conditions for 12 h. The media were then changed to the assay media with or without 25 nM EGF. Short-term adhesion with EGF was measured 30 min after addition of EGF. Adhesion was also measured at times comparable to the migration assay, 8 h after addition of EGF. The wells were filled with media and sealed using sealing tape avoiding air bubbles. The plates were then inverted and spun in a swing bucket SH-3000 rotor in a bench-top Sorvall centrifuge for 10 min at 25°C at 400, 600, or 800 × g. During each experiment, one plate at 3 μg/ml Fn coating concentration without EGF was kept at 1 g and used as a control. Cell number was quantified by manually counting cells in a defined well area. At least four wells were used at each condition and four fields were counted per well with each field in the control containing 300–400 cells. The cell number per well was normalized to the average cell number in the control well to obtain fraction adherent cells. The amount of centrifugal force required to detach 50% of the cells (F_{50}) was obtained from a plot of fraction adherent cells versus centrifugal force. The mean detachment force was calculated using the equation $f = RCF \cdot V \cdot (\rho_c - \rho_m)$, where f is the force on a cell, RCF is the relative centrifugal force, V is the cell volume, ρ_c is the density of the cell, and ρ_m is the density of the medium.

Membrane extension assay

The incubation protocol followed was identical to that described for the migration assay. The cells were videotaped using a 32× objective. Cell outlines were obtained every 15 min for a 1-h period. The protraction area was defined as the additional area extended by the cell at time $t = t_n + 15$ min when overlaid on the cell outline at time $t = t_n$. An average of four such areas over an hour divided by 15 min was defined as the absolute protrusion rate. Fractional protrusion activity was defined as the average rate of change of cell protrusion area normalized to the average cell area. The retraction area was defined as the area that was retracted by the cell at time $t = t_n + 15$ min when overlaid on the cell outline at time $t = t_n$. Similarly, fractional retraction activity was also measured by calculating the absolute retraction rate divided by the average cell area; 20–30 cells were analyzed at each condition.

RESULTS

EGF-stimulated cell migration requires a 6–8-h induction period for maximal migratory response

WT NR6 cells exhibit increased membrane activity, reorganization of the actin cytoskeleton, and disassembly of focal adhesions within 10 min of exposure to EGF (Xie et al., 1998). However, with prolonged exposure to EGF, these phenomena are less dramatic, and it has not been previously established how these changes correlate with the long-term motility response to EGF. We have previously observed that mean-squared displacements of WT NR6 cells measured every 30 min in the presence of EGF on Amgel, a biologically active extracellular matrix, increase with time of exposure to EGF up to a maximal value (Ware et al., 1998). Hence, we decided to examine whether the effect of EGF on cellular motility on fibronectin is dependent on the time of exposure to EGF. Cells were plated on 1 μg/ml Fn and incubated in serum-free media for 12 h before addition of EGF. We quantified cell speed over a 20-h period after addition of EGF. Cell speed was calculated at 15-min intervals by determining the change in cell centroid position during that time. The cell speed measured immediately after addition of EGF was lower than that in its absence. However, while the cell speed in the absence of EGF remained constant at its initial value of 18 μm/h, cell speed in the presence of EGF increased steadily over time for the first ~400 min (~7 h) after which it remained at its average maximal value of 40 μm/h for the rest of the course of the experiment (Fig. 1). This indicates that there is an induction time of ~6–8 h before a maximal migratory response is obtained in this cell type. Hence, all subsequent cell migration measurements with EGF were performed after the cells had been exposed to EGF for 8 h. For consistency, speed measurements in the absence of EGF were also performed following an 8-h incubation.

EGF regulation of cell speed is dependent on the surface fibronectin concentration

Because haptokinetic and haptotactic motility are modulated by substratum density (Aznavoorian et al., 1996; Dickinson and Tranquillo, 1993; Keely et al., 1995), we inves-

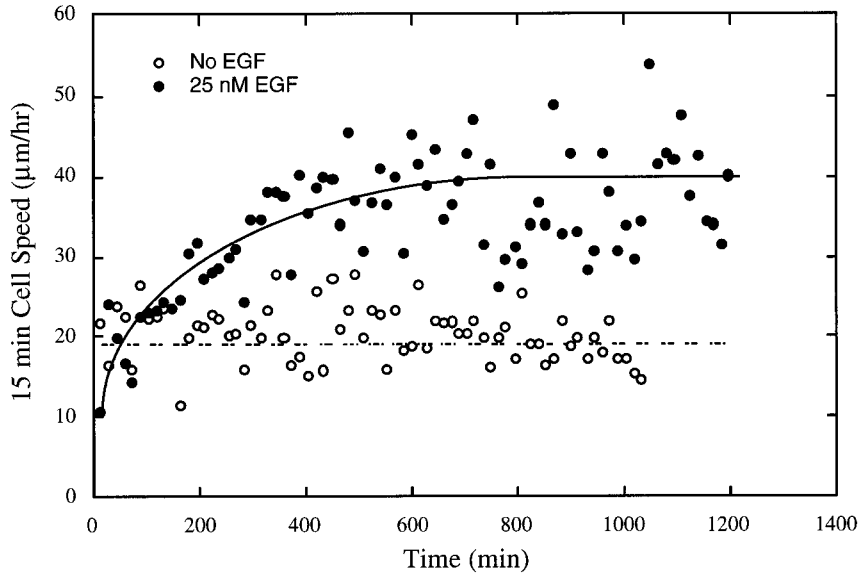

FIGURE 1 EGF-enhanced motility increases over an initial 6–8-h period. The effect of EGF on 15-min cell speeds of WT NR6 cells was determined as a function of time on 1 μg/ml Fn coating concentration. Mean centroid displacements were calculated every 15 min immediately upon addition of EGF for 20 h. Cells were plated in serum-free conditions for 12 h before addition of EGF.

tigated whether the effect of EGF on the motility was also dependent on the surface concentration of Fn. Cell speed was measured at varied surface Fn concentrations in the presence of saturating concentration of EGF and in the absence of EGF. The average cell speed of WT NR6 cells in the absence of EGF is roughly constant, independent of Fn coating concentration, at ~18 μm/h (Fig. 2). However, in the presence of EGF, cell speed demonstrated a biphasic dependence on the surface Fn concentration, with maximal cell speed of 42 μm/h occurring at an intermediate Fn concentration of 1 μg/ml. At the lowest Fn concentration of 0.1 μg/ml, cell speed in the presence of EGF actually was drastically reduced to 0.1 μm/h as compared to 16 μm/h in the absence of EGF. At Fn concentrations of 0.3 and 3 μg/ml, cell speeds in the presence of EGF were increased, though the increase was not as great as that observed at 1 μg/ml Fn concentration. Thus, EGFR-mediated cell motility is strongly dependent on Fn concentration, suggesting an interaction of the growth factor-stimulated pathways for cell motility regulation and those initiated upon ECM binding. Qualitatively similar results have been reported in a study of migration of this cell type under the influence of EGF on varying concentrations of Amgel (Ware et al., 1998).

EGF reduces cell adhesion at all surface fibronectin concentrations

In theoretical models, cell speed is predicted to depend on biophysical processes such as cell-substratum adhesion, contractile force generation, and membrane extension activity (Lauffenburger, 1989; DiMilla et al., 1991). Palecek et al. (1997) demonstrated that the variation of CHO cell speed with cell-substratum affinity, integrin level, and ECM substratum concentration could be explained by a variation in the strength of the cell-substratum adhesion. It has also been reported that short-term EGF exposure of WT NR6 cells causes a dramatic change in their morphology, resulting in decreased cell spread area and disassembly of focal adhesions (Ware et al., 1998; Welsh et al., 1991; Xie et al., 1998). Hence, we investigated whether the variation in EGF-stimulated migration on Fn could be explained by a variation in the cell-substratum adhesivity. The centrifugal force required to detach the cells is a measure of the strength of the cell-substratum adhesivity; the greater the F_{50} (see Materials and Methods), the stronger is the cell-substratum adhesivity. The cell adhesivity both in the presence and absence of EGF increased with increasing Fn coating concentration (Fig. 3). At each concentration of Fn, cell adhesion dropped substantially by 30 min after addition of EGF. By 8 h after addition of EGF, the cell adhesivity had risen to a value still significantly lower than that in the absence of EGF. Decreased adhesivity was most dramatic at the lowest Fn concentration of 0.1 μg/ml, where the cell-substratum adhesivity did not rise significantly even 8 h after addition of EGF following the fourfold decrease in F_{50} 30 min after addition of EGF.

In order to determine how locomotion speed in the presence and absence of EGF correlate with cell adhesivity, speed was determined as a function of the mean detachment force at each experimental condition by eliminating the Fn concentration as a common variable (Fig. 4). We observed that cell speed in the absence of EGF is not a function of cell adhesivity in the measured range of Fn concentration. However, under the influence of EGF, cell speed exhibits a biphasic dependence on cell adhesivity. Upon addition of EGF at 0.1 μg/ml Fn concentration the cell adhesivity dropped, indicating that the cells were so weakly adhered that they were unable to generate sufficient traction for locomotion. Cell morphology appeared rounded in the presence of EGF at this lowest surface Fn concentration, in

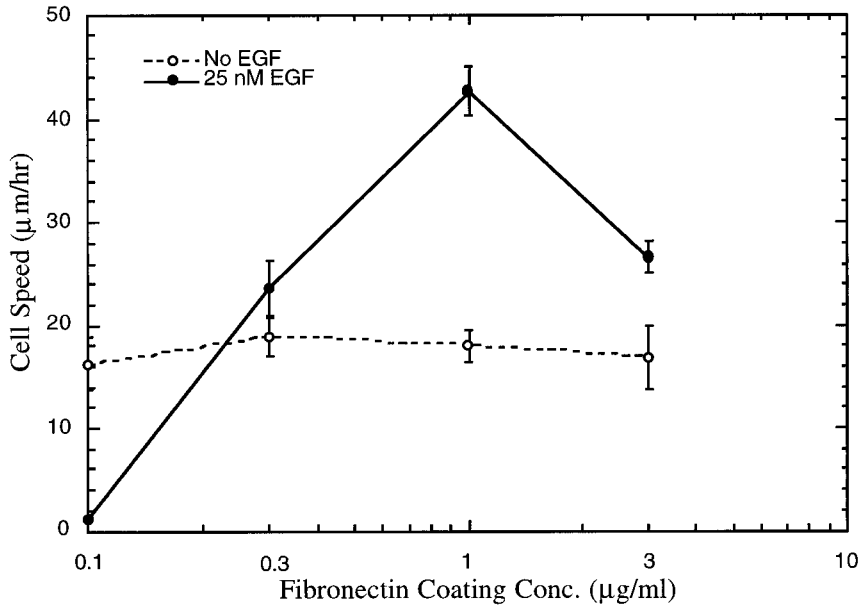

FIGURE 2 EGF differentially affects cell speed depending on fibronectin concentration. Single cells were tracked for 12 h after incubation with (*filled circles*) or without (*open circles*) EGF for 8 h on varying surface concentrations of Fn. The cell speeds are an average of 70–100 cells at each experimental condition over a 12-h time period.

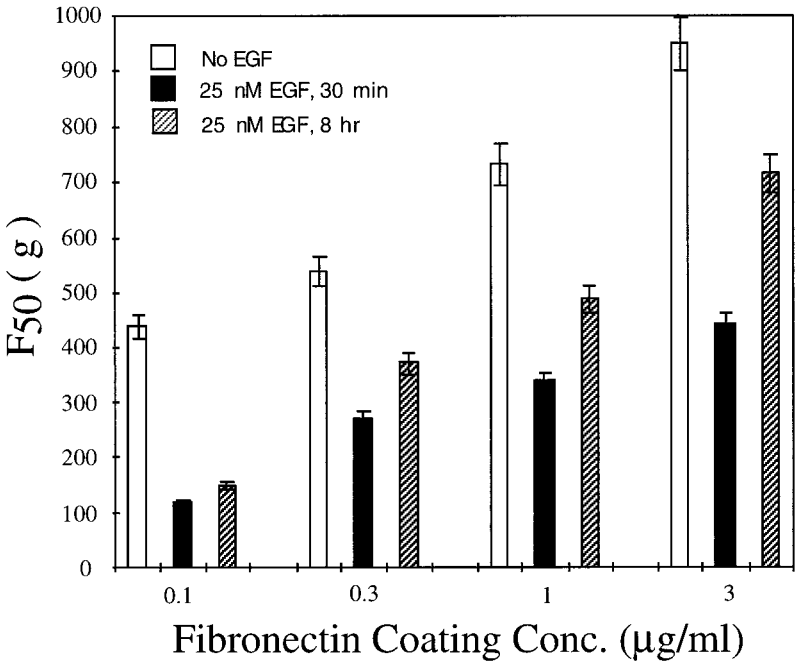

FIGURE 3 Cell adhesiveness is altered by both fibronectin concentration and EGF. Cells were plated in serum-free conditions on varying surface concentration of Fn in 24 well plates. In the absence of EGF, centrifugal force required to detach 50% of the plated cells (F_{50} in *g* units) was determined 12 h postplating (*open bars*). EGF was added 12 h after plating and F_{50} was determined 30 min after addition of EGF (*filled bars*). Adhesion was also quantified after 8 h of incubation with EGF (*hatched bars*). F_{50} was calculated from plots of fraction adherent cells as a function of centrifugal force (data not shown). These experiments were performed at centrifugation forces of 400, 600, and 800 × *g*. Bars represent standard errors.

contrast to well-spread cells in the absence of EGF (not shown). When the Fn concentration was increased to 3 μg/ml in the presence of EGF, locomotion speed decreased from its maximal value of 42 μm/h, indicating that the highly adherent cells were unable to generate sufficient contractile force to break cell-substratum bonds for detachment. At the Fn concentrations considered here, cell adhesion in the absence of EGF did not reach either extreme of cell-substratum adhesion levels where locomotion is completely inhibited. If adhesion were the sole physical process regulating cell speed in this situation, we would expect the plots of locomotion speed versus adhesivity in the presence and absence of EGF to collapse onto a single curve (Palecek et al., 1997). Instead, we observe that EGF reduces adhesion at all Fn concentrations, but that along with this reduction in adhesion an increase occurs in the maximal locomotion speed in the presence of EGF. Hence, the changes in cell speed induced by EGF can be explained only in part by effects on Fn-mediated adhesion.

Fractional membrane protrusion and retraction activity vary with surface fibronectin concentration in the presence of EGF but not in its absence

Since adhesion alone cannot fully explain the effects of EGF and Fn on WT NR6 motility, we investigated their

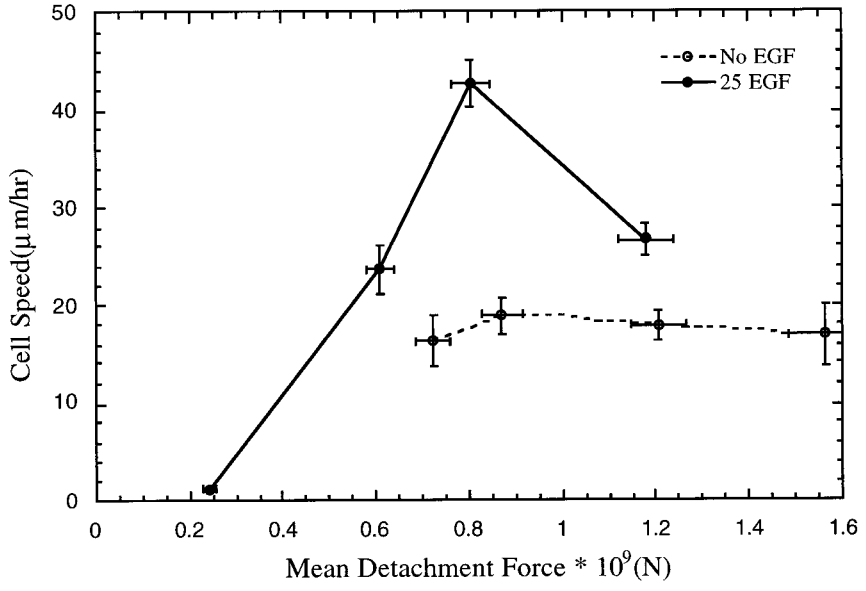

FIGURE 4 Cell speed as a function of mean detachment force. Data from Figs. 2 and 3 are replotted by eliminating the Fn concentration as a common variable. Mean detachment force is calculated from values of F_{50} (see Materials and Methods). Open circles represent no EGF preincubation, filled circles represent 8 h incubation in 25 nM EGF.

TABLE 1 Effect of EGF and Fn on cell area changes

Fibronectin Concentration (μg/ml)	Cell Spread Area (μm^2)		Absolute Protrusion Rate (μm^2/15 min)		Absolute Retraction Rate (μm^2/15 min)	
	0 nM EGF	25 nM EGF	0 nM EGF	25 nM EGF	0 nM EGF	25 nM EGF
0.1	790 ± 26	249 ± 12	107 ± 13	11 ± 0.5	96 ± 10	11 ± 0.5
0.3	1247 ± 29	857 ± 52	170 ± 26	273 ± 42	148 ± 27	262 ± 28
1.0	1352 ± 42	982 ± 35	190 ± 14	306 ± 21	170 ± 9	303 ± 28
3.0	1908 ± 68	1623 ± 39	196 ± 25	199 ± 28	213 ± 30	205 ± 30

Cell spread area was measured by outlining cells every 15 min over a 1-h interval; 20–30 cells were analyzed at each condition. Absolute protrusion and retraction rates were measured over a 1-h interval by calculating the change in cell spread area between subsequent cell outlines every 15 min. Refer to Materials and Methods for the description of individual measurements. The errors represent SEM.

effects on the cell membrane activity. In order to further understand the increase in maximal cell speed in the presence of EGF, we quantified the average membrane protrusion and retraction activity in the presence and absence of EGF on varied surface Fn concentrations. Cell spread area was also quantified under all the above conditions. The cell spread area increased with increasing surface Fn density in the presence as well as the absence of EGF, although the cell area in the presence of EGF was lower than that in its absence at each of the Fn concentrations. At intermediate concentrations of 0.3 and 1 μg/ml, the absolute protrusion rate was significantly greater in the presence of EGF than that in its absence. At the lowest Fn concentration of 0.1 μg/ml, EGF drastically reduced the absolute protrusion rate. However, at 3 μg/ml of Fn, EGF had no significant effect on the absolute protrusion rate. Identical trends were observed in the absolute retraction rates (Table 1).

To gain better insight into the effect of EGF on membrane activity, the absolute protrusion and retraction rates were normalized to the respective cell spread areas to account for the EGF and Fn effects on spreading (see Materials and Methods). The fractional protrusion activity was essentially independent of Fn concentration in the absence of EGF (Fig. 5). However, in the presence of EGF, the fractional protrusion activity was the highest at Fn concentrations of 0.3 and 1.0 μg/ml and was also significantly greater than that in the absence of EGF. At 0.1 μg/ml Fn, EGF significantly decreased the fractional protrusion activity. There was no observable effect on activity in the presence and absence of EGF at the highest Fn concentration of 3 μg/ml. Fractional retraction activity was similar to the fractional protrusion activity (Table 1). Hence, we find that EGF alters membrane activity of these cells in a manner which again depends upon Fn concentration. However, it is important to note that the variation of membrane activity does not precisely parallel the variation of locomotion speed with Fn concentration in the presence of EGF. At 0.3 μg/ml Fn, membrane activity is enhanced by EGF but locomotion speed is not. This discrepancy is significant for interpretation of the rate-limiting steps of migration, as will be discussed below.

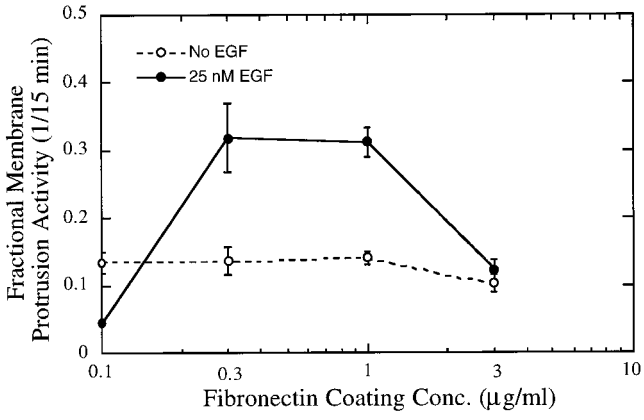

FIGURE 5 Membrane activity is altered by EGF depending on Fn concentration. Fractional membrane protrusion activity was determined as a function of Fn coating density with (*filled circles*) and without (*open circles*) EGF. Fractional membrane protrusion activity was calculated by normalizing the average absolute protrusion rate (see Table 1) by the average cell area at each experimental condition. Bars represent standard errors.

DISCUSSION

Cell migration is controlled by the concerted action of both growth factor receptors and integrins. We analyzed this integrated regulation at the level of underlying biophysical processes that govern cell movement, beginning with membrane protrusion activity and cell-substratum adhesion. Previously, there has been little information concerning how growth factors and extracellular matrix coordinately influence the biophysical processes that integrate to govern migration.

We find that sustained cell speed in the presence of EGF is a function of Fn concentration with the maximal cell speed occurring at intermediate Fn levels. A surprising result is that at low Fn concentration addition of EGF drastically reduces cell speed to levels well below that in the absence of EGF. At the same time, our centrifuge assay showed that EGF decreases adhesion both acutely and at the longer time scales relevant to migration. It is important to note again that more extensive experimental protocols than are typically employed for cell migration and adhesion studies are necessary in order to not miss these effects. For instance, if comparisons of cell locomotion speed in the absence and presence of EGF had been made here at only a single ECM concentration, we would have been led to an

incomplete understanding of its effect. Similarly, if adhesion had been measured here by a simple washing assay alone in which minimal distractive forces are applied, we would have incorrectly concluded that EGF had no effect on adhesivity.

Because EGF did reduce cell-substratum adhesivity, we might have initially speculated that EGF induces motility by reducing adhesion to substratum, with a threshold being required for motility (Xie et al., 1998). However, upon analyzing cell speed as a function of adhesivity (Fig. 4), it is clear that a change in adhesion alone cannot explain the variation in cell speed with Fn concentration in the presence of EGF. Hence, we also quantified the membrane extension activity to determine whether this is an additional biophysical phenomenon that helps govern locomotion speed. We find that EGF does indeed increase the absolute membrane activity at intermediate Fn concentrations and decreases the cell spread area at all the Fn concentrations studied. The trends and values of the absolute protrusion rate and the absolute retraction rate are identical, indicating that overall cell spread area is maintained at a constant level during the course of cell body translocation despite noticeable changes in cell shape. This is consistent with previous reports of a relationship between events at the cell front and rear (Chen, 1979; Weber et al., 1995). Fractional protrusion and fractional retraction (i.e., membrane activities normalized to spread area) are significantly elevated in the presence of EGF at intermediate Fn concentrations, though their levels decrease at extremes of the substratum density.

Analyzing locomotion speed as a function of membrane activity by eliminating Fn concentration as an independent variable permits elucidation of how membrane activity and adhesivity integrate to yield locomotion (Fig. 6). Locomotion speeds in the absence of EGF do not vary with fractional membrane protrusion activity. However, in the presence of EGF locomotion speed is affected by fractional membrane protrusion activity, but differently within three sequential adhesivity regimes. At the lowest Fn concentration, speed was the lowest along with the lowest membrane activity and adhesivity. At the slightly higher Fn concentration of 0.3 μg/ml, speed increased with a concomitant increase in fractional membrane protrusion activity. When the Fn concentration was further increased to 1.0 μg/ml, speed increased to its maximum value without a significant change in the fractional membrane protrusion activity, suggesting that at 0.3 μg/ml the cell speed had been limited by insufficient adhesive traction at the cell front. However, at the highest Fn concentration locomotion speed decreased, accompanied with a decrease in fractional membrane protrusion activity, probably due to an inability for cells to detach dynamically from the substratum; alternatively, high Fn concentrations may generate signals suppressing membrane activity.

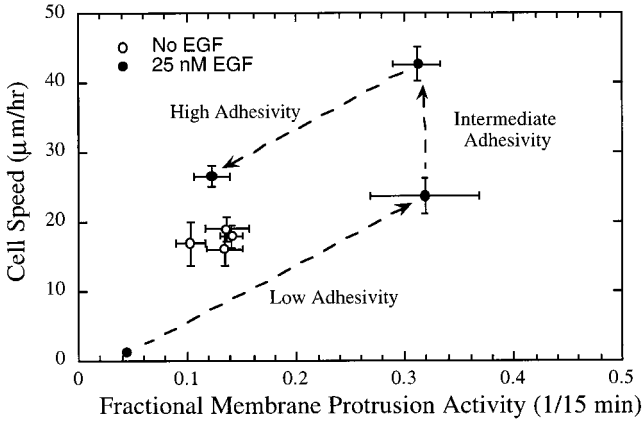

FIGURE 6 Cell speed can be considered a function of fractional membrane protrusion activity. Data from Figs. 2 and 5 were used to calculate the variation of cell speed with fractional membrane protrusion activity by eliminating the Fn concentration as a common variable. Cell speed in the absence of EGF (*open circles*) does not show significant variations with fractional membrane protrusion activity. Cell speed in the presence of EGF (*filled circles*) varies with fractional membrane protrusion activity through three regimes of adhesivity.

A different angle from which to illustrate the mechanisms by which EGF and Fn coordinately govern locomotion is seen by analyzing speed as a function of adhesion strength, again eliminating Fn concentration as an independent variable (Fig. 7). We find that there is no significant variation in either speed or membrane activity in the absence of EGF (Fig. 7*a*). However, both speed and membrane activity vary with adhesion strength in a biphasic manner in the presence of EGF (Fig. 7*b*). At the low adhesion condition of 0.1 μg/ml Fn, both membrane activity and locomotion speed are lower in the presence than in the absence of EGF, likely due to the inability of membrane protrusions to form stable attachments with the substratum. At 3 μg/ml Fn, both membrane activity and locomotion speed are roughly similar in the presence and absence of EGF, probably due to the inability of cells to detach dynamically under this strong adhesion condition or alternatively due to signals suppressing membrane activity. At 0.3 μg/ml Fn, however, membrane activity in the presence of EGF is highly stimulated, while locomotion speed is not significantly increased. This discrepancy may be caused by an ability of extended membrane to form a stable attachment, but one that is not sufficiently strong in traction for contractile forces to give rise to cell body translocation. At the condition of 1 μg/ml Fn, though, adhesivity has increased enough for this translocation to occur.

The increase in membrane extension activity with EGF at certain Fn concentrations is consistent with the increased membrane ruffling and filopodia formation (Segall et al., 1996) accompanied with increased activity of rac and cdc42, respectively (Ridley and Hall, 1992). The initial decrease in adhesion upon addition of EGF is expected based on the observation that EGF causes short-term disassembly of focal adhesions and loss of stress fibers, and results in a rounded cell morphology (Welsh et al., 1991; Xie et al., 1998). It remains to be determined exactly what causes this decrease in effective adhesion; possible candidates include integrin downregulation, affinity decrease in

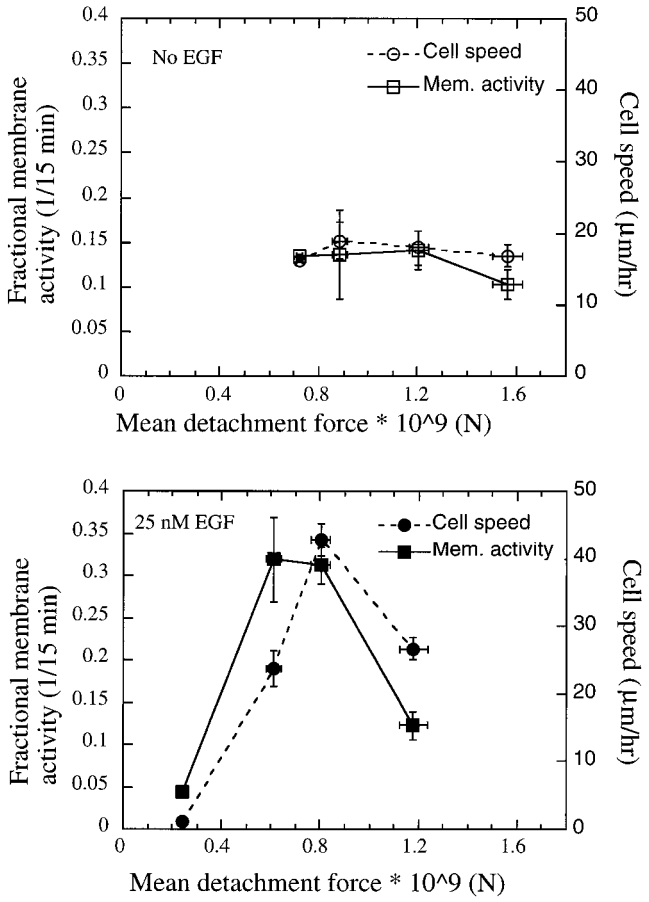

FIGURE 7 Cell speed and fractional membrane protrusion activity are modulated in a biphasic manner by the strength of cell-substratum adhesion. (*a*) Cell speed (*open squares*) and fractional membrane protrusion (*open circles*) activity do not vary significantly with the strength of cell-substratum adhesion in the absence of EGF. (*b*) In the presence of EGF, both cell speed (*filled squares*) and fractional membrane protrusion activity (*filled circles*) vary with cell/substratum adhesiveness in a biphasic manner. High membrane activity as well as optimal adhesion are essential for maximal cell speed.

integrin-ECM interactions, induced proteolysis of adhesion components, or increased contractile force generated within the cell. We quantified $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin expression in the absence of EGF 30 min after exposure to EGF and 8 h after exposure to EGF, and found no variation in their expression (data not shown); this strongly suggest that downregulation of at least $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins is not involved. Future work will define the cell signals that alter integrin function to accomplish the biophysical process of membrane extension.

We note that a rigorous investigation of the time-dependence of growth factor-induced cell motility has not been reported. Knowing when cells start to move and how long it takes to reach maximal speed is required to link biochemical events to the physiological consequences. Activity of signaling pathways, often assayed within minutes of growth factor exposure, usually declines with prolonged EGF exposure (Chen et al., 1994b; Waters et al., 1996). We found that despite the diminished membrane activity over time, cell motility continued to increase significantly, not approaching maximal speed until 6–8 h after EGF addition (Fig. 1). Clear implications are that motility must be studied either after an induction period or over an extended period of time of which the induction time is a small fraction, and that candidate causal signaling activities must be assayed during active motility, unless it is the acute activities—which may be ultimately unrelated to sustained migration—that are of central focus. The gradual increase in motility insinuates cellular adaptation or reprogramming for maximal responsiveness to EGF. That cell locomotion can be noted even at the earliest time periods, however, does suggest that the basic response and motility machinery are in place and, at least, partially activatable. New protein synthesis or transcription of specific genes are speculated as being important for full response. This would be consistent with observations that low doses of actinomycin-D block cell motility (Bauer et al., 1992; Chen et al., 1994a; Gordon and Staley, 1990).

The finding (Fig. 2) that migration speed in the absence of exogenous EGF is essentially independent of Fn coating concentration is an interesting finding in itself, although consistent with at least one recent report (Ware et al., 1998). This kind of situation has not been investigated in detail in previous literature. One reason is that studies of cell migration are typically performed in the presence of substantial levels of serum or a protein-containing medium even when not exploring effects of specific growth factors. Another is that studies of cell migration generally do not explore function over a wide range of substratum ligand concentrations. An intriguing possible explanation is that at low levels of soluble exogenous stimuli, the diverse effects of integrin-mediated signals [e.g., force generation (Klemke et al., 1997), affinity modulation (Hughes et al., 1997), and membrane activity (Lin et al., 1997)] coordinate intracellularly to maintain a relatively constant balance of membrane extension activity, adhesion, and force generation.

We believe that our study provides new insight into the integration of signaling pathways initiated from growth factors and ECM through the biophysical readouts of cell migration, cell-substratum adhesion, and membrane activity. We suggest that the regulation of locomotion by Fn and EGF overall can be understood in terms of membrane extension activity, attachment, and detachment as providing rate-limiting steps in sequential regimes of increasing cell/substratum adhesivity (see Figs. 6 and 7). Membrane extension is limiting at lowest adhesivity, then membrane attachment for traction is limiting at intermediate adhesivity, and finally, membrane detachment is limiting at highest adhesivity. Whether other cellular factors such as contractile force, front-versus-rear asymmetry, and mechanical properties are additionally affected by synergistic regulation by EGF and Fn—or, more generally, growth factors and ECM—remains to be investigated, as they are also implicated in the theoretical models for cell migration (Lauffenburger, 1989; DiMilla et al., 1991). We believe that bio-

physical analysis analogous to that presented here offers a useful framework for understanding the action of various components involved in and regulated by biochemical signaling pathways.

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