Receptor Tyrosine Kinase Signaling Required for Integrin αvβ5-directed Cell Motility but Not Adhesion on Vitronectin

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Abstract. FG human pancreatic carcinoma cells adhere to vitronectin using integrin αvβ5 yet are unable to migrate on this ligand whereas they readily migrate on collagen in an α2β1-dependent manner. We report here that epidermal growth factor receptor (EGFR) activation leads to de novo αvβ5-dependent FG cell migration on vitronectin. The EGFR specific tyrosine kinase inhibitor tyrphostin 25 selectively prevents EGFR autophosphorylation thereby preventing the EGF-induced FG cell migration response on vitronectin without affecting constitutive migration on collagen. Protein kinase C (PKC) activation also leads to αvβ5-directed motility on vitronectin; however, this is not blocked by tyrosine kinase inhibitors. In this case, PKC activation appears to be associated with and downstream of EGFR signaling since calphostin C, an inhibitor of PKC, blocks FG cell migration on vitronectin induced by either PKC or EGF. These findings represent the first report implicating a receptor tyrosine kinase in a specific integrin mediated cell motility event independent of adhesion.

CELL migration plays a critical role in a variety of biological processes including embryonic development, angiogenesis, wound repair, and tumor cell metastasis. Though the detailed mechanisms of cell migration are poorly understood, it is clear that adhesion events are crucial to this process. The adhesive interactions between cells and the extracellular matrix are mediated by integrins, a family of cell surface receptors that bind to extracellular matrix proteins including vitronectin, collagen, and fibronectin (Hynes, 1992). While many cells express integrin receptors and are capable of adhesion their capacity to promote cell motility is likely to be regulated independently by growth factors or cytokines. In fact, cytokines such as epidermal growth factor (EGF) and insulin-like growth factor one (IGF-1)[1] induce cell motility of a wide variety of cells (Stracke et al., 1989; Stoker and Gherardi, 1990; Chen et al., 1993; Matthay et al., 1993). However, the mechanism regulating cytokine-induced cell motility has not been clearly defined.

FG human carcinoma cells provide a useful model for assessing the role of physiological factors that regulate cell migration. Specifically, FG cells attach to vitronectin using integrin αvβ5 yet are unable to migrate on this ligand whereas they are competent to migrate on collagen in an α2β1-dependent manner (Leavesley et al., 1992). These findings imply that FG cell motility on collagen is regulated by intrinsic intracellular events whereas αvβ5-dependent migration on vitronectin requires an exogenous stimulus. In this report, we provide direct evidence that the EGF receptor (EGFR) stimulates a protein kinase C-dependent signaling pathway leading to the induction of αvβ5-dependent FG cell migration on a vitronectin substrate. These findings provide the first link between the activation of a tyrosine kinase receptor, protein kinase C, and a specific integrin-mediated motility event. Moreover, evidence is presented that this signaling pathway leads to αvβ5-directed motility but not adhesion to vitronectin.

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1. *Abbreviations used in this paper*: FBM, fibroblast basal medium; IGF-1, insulin-like growth factor one; PKC, protein kinase C.

Materials and Methods

Cells and Cell Culture

FG human pancreatic carcinoma cells were provided by Dr. Shama Kajiji and Vito Quaranta (The Scripps Research Institute, La Jolla, CA). FG-B is a subline stably transfected with a full-length cDNA encoding human β_3 gene and, thus, expresses functional αvβ3 (Leavesley et al., 1992). All cells were grown in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FBS and 50 μg/ml gentamycin and were free from mycoplasma during these studies. Before testing all cells were starved for 24 h by replacing serum containing culture media with FBS-free RPMI.

Monoclonal Antibodies and Reagents

Integrin-specific mAbs P3G2 (anti-αvβ5; Wayner et al., 1991), 6F1 (anti-α2β1; Coller et al., 1990), LM609 (anti-αvβ3; Cheresh and Spiro, 1987), P4C10 (anti-β1; Carter et al., 1990), and AP3 (anti-β3; Dr. P. Newman, Blood Center of Southwestern Wisconsin, Milwaukee, WI) 661 (anti-vitronectin, Nip et al., 1992) were purified from ascites on protein A-Sepharose. MAbs to the epidermal growth factor and insulin-like growth factor receptors were obtained from Oncogene Science (Uniondale, NY) and PY20 (biotinylated anti-phosphotyrosine) was from ICN Biomedicals,

Inc. (Costa Mesa, CA). Horseradish peroxidase-conjugated strepavidin/biotin complex was from Amersham Corp. (Arlington Heights, IL). Tyrphostin 25 ([3,4,5-trihydroxyphenyl]-methylene propanedinitrile) was purchased from Calbiochem (San Diego, CA). Tyrphostin 63 ([4-hydroxybenzyl] malonitrile) an analogue that does not inhibit EGFR kinase activity was obtained from LC Laboratories (Woburn, MA). Phorbol, 12-myristate, 13-acetate (PMA), genistein, and calphostin C were acquired from Calbiochem. Human recombinant EGF and IGF-1 were obtained from Genzyme (Cambridge, MA).

Adhesive Ligands

Vitronectin was prepared as described by Yatohgo et al. (1988). Collagen type 1 was obtained from Upstate Biotechnology Inc. (Lake Placid, NY).

Cell Migration Assay

Cell migration assays were performed using modified Boyden chambers containing polycarbonate membranes (tissue culture treated, 6.5-mm diameter, 10-μm thickness, 8-μm pores, Transwell®; Costar, Cambridge, MA). The under surface of the membrane was coated with vitronectin or collagen (10 μg/ml in PBS, pH 7.4, unless otherwise noted) for 2 h at 37°C. Excess ligand was removed and the lower chamber filled with 0.5 ml of fibroblast basal medium (FBM)[1] containing 0.5% BSA (FBM-BSA; Clonetics, San Diego, CA). Cells were harvested with trypsin/EDTA (GIBCO BRL) and the trypsin inactivated with soybean trypsin inhibitor (Sigma Chem. Co., St. Louis, MO). Cells were washed with FBM-BSA then added to the upper chamber (100,000 cells/0.1 ml FBM-BSA) and allowed to migrate in the presence or absence of the appropriate cytokine or PMA for various times at 37°C in 6% CO_2. To evaluate the effects of kinase inhibitors on migration, cells were pretreated with tyrphostin 25 (50 μM for 12–16 h, genistein (50 μM for 1 h) or calphostin C (40 nM for 30 min) before being treated with cytokine or PMA for 1 h and then washed and allowed to migrate as described above. The upper surface of the membrane was then wiped with a cotton-tip applicator to remove nonmigratory cells and the migrant cells on the under surface fixed and stained for 20 min with 1% crystal violet in 0.1 M borate, pH 9.0, and 2% ethanol. The number of stained cells per 40× field were counted with an inverted microscope or the dye was eluted with 10% acetic acid and its absorbency determined at 600 nm. Random migration was determined by coating both the lower and upper membrane with the appropriate adhesive protein. In a typical experiment, only a few cells (i.e., 1–5) randomly migrate to the lower membrane. Nonspecific or background migration was evaluated on BSA-coated membranes and subtracted from all data points. In all cases, cell migration on BSA was less than 1% of the values obtained with adhesive proteins. Each determination represents the average of three individual wells and error bars represent the standard error of the mean (SEM).

Adhesion Assay

Cell adhesion was performed according to Leavesley et al. (1992) with minor modifications. Briefly, 48-well cluster plates (polystyrene, non-tissue culture treated; Costar, Cambridge, MA) were coated with 10 μg/ml vitronectin or collagen 1 in PBS, pH 7.4. Proteins were allowed to bind for 2 h at 37°C before the wells were rinsed and blocked for 2 h with 2% heat-denatured BSA (RIA grade; Sigma) in PBS, pH 7.4. Cells were harvested as for the migration assay and added to the wells at a concentration of 100,000 cells/0.1 ml FBM-BSA. Nonadherent cells were removed with gentle washing and cell attachment determined with a colorimetric cell titer assay (Cell Titer 96™; Promega Corp., Madison, WI). Each data point was calculated from triplicate wells and was expressed as the mean ± SE. Nonspecific cell adhesion as measured on BSA-coated wells has been subtracted.

Flow Cytometric Analysis

Cells pretreated in the presence or absence of EGF (100 ng/ml) for 24 h were harvested as for the migration assay, rinsed twice in ice-cold FACS buffer (PBS with 2% FBS and 0.1% sodium azide, pH 7.4) then incubated with primary antibodies (10 μg/ml) on ice for 1 h. Cells were washed twice with excess FACS buffer and then incubated with secondary antibody (phycoerytherin-conjugated goat anti-mouse immunoglobulin [diluted 1/100]; Fischer, Pittsburgh, PA) for 45 min on ice. Cells were analyzed with a Becton-Dickinson FACScan flow cytometer. Cell analysis was gated on forward and size scatter intensities. The results were presented as single parameter histograms.

Immunoprecipitation and Antiphosphotyrosine Blotting of EGF and IGF-1 Receptors

FG cells were grown to 80% confluency in T75 flasks (~10–15 × 10^6 cells/flask with RPMI containing 10% FBS. The culture media was removed and the cells incubated in serum-free RPMI for 12–16 h in the presence or absence of 50 μM tyrphostin. EGF (100 ng/ml) or IGF-1 (20 ng/ml) was added directly to the flask containing the labeled cells for 1 h at 37°C then rinsed twice with ice-cold Hanks balanced salt solution (pH 7.4) containing 2 mM sodium vanadate and isolated with a rubber policeman. Cells were lysed with RIPA buffer 100 mM Tris, 0.15 M NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 1% aprotinin, 2 mM PMSF, 10 μg/ml leupeptin, and 5 mM EDTA supplemented with 2 mM sodium vanadate for 1 h at 4°C. The lysate was clarified by centrifugation at 14,000 rpms for 20 min and the amount of total protein determined using the BCA protein assay reagent (Pierce Chem. Co., Rockford, IL). Protein concentration was normalized to 500 μg/ml with RIPA buffer then preabsorbed with an equal volume of pansorbin (Calbiochem) for 2–4 h at 4°C. The pansorbin was removed by centrifugation at 14,000 rpms for 10 min and the receptors precipitated with anti-EGF and anti-IGF-1 receptor antibodies bound to protein G (10 μg antibody/10 μl stock bead suspension; Pierce) for 16 h at 4°C. The beads were then washed three times with each of the following buffers: RIPA, PBS with 0.5% tween 20 and 1 mg/ml ovalbumin, and PBS 0.5% Tween 20. Immunoprecipitates were resuspended in SDS electrophoresis sample buffer with 5% 2-mercaptoethanol and then heated to 100°C for 5 min. Receptors were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 6% gels under reducing conditions. Western analysis of immunoprecipitates was performed using biotinylated antiphosphotyrosine monoclonal antibody (PY20) and horseradish peroxidase-conjugated strepavidin/biotin complex and the ECL system (Bronstein et al., 1992).

Results

EGF Specifically Promotes FG Cell Migration on Vitronectin

FG human carcinoma cells attach to vitronectin but are unable to migrate on this ligand whereas they readily attach to and migrate on collagen (Leavesley et al., 1992). These findings suggest that migration on vitronectin requires an additional stimulus. Because EGF and other cytokines are known to promote migration of a wide variety of cell types, we investigated the possibility that FG cell migration on vitronectin could be induced with EGF. As shown in Fig. 1, EGF promotes FG cell migration on vitronectin in a dose-dependent manner. Migration was maximal at 100 ng/ml and could be inhibited with a monoclonal antibody to the EGF receptor. Similar results were observed when cells were pretreated with EGF (100 ng/ml) for 1 h, rinsed and then allowed to migrate for 24 h (data not shown). As previously reported (Leavesley et al., 1992), FG cell migration on collagen takes place in the absence of EGF and as we show here is not stimulated by this cytokine (Figs. 1 and 2) indicating that FG cell migration on these matrix proteins are regulated by distinct intracellular pathways. Furthermore, cell migration on vitronectin and collagen showed the same dose response where the half maximal ligand coating concentration is 2–3 μg/ml (Fig. 2). It is important to note that in the absence of EGF FG cells are incapable of cell migration on vitronectin even at the superoptimal dose of 20 μg/ml (Fig. 2).

Integrin $\alpha v\beta 5$ Mediates EGF-induced FG Cell Migration on Vitronectin

FG cells utilize integrin $\alpha v\beta 5$ as their primary vitronectin receptor since they fail to express $\alpha v\beta 3$ (Cheresh et al., 1989; Leavesley et al., 1992). To investigate whether $\alpha v\beta 5$

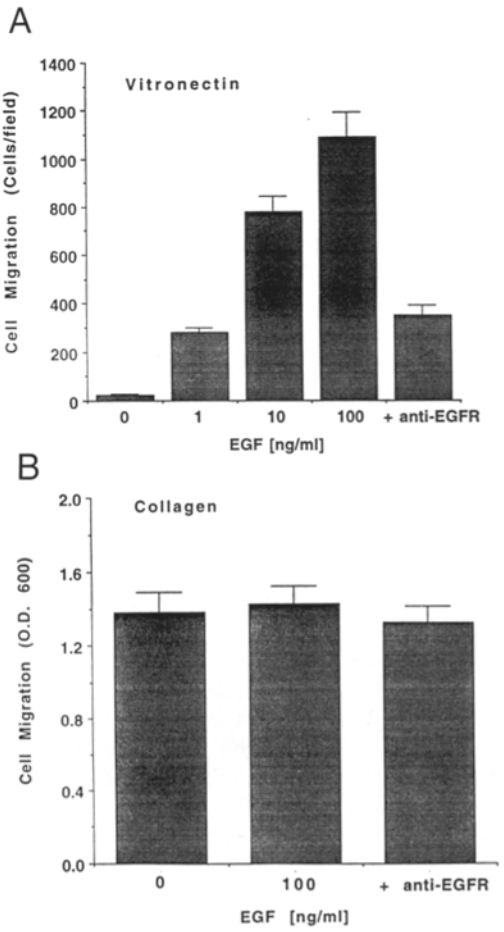


Figure 1. Effect of EGF on FG cell migration on vitronectin or collagen. Cell migration on vitronectin (*A*) or collagen (*B*) was determined using a modified Boyden chamber with a 8-μm porous membrane coated with the appropriate extracellular matrix protein (10 μg/ml). Cells were allowed to traverse the membrane for 24 h in the presence of various concentrations of EGF or EGF (100 ng/ml) plus anti-EGFR antibodies (10 μg/ml). The cells were then stained and counted or enumerated from dye uptake as described in Materials and Methods. Each bar represents the mean ± SE of three replicate wells.

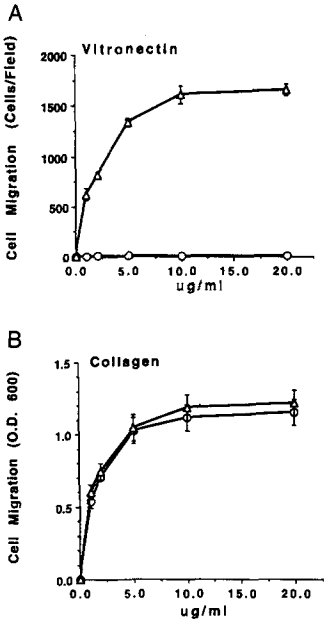


Figure 2. Effect of EGF on FG cell migration on various concentrations of vitronectin or collagen. Cell migration was evaluated using a modified Boyden chamber with a porous membrane coated with various concentrations of vitronectin (*A*) or collagen (*B*). Cells were allowed to traverse the membrane for 24 h in the presence (*triangles*) or absence (*circles*) of EGF (100 ng/ml). Cells were then stained and counted or enumerated from dye uptake as described in Materials and Methods. Each point represents the mean ± SE of three replicate wells.

was responsible for mediating EGF-induced FG cell migration on vitronectin, cell migration assays were performed in the presence of specific anti-integrin monoclonal antibodies. As shown in Fig. 3, mAb P3G2 to a functional epitope on integrin $\alpha v\beta 5$ inhibited EGF-induced FG cell migration on vitronectin but had no effect on collagen-dependent migration. Similarly, mAb 661 to vitronectin specifically inhibited migration on this ligand. In contrast, mAb P4C10 to the $\beta 1$ integrin subunit inhibited migration on collagen but failed to affect EGF-induced migration on vitronectin. As expected, mAb LM609 directed to $\alpha v\beta 3$, not expressed by these cells, had no effect on migration on either ligand. These data demonstrate that EGF-induced migration on vitronectin is mediated by the integrin $\alpha v\beta 5$.

EGF Does Not Alter Integrin Expression on FG Cells

To determine whether EGF-induced cell migration on vitronectin was the result of a quantitative change in the number of surface receptors, cells were treated in the presence or absence of EGF for 24 h and examined for cell surface integrin $\alpha v\beta 5$ and $\alpha v\beta 3$ or the EGFR. As shown in Fig. 4, exposure of FG cells to EGF dramatically decreases the number of EGF receptors on the cell surface as previously reported for cells exposed to EGF (Das and Fox, 1978). However, this treatment did not influence the expression of integrins $\alpha v\beta 5$ or $\alpha v\beta 3$ (Fig. 4). Thus, EGF-induced cell migration on vitronectin is not the result of increased surface expression of $\alpha v\beta 5$ or de novo expression of the vitronectin receptor $\alpha v\beta 3$.

EGF Has No Quantitative Effect on FG Cell Adhesion to Vitronectin but Promotes Cell Spreading

We investigated whether EGF-induced cell motility on vitronectin was due to activation of $\alpha v\beta 5$ leading to a quantitative change in FG cell adhesion to vitronectin. Cells incubated in the presence or absence of EGF were allowed to

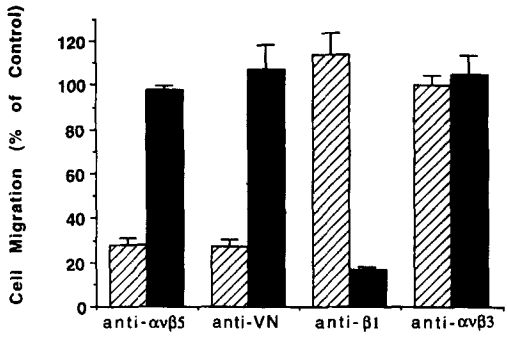


Figure 3. Role of $\alpha v\beta 5$ in EGF-induced cell migration on vitronectin. FG cell migration on vitronectin (*hatched bar*) or collagen (*shaded bar*) was performed in the presence of monoclonal antibodies (50 μg/ml) to functional epitopes on vitronectin (VN; 661) or integrins $\alpha v\beta 5$ (P3G2), $\alpha v\beta 3$ (LM609), and $\beta 1$ (P4C10). Cell migration was performed as described in Materials and Methods. Each bar represents the mean percentage migration relative to an EGF-stimulated control in the absence of antibody ± SE of three replicate wells.

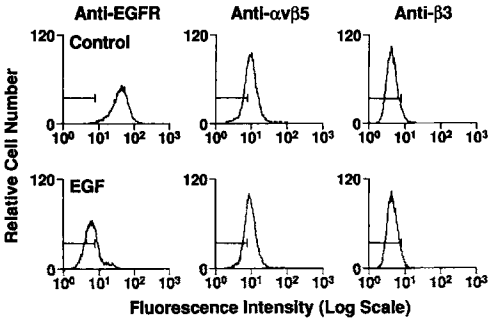


Figure 4. Effect of EGF on EGF receptor and integrin expression. Cells pretreated with EGF (*bottom*) or control buffer (*top*) for 24 h were stained with either anti-EGFR or anti-integrin antibodies to αvβ5 (P3G2), α2β1 (6F1), and β3 (AP3). The cells were rinsed, stained with phycoerytherin-conjugated secondary antibody, and analyzed with a FACScan flow cytometer. The bar represents background fluorescence of cells treated with an irrelevant antibody. The results are presented as single parameter histograms.

attach to wells coated with vitronectin or collagen for various times. As shown in Fig. 5, *A* and *B*, EGF did not alter the rate or number of FG cells attached to vitronectin or collagen compared to those cells not treated with EGF. Thus, EGF-induced FG cell migration on vitronectin was not the result of a general increase in cell adhesiveness to this ligand.

Cell migration not only depends on adhesion but also on the ability of cells to change shape in response to their substrate. Because cell spreading is a prominent feature of motile cells, we investigated the possibility that EGF induces FG cell spreading on vitronectin. Cells were incubated in the presence or absence of EGF and then allowed to attach and spread on coverslips coated with vitronectin or collagen. As shown in Fig. 5 *C*, FG cells not treated with EGF attach yet fail to spread on vitronectin whereas they readily attach and spread on collagen. However, pretreatment of cells with EGF promoted measurable spreading on vitronectin (Fig. 5 *C*). In this case, a significant number of these cells (40–50%) spread after 2–4 h. Thus, FG cell spreading and migration on vitronectin requires activation with a cytokine such as EGF while collagen-dependent spreading and migration does not. These results suggest that the ability of EGF to promote FG cell migration on vitronectin may be linked to its capacity to induce spreading on this ligand.

EGF-induced FG Cell Migration on Vitronectin Requires Autophosphorylation of the EGFR

Autophosphorylation of tyrosine kinase receptors by their ligands represent an initial signaling event in a pathway leading to intracellular kinase activity resulting in cellular responses including proliferation and motility (Ullrich and Schlessinger, 1990). To determine if phosphorylation of the EGFR was necessary for EGF-induced FG cell migration on vitronectin, we utilized tyrphostin 25, a specific inhibitor of the EGFR tyrosine kinase. FG cells pretreated with or without 50 μM tyrphostin 25 were stimulated to migrate on vitronectin with EGF. As shown in Fig. 6, tyrphostin 25 significantly inhibited EGF-induced migration on vitronectin while having no effect on collagen migration. This effect was dose-dependent reaching maximum inhibition at 50–100 μM (data not shown). Moreover, cells washed free of the inhibitor and cultured for 24 h showed restored EGF-dependent migration on vitronectin demonstrating that the effects of tryphostin 25 were fully reversible and not toxic (data not shown). In addition, an analogue of tyrphostin (tyrphostin 63) that does not block autophosphorylation of the EGFR had no effect on EGF-induced migration on vitronectin at similar concentrations (data not shown).

In a parallel experiment, the effect of tyrphostin 25 on cell attachment was examined. In this case, the drug had no effect on cell attachment to vitronectin or collagen (Fig. 6). Interestingly, cell spreading on vitronectin but not on collagen was blocked by tyrphostin 25 indicating that the ability of EGF to promote FG cell migration may be linked to activation of the EGFR tyrosine kinase (data not shown). In addition, tyrphostin 25 did not inhibit cell spreading and migration on vitronectin of FG cells transfected with the β_3 gene as these events are constitutive and depend on de novo expression of integrin αvβ3 (Fig. 7) as shown previously (Leavesley et al., 1992). Taken together, these results indicate that tyrphostin 25 specifically inhibits αvβ5-dependent EGF-induced cell spreading and migration on vitronectin without affecting cell attachment to this ligand.

EGF and other cytokines activate specific as well as common intracellular signaling pathways leading to a number of important biological processes. In fact, both EGF and IGF-1 activate protein kinase C (PKC) which is thought to mediate signaling events involved in various cellular processes (Cochet et al., 1984; Lin et al., 1986; Czech, 1989). Therefore, to establish the specificity of the EGF-signaling pathway we examined the ability of tyrphostin 25 to inhibit cell migration on vitronectin induced by activation of IGF-1R another tyrosine kinase receptor or PMA, a potent activator of PKC. FG cells treated with these agonists for 1 h were washed, rinsed, and allowed to migrate for various times. As shown in Fig. 8 *A*, EGF, IGF-1, and PMA all stimulate FG cell migration on vitronectin. Increasing the concentration or length of exposure to the agonists did not increase their migration (data not shown). It is important to note that IGF-1 and PMA-induced cell motility on vitronectin is also mediated by αvβ5 since anti-αvβ5 antibodies specifically blocked migration on this ligand (data not shown). As shown in Fig. 8 *B*, tyrphostin 25 only blocked EGF-dependent motility since it failed to inhibit motility induced by IGF-1 or PMA. These results demonstrate that the effects of tyrphostin 25 are due to its ability to specifically block migration induced by the EGFR. However, direct activation of PKC by phorbol ester can apparently bypass the tyrphostin 25 inhibitable signaling event initiated by the EGFR. In support of this contention, the PKC inhibitor calphostin C blocks EGF, IGF-1, as well as PMA-induced αvβ5-directed FG cell migration on vitronectin (Table I), yet has no effect on cell migration on collagen (data not shown). Also, genistein (an inhibitor of EGFR as well as IGF-1R tyrosine kinase activity) blocks motility induced by either cytokine, yet has no effect on PMA-induced cell migration. These results indicate that PKC is associated with and downstream from the EGF or IGF-1 signaling pathway leading to αvβ5-dependent motility (Table I).

Tyrosine kinase receptor signaling events are typically initiated by autophosphorylation of the receptor. As shown in Fig. 9 *A*, EGF and IGF-1 readily stimulate autophosphorylation of their respective receptors. However, cells pretreated with tyrphostin 25, then stimulated with EGF showed basal

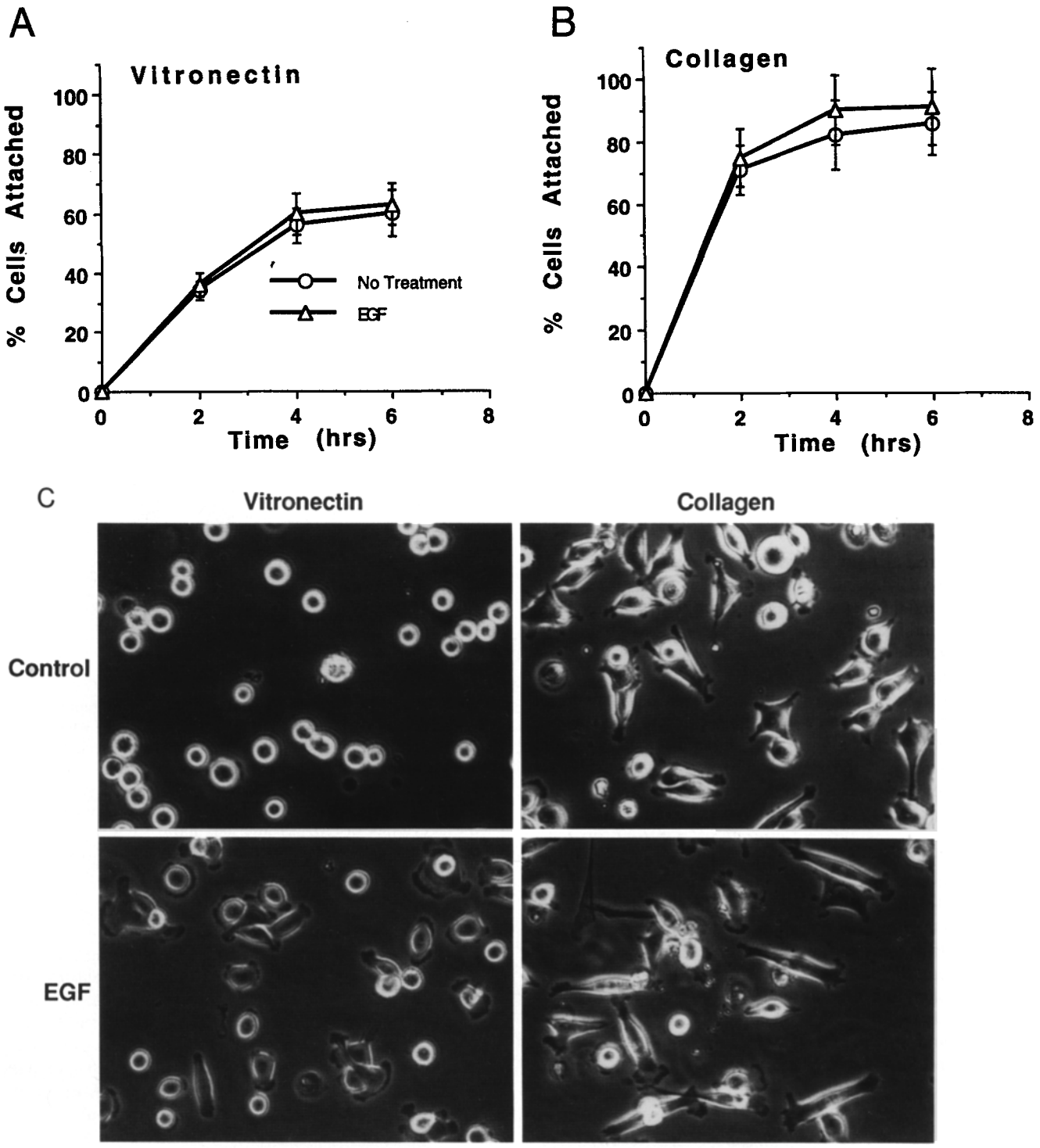


Figure 5. Effect of EGF on FG cell adhesion and spreading on vitronectin and collagen. (*A* and *B*) Cells pretreated in the presence (*triangles*) or absence (*circles*) of EGF (100 ng/ml) for 24 h were allowed to adhere to vitronectin or collagen for various times. Unattached cells were removed by washing and the remaining cells quantified by measuring the conversion of MTT tetrazolium salt to formazan dye (see Materials and Methods). Cell attachment to BSA-coated wells was subtracted as background. Each point represents the mean ± SE of three replicates. (*C*) Cells pretreated in the presence or absence of EGF (100 ng/ml for 24 h) were allowed to attach and spread on glass coverslips coated with vitronectin or collagen (40 μg/ml) for 4 h before being photographed at 300× magnification as described in Materials and Methods.

levels of EGFR autophosphorylation. In contrast, tyrphostin 25 had no effect on IGF-1–induced IGF-1R autophosphorylation. These results demonstrate that tyrphostin 25 specifically inhibits EGFR activation while having no effect on IGF-1R tyrosine kinase and this likely accounts for its selective effect on EGF-induced FG cell migration on vitronectin (Fig. 8). We also examined the ability of the anti-EGFR antibody to block autophosphorylation of the EGFR. This anti-

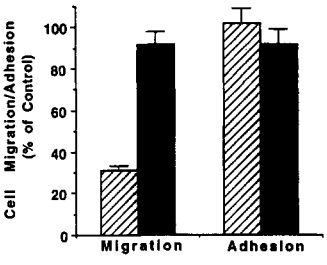


Figure 6. Effect of the tyrosine kinase inhibitor tyrphostin 25 on FG cell migration and adhesion. Cells were pretreated with tyrphostin 25 (50 μM) for 12–14 h before being stimulated with EGF (100 ng/ml) for 1 h. The cells were rinsed and then either allowed to migrate on vitronectin (*hatched bar*) or collagen (*shaded bar*) for 12 h or tested in a 2-h adhesion assay as described in Materials and Methods. Each bar represents the mean percentage migration/adhesion relative to an EGF stimulated control in the absence of tyrphostin 25 ± SE of triplicate wells.

body blocks EGF-induced motility on vitronectin (Fig. 1) and as shown as in Fig. 9 *B*, also blocks EGFR autophosphorylation. Together these findings demonstrate that αvβ5-directed motility of FG cells depends on a signaling pathway mediated by the activation of receptor tyrosine kinases and protein kinase C.

Discussion

Evidence presented in this study demonstrates that EGF promotes αvβ5-dependent carcinoma cell migration on vitronectin and this depends on activation of the EGFR tyrosine kinase and PKC. These findings provide the first link between activation of a receptor tyrosine kinase, PKC, and an integrin-dependent motility event.

Previous studies from our laboratory have shown that FG cells express integrin αvβ5 as their primary vitronectin receptor yet they are unable to migrate on this ligand even though they readily migrate on collagen using integrin α2β1 (Leavesley et al., 1992). Moreover, FG cells transfected with a cDNA encoding the β3 subunit express integrin αvβ3 enabling them to constitutively migrate on a vitronectin substrate. These findings suggest that integrin αvβ5 requires an exogenous stimulus to engage the motility machinery whereas integrins α2β1 and αvβ3 on these cells do not.

It is well documented that EGF as well as other cytokines induce motility of a variety of cell types, however, little is known about the intracellular factors that mediate these events (Stoker and Gherardi, 1991). Results from recent studies imply that integrins may function cooperatively with cytokines and growth factors to integrate and transmit signals required for cell attachment and motility (Chen et al., 1993; Matthay et al., 1993). Therefore, we examined the ability of various cytokines to promote FG cell migration on vitronectin. EGF and IGF-1, two activators of receptor tyrosine kinases, were found to specifically promote FG cell migration on vitronectin since they had no effect on FG cell migration on collagen. In addition, PKC activation promoted FG cell migration on vitronectin. It is of interest to note that EGF and IGF-1 are cytokines whose activity can result in PKC activation (Rosen et al., 1991; Tramm et al., 1991; Halaban et al., 1992). Our results demonstrate that PKC is downstream from the EGFR stimulation since calphostin C blocks EGF, IGF-1, and PMA-induced motility without effecting cell adhesion to vitronectin. This contention is also supported by the finding that tyrosine kinase inhibitors block migration induced by EGF and IGF-1 but not PMA. Thus, αvβ5-dependent migration of FG cells requires activation of a receptor tyrosine kinase and PKC while α2β1-dependent motility of these cells involves a completely distinct signaling pathway.

The specific mechanism responsible for EGF-induced FG cell migration on vitronectin remains to be elucidated. However, it is clear that this motility event is not the result of a

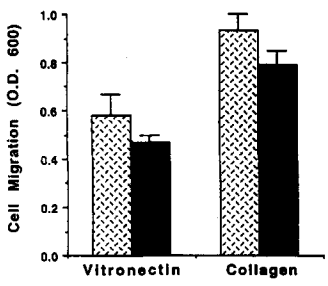
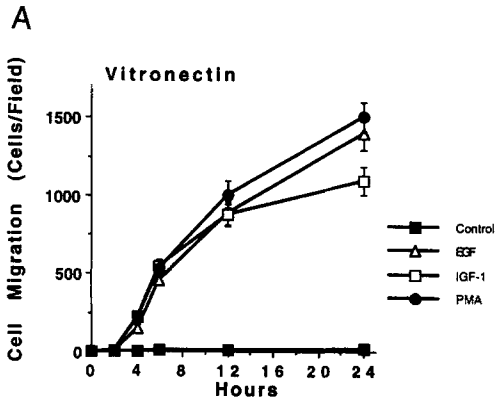


Figure 7. Effect of tyrphostin 25 on FG-B cell migration. FG cells stably transfected with a cDNA encoding β3 (FG-B cells express integrin αvβ3, Leavesley et al., 1992) were pretreated for 12–14 h in the presence (*shaded bar*) or absence (*hatched bar*) of tyrphostin 25 (50 μM) and then allowed to migrate on vitronectin or collagen for 24 h. Cell migration was enumerated from dye uptake as described in Materials and Methods. Each bar represents the mean ± SE of triplicate wells.



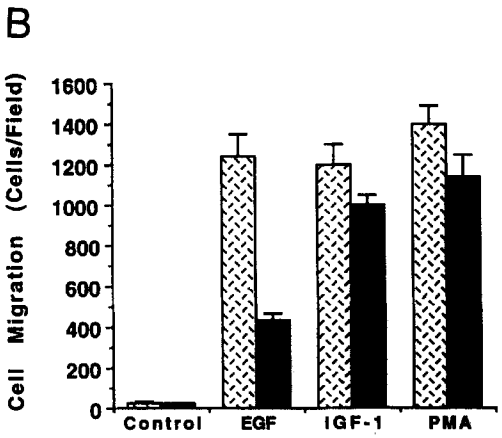


Figure 8. Effect of tyrphostin 25 on PMA and IGF-1 stimulated FG cell migration on vitronectin. (*A*) Cells were pretreated with buffer (*control*), EGF (100 ng/ml), PMA (5 ng/ml), or IGF-1 (20 ng/ml) for 1 h then washed and allowed to migrate for various times. (*B*) Cells were pretreated in the presence (*filled bar*) or absence (*stippled bar*) of 50 μM tyrphostin 25 for 12–16 h before being stimulated with cytokine or PMA as described above. Cells were allowed to migrate for 18–24 h. Each bar or point represents the mean ± SE of triplicate wells.

Table I. Effect of Tyrosine Kinase and Protein Kinase C Inhibitors on Agonist-induced Migration

Inhibitor	No treatment	EGF	IGF-1	PMA
No inhibitor	16.3 ± 4*	1638 ± 31	991 ± 116	1553 ± 42
Calphostin C‡	NT¶	334 ± 54	131 ± 53	584 ± 55
Tyrphostin 25§	NT	463 ± 36	763 ± 64	1495 ± 25
Genistein‖	NT	584 ± 55	226 ± 56	1474 ± 49

* FG cells were either pretreated in the presence or absence of inhibitors and then treated with buffer (no treatment), EGF (100 ng/ml), IGF-1 (20 ng/ml) or PMA (5 ng/ml) for 1 h. Cells were washed and then allowed to migrate for 24 h before being stained and counted as described in the Materials and Methods. Values represent the mean ± SE of the number of cells migrating per field of three replicate wells.
‡ Specific inhibitor of protein kinase C.
§ Specific inhibitor of EGF receptor tyrosine kinase.
‖ General inhibitor of tyrosine kinase.
¶ *NT*, not tested.

general increase in the number of cell surface vitronectin receptors since EGF did not alter expression of $\alpha v\beta 5$ or promote de novo expression of $\alpha v\beta 3$. Furthermore, FG cell adhesion to vitronectin was not quantitatively affected by EGF treatment. Interestingly, EGFR activation did promote FG cell spreading on vitronectin which may facilitate migration of these cells. The ability of $\alpha v\beta 5$ to respond to EGFR signaling may be due to direct phosphorylation of the αv or $\beta 5$ subunit (Freed et al., 1989). Alternatively, after EGFR activation, other cytoplasmic factors may become associated with $\alpha v\beta 5$ or influence its ability to interact with the actin cytoskeleton which, in turn, may promote spreading and motility of FG cells on vitronectin.

The EGF receptor possesses intrinsic tyrosine kinase activity that is central to the regulation of a wide range of biological responses including cell proliferation and motility (Ullrich and Schlessinger, 1990; Chen et al., 1994). It is now clear that EGF binding leads to phosphorylation of the receptor and to various intracellular substrates that are important for transmitting intracellular signals (Ullrich and Schlessinger, 1990). Tyrphostins are a family of potent tyrosine kinase inhibitors that have been used as indicators of the involvement of the tyrosine kinases in a variety of cellular processes (Yaish et al., 1988; Faaland et al., 1991; Merkel et al., 1993). In fact, one such compound, tyrphostin 25, has been shown to selectively inhibit the EGFR tyrosine kinase activity. (Yaish et al., 1988; Gazit et al., 1989; Lyall et al., 1989). Moreover, this inhibitor is known to selectively block EGFR kinase activity without affecting EGF binding or EGF-induced degradation and down regulation of EGFRs in intact cells (Lyall et al., 1989; Faaland et al., 1991). Therefore, we utilized tyrphostin 25 to examine the role of EGFR stimulated cell migration on vitronectin. Several lines of evidence indicate that this kinase inhibitor specifically impacted the EGFR signaling pathway leading to FG cell migration on vitronectin. First, tyrphostin 25 inhibited migration on vitronectin without affecting the intrinsic ability of FG cells to migrate on collagen. Also, transfection of FG cells with a cDNA encoding $\beta 3$ promoted expression of $\alpha v\beta 3$ enabling these cells to constitutively migrate on vitronectin and this was not inhibitable with tyrphostin 25. Second, tyrphostin 25 had no effect on FG cell adhesion to vitronectin or collagen indicating the effects were restricted to $\alpha v\beta 5$-dependent cell motility. Third, two other activators of FG cell motility, namely, IGF-1 and PMA, were not affected by this inhibitor. Finally, tyrphostin 25 selectively inhibited ligand-induced autophosphorylation of the EGFR but not the IGF-1R within FG cells. These results suggest that the EGFR kinase is critical for the EGF-induced FG cell migration on vitronectin via integrin $\alpha v\beta 5$. The recent finding that EGF-induced receptor autophosphorylation and tyrosine kinase activity was required for motility and dispersion of murine fibroblast cells transfected with the human EGFR is consistent with this notion (Chen et al., 1994). Thus, our results demonstrate that cytokine receptor tyrosine kinases play an important role in the regulation of a signaling pathway leading to the induction of cell motility. The specific activation of $\alpha v\beta 5$ and its engagement with the motility machinery may impact the biological properties of cells expressing this integrin which include most if not all epithe-

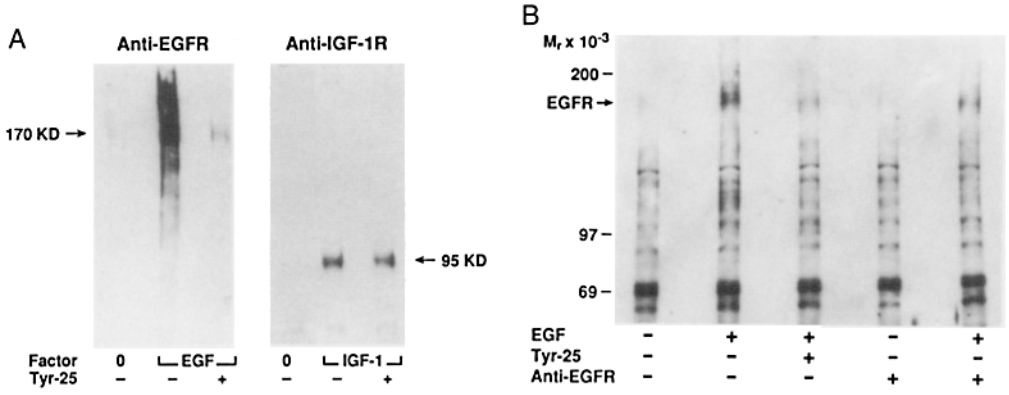


Figure 9. Effect of tyrphostin 25 on EGF and IGF-1 induced receptor tyrosine phosphorylation. Detergent lysates from FG cells were resolved on 6% gels, transferred to nitrocellulose, and Western blotted using antiphosphotyrosine antibodies as described in Materials and Methods. (*A*) Antiphosphotyrosine blot of EGF and IGF-1 receptors isolated by immunoprecipitation from FG cells treated as in Fig. 8 *B*. (*B*) Antiphosphotyrosine blot of proteins (10 μg/lane) from FG cells treated as in Fig. 1.

lial cells (Pasqualini et al., 1993). Moreover, our findings provide the first link between the activation of a tyrosine kinase receptor and an integrin mediated motility event. These results support the contention that cross-talk between cytokines and specific integrins may be important for the induction of cell motility during embryonic development, angiogenesis, and wound repair.

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