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Integrin-mediated Signaling Events in Human Endothelial Cells

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Vascular endothelial cells are important in a variety of physiological and pathophysiological processes. The growth and functions of vascular endothelial cells are regulated both by soluble mitogenic and differentiation factors and by interactions with the extracellular matrix; however, relatively little is known about the role of the matrix. In the present study, we investigate whether integrin-mediated anchorage to a substratum coated with the extracellular matrix protein fibronectin regulates growth factor signaling events in human endothelial cells. We show that cell adhesion to fibronectin and growth factor stimulation trigger distinct initial tyrosine phosphorylation events in endothelial cells. Thus, integrin-dependent adhesion of endothelial cells leads to tyrosine phosphorylation of both focal adhesion kinase and paxillin, but not of several growth factor receptors. Conversely, EGF stimulation causes receptor autophosphorylation, with no effect on focal adhesion kinase or paxillin tyrosine phosphorylation. Adhesion to fibronectin, in the absence of growth factors, leads to activation of MAPK. In addition, adhesion to fibronectin also potentiates growth factor signaling to MAPK. Thus, polypeptide growth factor activation of MAPK in anchored cells is far more effective than in cells maintained in suspension. Other agonists known to activate MAPK were also examined for their ability to activate MAPK in an anchorage-dependent manner. The neuropeptide bombesin, the bioactive lipid lysophosphatidic acid (LPA), and the cytokine tumor necrosis factor α, which signal through diverse mechanisms, were all able to activate MAPK to a much greater degree in fibronectin-adherent cells than in suspended cells. In addition, tumor necrosis factor α activation of c-Jun kinase (JNK) was also much more robust in anchored cells. Together, these data suggest a cooperation between integrins and soluble mitogens in efficient propagation of signals to downstream kinases. This cooperation may contribute to anchorage dependence of mitogenic cell cycle progression.

INTRODUCTION

The vascular endothelium represents an important interface between the blood vessel lumen and tissues, is central to early stages of development, and is involved in the structural and functional regulation of homeostasis and in tissue perfusion including regulation of vascular tone and permeability (Folkman and Shing, 1992; Wagner and Risau, 1994). It is also important to the processes of wound healing, inflammation, and angiogenesis (Clark *et al.*, 1982; Folkman, 1992), and plays a critical role in various disease processes, such as atherosclerosis, the growth of solid tumors, and metastasis (Blood and Zetter, 1990; Folkman, 1992, 1995; Ross, 1993). A wide range of factors are known to regulate endothelial function; these factors include vasoconstrictors, vasodilators, anticoagulants, growth factors, and cytokines [basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), TGF-β, and tumor necrosis factor α (TNFα)]. In addition, endothelial cells are influenced by expression of engagement of adhesion molecules such as the β_1 integrins. Integrin binding, cytoskeletal organization, and consequent changes in cell shape are inti-

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mately involved in the process whereby cell adhesion to the extracellular matrix or adjacent cells regulates endothelial cell migration, proliferation, and differentiation, angiogenesis, and apoptosis (Hynes, 1992; Juliano and Haskill, 1993; Frisch and Francis, 1994; Clark and Brugge, 1995; Varner *et al.*, 1995). The extracellular matrix is required for endothelial cell survival and proliferation in response to growth factors, rendering cells apoptotic in the absence of matrix (Meredith *et al.*, 1993; Re *et al.*, 1994).

Remodeling of blood vessels and the concomitant reorganization of the cytoskeleton requires the involvement of integrins, which comprise a family of at least 20 different $\alpha\beta$ heterodimers in mammals (Hynes, 1992). In addition to playing a major role in angiogenesis and apoptosis, integrins are also believed to be important in maintaining endothelial cell polarity and in the sensing and responding to changes in blood vessel flow conditions. Several different members of the integrin family are expressed on the surface of endothelial cells, including the receptors for fibronectin, laminin, and collagen. Different combinations of integrin subunits on the cell surface allow cells to recognize and respond to a variety of extracellular matrix proteins under different physiological conditions. For example, the $\alpha_v\beta_1$ and $\alpha_5\beta_1$ fibronectin receptors are highly expressed in quiescent endothelial cells, whereas the $\alpha_v\beta_3$ fibronectin and vitronectin receptor is expressed only during the process of angiogenesis (Brooks *et al.*, 1994a). In fact, antagonist blocking of $\alpha_v\beta_3$ has been shown to induce apoptosis in proliferating endothelial cells (Brooks *et al.*, 1994b).

Binding of integrins with extracellular matrix proteins is characterized by extracellular domain conformational changes, cytoskeletal reorganization, and integrin clustering (Burridge *et al.*, 1988; Loftus *et al.*, 1994). In many cell types, clustering of integrins leads to formation of focal adhesion complexes containing cytoplasmic cytoskeletal proteins and also provides a framework for the assembly of catalytic signaling proteins (Burridge *et al.*, 1988; Yamada and Miyamoto, 1995). Integrin ligation has been shown to result in activation of a variety of cellular signaling pathways (Rosales *et al.*, 1995). In many cell types, integrin binding induces tyrosine phosphorylation of specific proteins, including focal adhesion kinase (FAK), a cytoplasmic nonreceptor tyrosine kinase, and paxillin, an adapter protein (Burridge *et al.*, 1992; Hanks *et al.*, 1992; Schaller *et al.*, 1992). FAK plays a key role in regulating the dynamic changes in actin cytoskeleton organization that are a prerequisite for cell migration. The tyrosine phosphorylation of FAK is stimulated by β1 and β3 integrins (Guan and Shalloway, 1992; Huang *et al.*, 1993; Ishida *et al.*, 1996), as well as by numerous regulatory peptides and lipids acting on G protein-coupled receptors (Zachary *et al.*, 1993; Seufferlein and Rozengurt, 1994). Paxillin, a 68-kDa protein that colocalizes to focal adhesions (Burridge *et al.*, 1992), has been shown to associate with FAK and is a putative substrate for FAK (Turner *et al.*, 1993; Hildebrand *et al.*, 1995).

Although integrins show no intrinsic protein kinase activity (Hynes, 1992), their engagement activates signal-transducing molecules usually associated with growth factor stimulation, including protein kinases, inositol lipid kinases, G proteins, and the Na^+/H^+ antiporter (Ingber *et al.*, 1990; Guan *et al.*, 1991; Guan and Shalloway, 1992; Kornberg *et al.*, 1992; Schwartz and Lechene, 1992; Kapron-Bras *et al.*, 1993; Chen and Guan, 1994; Chen *et al.*, 1994; Schlaepfer *et al.*, 1994). Accumulating evidence demonstrates overlap between signals generated by integrins and the consensus Ras-signaling pathway triggered by receptor tyrosine kinases that is important in cell proliferation and differentiation (Chen *et al.*, 1994; Schlaepfer *et al.*, 1994; Zhu and Assoian, 1995). Similar to stimulation of tyrosine kinase receptors, integrin-mediated cell adhesion has been shown to activate MAPK, a key downstream effector of the Ras-signaling pathway (Chen *et al.*, 1994). However, in fibroblasts, recent studies show that FAK activation and integrin-mediated MAPK activation are independent events (Lin *et al.*, 1997a,b). Since integrins stimulate many of the same signal transduction events as growth factors, including phosphorylation and activation of MAPK, numerous laboratories have explored the possibility that integrins and growth factors might function cooperatively with respect to MAPK activation (Assoian, 1997; Renshaw *et al.*, 1997; Schwartz, 1997). Most of the studies thus far have been performed in fibroblasts. However, there have also been valuable studies concerning integrin regulation of ionic transients and of polyphosphoinositide levels in endothelial cells (Ingber *et al.*, 1990; McNamee *et al.*, 1993; Chong *et al.*, 1994). Thus, it seems important to further develop our understanding of integrin regulation of signaling processes in vascular endothelial cells.

It has become increasingly apparent that, like receptor protein tyrosine kinases, G protein-coupled receptors and G proteins are also involved in the regulation of cell growth and differentiation (Pace *et al.*, 1995; Della Roca *et al.*, 1997). Many of these effects are mediated through the MAPK-signaling cascade. However, the coupling mechanisms between G protein-linked receptors and the MAPK kinase cascade are still poorly characterized. Interestingly, tyrosine phosphorylation has recently been implicated in the action of neuropeptide and bioactive lipids that act as cellular growth factors through G protein-coupled receptors (Rozengurt, 1995; Della Roca *et al.*, 1997). Very little is known about what role integrins might play in the regulation of G protein-coupled receptor signaling to MAPK. Cytokine signaling is also important in the biology of endothelial cells, with TNFα playing a par-

ticularly important role. The signaling pathways downstream of TNF are very complex (Liu *et al.*, 1996) and have not, to our knowledge, been investigated from the point of view of anchorage dependence.

In the present study, we examined whether integrin-mediated anchorage regulates growth factor-signaling events in human endothelial cells. We report that anchorage of endothelial cells to a fibronectin substratum is sufficient to induce FAK and paxillin tyrosine phosphorylation, but tyrosine kinase receptors are not autophosphorylated. Conversely, treatment with polypeptide growth factors is able to induce receptor phosphorylation, independent of adhesion to a matrix. However, we demonstrate a cooperation between integrins and soluble polypeptide growth factors in the activation of MAPK. In addition, we find that activation of MAPK by bombesin, lysophosphatidic acid, or TNFα is also anchorage dependent.

MATERIALS AND METHODS

Immunological Reagents

All antibodies used against integrin subunits were anti-human mouse monoclonals (Life Technologies, Gaithersburg, MD). Mouse mAbs against FAK (clone 2A7) and anti-phosphotyrosine (clone 4G10) antibody were from Upstate Biotechnology (Lake Placid, NY); anti-paxillin (clone 349) was obtained from Transduction Laboratories (Lexington, KY). Antibody to the active phosphorylated forms of p42/p44 MAPK was purchased from Promega (Madison, WI). Antibodies against p42 (MAPK2, sc-154), MAPKs 1 and 2 (sc-93), and JNK1 (sc-474) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit antibody against the EGF-receptor was from Upstate Biotechnology. R-phycoerythrin-conjugated secondary antibodies and either FITC- or TRITC-conjugated secondary antibodies were obtained from Sigma (St. Louis, MO). Myelin basic protein (MBP) was purchased from Life Technologies, Inc., and c-Jun–GST fusion protein was prepared by A. Aplin (University of North Carolina Chapel Hill). Protein G-Sepharose 4 Fast Flow was purchased from Pharmacia Biotech (Piscataway, NJ). [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from DuPont/New England Nuclear (Wilmington, DE). Bombesin was purchased from Peninsula Laboratories (Belmont, CA). Calyculin A was obtained from Calbiochem (San Diego, CA). EGF and bFGF were purchased from Upstate Biotechnology. Recombinant VEGF and TNFα were kindly provided by Genentech (South San Francisco, CA). All other reagents and chemicals were purchased from Sigma unless otherwise indicated.

Endothelial Cell Isolation and Culture, Preparation of Fibronectin-coated Dishes, and Cell Adhesion Studies

Primary human umbilical vein cells (HUVECs) were kindly provided by Dr. Lew Romer (Univeristy of North Carolina, Chapel Hill, Chapel Hill, NC) and were harvested and maintained as described previously (Romer *et al.*, 1994). HUVECs were also obtained from Clonetics (San Diego, CA) and were maintained according to the supplier's directions. For experiments, HUVEC cultures were grown to confluence and used between passages three and four. The human endothelial cell line ECV304 (American Type Culture Collection, Rockville, MD) was maintained in Medium 199 (Life Technologies) supplemented with 10% heat-inactivated FBS (HyClone, Logan, CT) and penicillin (50 units/ml)/streptomycin (50 μg/ml) in 5% CO_2/95% air at 37°C. For adhesion studies, 100-mm plastic Petri dishes were coated with 20 μg/ml fibronectin (Collaborative Research, Bedford, MA) in PBS at 4°C overnight. Dishes were washed twice with PBS and blocked with 2% BSA in serum-free medium for 1 h at 37°C. Blocking buffer was aspirated and cells were replated on fibronectin-coated dishes at a cell density of approximately 50%. For immunofluorescence, cells were fixed in 2% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS. Cells were stained with an anti-phosphotyrosine mAb to visualize focal contacts or with rhodamine-labeled phalloidin to visualize actin filaments. Cell spreading, focal contacts, and stress fibers were observed and recorded using an Olympus digital phase-fluorescence microscopy system.

Flow Cytometry Analysis

Near confluent cells (approximately 4×10^6/185-cm^2 flask) were detached by treatment with 0.05% trypsin/EDTA, washed, counted, aliquoted ($0.5–1 \times 10^6$cells/condition) and resuspended in 100 μl of cold PBS containing 1% BSA (1% BSA/PBS). Cells were incubated with primary antibodies (2 μg/ml or 10 μg/ml) for 60 min at 4°C, washed, and incubated with either goat anti-mouse or anti-rabbit IgG conjugated to R-phycoerythrin (20 μg/ml) for 30 min at 4°C. Following three washes in 1% BSA/PBS, cells were briefly fixed in 2% paraformaldehyde in PBS, washed, and resuspended in 500 μl of PBS and analyzed for fluorescence using a flow cytometer (Becton Dickinson, San Jose, CA). Typically, 2×10^4 cells were analyzed. Omitting the primary antibody assessed background fluorescence. For both cell types, the relative fluorescence intensity was expressed as a percentage of β_1 fluorescence.

Cell Treatment, Immunoprecipitation, Western Blots, and Immune Complex Kinase Assays

Confluent cells were serum starved for 6 h prior to detachment with 0.05% trypsin/0.33 mM EDTA. Trypsin was inactivated with soybean trypsin inhibitor (1 mg/ml), and cells were pelleted and resuspended in Medium 199 containing 2% BSA, and rocked for 60 min at 37°C to allow kinases to become quiescent. Cells were replated on plastic dishes precoated with fibronectin (20 μg/ml) and incubated at 37°C for the indicated times. Mitogens were added where indicated for 5 min prior to cell harvest. Cells were washed three times on ice with cold PBS and lysed in a modified RIPA buffer containing 50 mM Tris (pH 7.5), 1% Nonidet P-40, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM sodium fluoride, 1 mM sodium pyrophosphate, 1 mM sodium *o*-vanadate, 1 mM nitrophenolphosphate, 5 mM benzamidine, 0.2 μM calyculin A, 2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin. Cells were kept on ice for 10 min in lysis buffer, and cell lysates were prepared by scraping, followed by vortexing, and centrifuging for 5 min at 15,000 $\times$ *g* at 4°C. Cell lysates were stored at −70°C until use. Lysate protein concentration was determined using the bicinchonic acid assay (Pierce, Rockford, IL).

For immunoprecipitation cell lysates were incubated with primary antibody for either 2 h (FAK, MAPK2, and JNK1) or overnight (for growth factor receptors) at 4°C with rocking, followed by incubation with protein G-Sepharose for an additional 2 h at 4°C. Bead complexes were washed three times with cold RIPA buffer (without added protease inhibitors) and boiled with SDS-PAGE sample buffer. For immune complex kinase assays, the precipitates were washed once with cold RIPA buffer and three times with cold kinase wash buffer (0.25 M Tris, pH 7.5, 0.1 M NaCl). Immunocomplexes were incubated for 30 min in a kinase assay buffer (10 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 10 μM ATP, 5 μCi [^{32}P]ATP) containing either 10 μg of MBP or c-Jun-GST. Reactions were stopped with the addition of hot 3× SDS sample buffer, boiled for 5 min, and centrifuged. Proteins were electrophoresed on 15% SDS-polyacrylamide gels, dried, and exposed to x-ray film. The ^{32}P-labeled MBP or c-Jun-GST substrate bands, corresponding to MAPK and JNK, respectively, were quantitated using a Storm 840

PhosporImager with Image-Quant software (Molecular Dynamics, Sunnyvale, CA).

For Western blot analysis, total cell lysates or immunoprecipitated proteins were separated by SDS-PAGE under reducing conditions. Proteins were transferred electrophoretically onto polyvinylidene fluoride membranes (Immobilon P, Millipore Corp., Bedford, MA), blocked with 1% BSA/PBS/0.1% Tween 20 for 1 h (or overnight at 4°C) ,and subsequently incubated with primary antibody (1 μg/ml) in 1% BSA/PBS/0.1% Tween 20 for 1 h at room temperature. Membranes were washed briefly and incubated with goat anti-mouse IgG or goat anti-rabbit IgG peroxidase conjugates for 30 min. Following extensive washing, immunoreactivity was detected on Hyperfilm using ECL (ECL kit, Amersham Corp., Arlington Heights, IL). Images from ECL autoradiograms were captured using NIH Image and Adobe software.

RESULTS

Cell Morphology and Integrin Subunit Expression in HUVECs and ECV304 Cells

In this study, we used adhesion to fibronectin to evaluate integrin-mediated signaling events in primary HUVECs and an endothelial cell line, ECV304. We first examined the morphology of these cells, and evaluated their display of integrin subunits at the cell surface. Morphologically, both the ECV304 cells and HUVECs grew as contact-inhibited cobblestone monolayers; in addition, both cell types displayed well-developed focal contacts and stress fibers (Figure 1A). A typical integrin flow cytometry profile is given in Figure 1B, whereas Table 1 compiles the results of a survey of several integrin subunits. Several α subunits known to complex with β_1 were expressed, with HUVECs having relatively higher levels of $\alpha_5\beta_1$, a fibronectin receptor, while ECV304 cells seemed to preferentially express $\alpha_3\beta_1$. As expected, α_v integrins were also detected, suggesting that the $\alpha_v\beta_1$ fibronectin receptor might also be present. Thus, both cell types displayed typical endothelial cell morphological characteristics and a number of commonly expressed integrins, including one or more potential receptors for fibronectin.

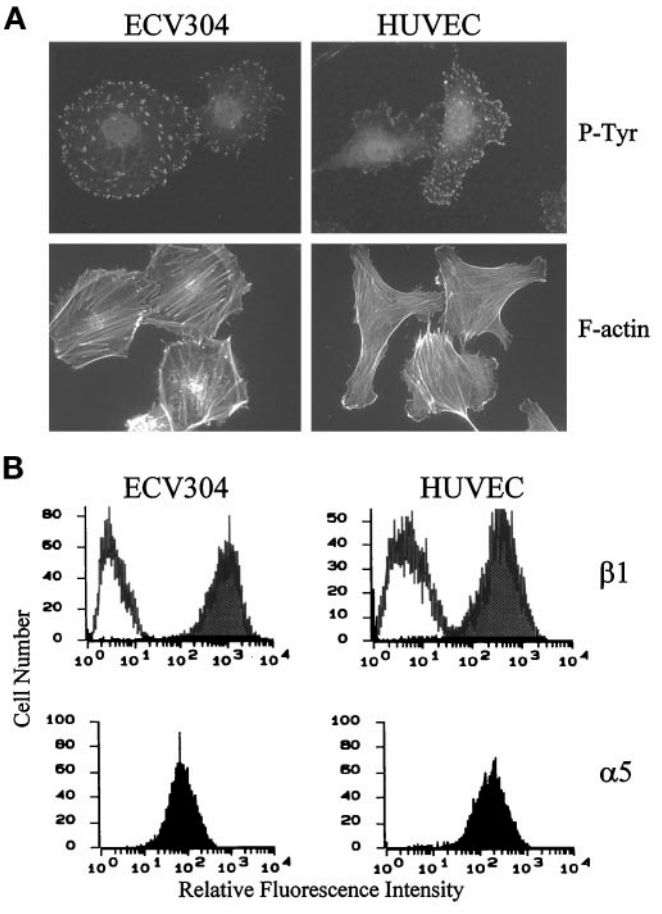

Figure 1. Morphology and integrin expression of ECV304 cells and HUVECs. (A) Morphology. Cells were maintained in culture as described in MATERIALS AND METHODS. Fixed, permeabilized cells were stained for phosphotyrosine to visualize focal contacts and for actin to visualize stress fibers. (B) Flow cytometry. For the analysis of integrin expression, viable cells were stained with anti-β_1 (top) or anti-α_5 (bottom) antibodies. Shaded histograms represent cells treated with anti-integrin subunit antibodies; open histograms show the staining of negative controls (no primary antibody). The ordinate displays cell number, and the abscissa displays relative fluorescence intensity on an arbitrary scale.

Cell Adhesion and Mitogen Stimulation Trigger Distinct Initial Tyrosine Phosphorylation Events

Attachment of ECV304 cells to a fibronectin substratum stimulated the tyrosine phosphorylation of several protein bands as detected by anti-phosphotyrosine blots; the major bands were approximately 125 kDa and 68 kDa (Figure 2A). As reported for other cell types (Lin *et al.*, 1997a,b), adhesion of endothelial cells to fibronectin induced tyrosine phosphorylation of FAK, as seen by both anti-phosphotyrosine blots of total cell lysates (Figure 2A, top panel) and after immunoprecipitation with an anti-FAK antibody (Figure 2C, top panel). Cell adhesion to fibronectin was also accompanied by tyrosine phosphorylation of the 68-kDa focal adhesion-associated protein paxillin, a putative substrate for FAK (Figure 2, A and D). Therefore, major components of the adhesion-dependent tyrosine phosphorylated 68-kDa and 125-kDa bands were identified as paxillin and FAK, respectively. Tyrosine phosphorylation of paxillin and FAK was not modified by EGF stimulation (Figure 2, C and D).

To compare the effects of integrin receptor-mediated tyrosine phosphorylation events to the known growth factor tyrosine kinase cascade, we used EGF as an agonist. Treatment of ECV304 cells with EGF induced autophosphorylation of the 180-kDa EGF receptor, independent of adhesion, as seen in both the total cell lysates (Figure 2A) and in lysates immunoprecipitated for the EGF receptor (Figure 2B). These results clearly show that integrin engagement and growth factor

Table 1. Integrin subunit expression in ECV304 cells and HUVECs

	% Maximal mean fluorescence	
Integrin subunit	ECV304	HUVEC
$\beta1$	1.00	1.00
$\alpha v\beta5$	0.40	0.27
$\alpha2$	0.39	0.59
$\alpha3$	1.40	0.40
$\alpha4$	0.10	0.09
$\alpha5$	0.22	0.68
αv	0.52	0.32

Cells (0.5×10^6) were incubated with primary antibodies, washed, incubated with an anti-mouse IgG conjugated to R-phycoerythrin, fixed, and analyzed for fluorescence using a flow cytometer. The relative fluorescence intensity is expressed as a percentage of β_1 fluorescence.

stimulation are able to trigger a discrete set of early signaling events.

MAPK and FAK Are Activated by Adhesion to Fibronectin

Integrin-dependent adhesion of ECV304 cells to a fibronectin-coated substratum leads to rapid activation of the cytoplasmic serine/threonine kinase MAPK. Using an antibody specific for the activated phosphorylated forms of MAPK, we show that adhesion to fibronectin increased levels in both the p42 and p44 forms of MAPK (MAPK 1 and 2, respectively). However, in ECV304 cells, the activation of the 42-kDa form of MAPK was consistently more prominent than the p44 form. Both the p42 and p44 isoforms of MAPK are present in ECV304, with p42 being more abundant (Figure 3A, bottom panel). Integrin-mediated activation of MAPK was transient and showed a maximal activation at 15–30 min with a gradual decline over a period of 2–3 h (Figure 3A, top panel). In contrast, antiphosphotyrosine Western blots of FAK immunoprecipitates showed FAK tyrosine phosphorylation with a somewhat delayed, but clearly more sustained, activity in comparison to the MAPK activation time course (Figure 3B, top panel). In ECV304 cells, the maximal activation of MAPK occurred with minimal evidence of cell spreading, whereas the maximal increase in FAK tyrosine phosphorylation appears to be coincident with cell spreading on fibronectin (Figure 3C).

Cytochalasin D, a drug known to disrupt the actin cytoskeleton and to interfere with integrin-mediated activation of MAPK and FAK in fibroblasts, was also examined in adherent endothelial cells. A 30-min pretreatment of ECV304 cells with 1 μM cytochalasin D largely blocked the activation of both MAPK and FAK when endothelial cells were plated on fibronectin for 30 min (Figure 3D). MAPK activation was also inhibited at later time points and no MAPK activation was observed in nonadherent cells (our unpublished results). Care was taken to obtain a concentration of cytochalasin D that was not toxic to endothelial cells, which might have prevented secure adhesion to fibronectin. At all time points, cells were well anchored

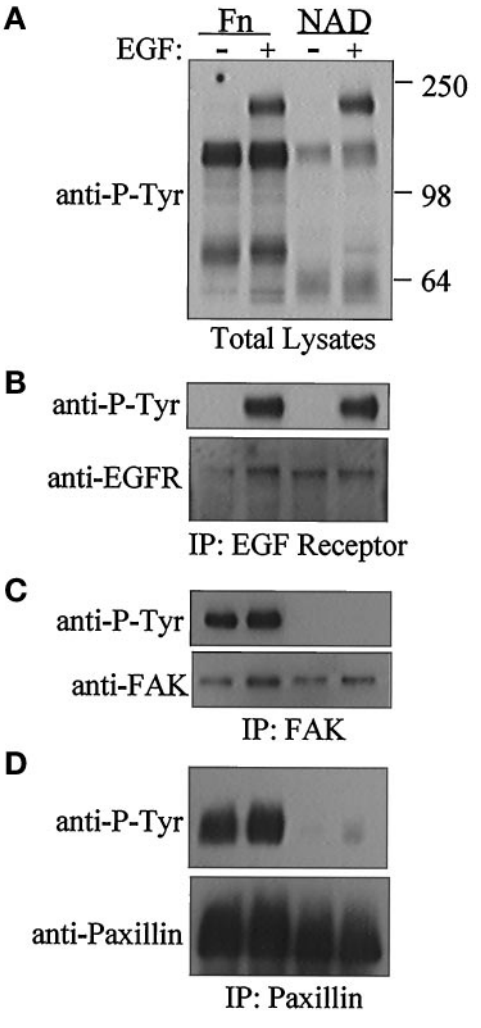

Figure 2. Cell adhesion and EGF stimulation trigger distinct initial tyrosine phosphorylation events in ECV304 cells. Confluent cells were serum starved for 6 to 8 h, trypsinized, resuspended in 2% BSA, and rocked in suspension for 1 h. Tyrosine phosphorylation levels were measured in cells either replated on fibronectin-coated dishes (Fn) or maintained in nonadherent suspension culture (NAD) for 2 h. Where indicated (+) EGF (1 ng/ml) was added to medium 5 min before cell harvest. Samples were Western blotted with a monoclonal anti-phosphotyrosine antibody and then reprobed with an antibody to the protein of interest. (A) Total cell lysates; blotted with anti-P-tyr. (B) lysates immunoprecipitated with a monoclonal anti-EGF receptor antibody; blotted with anti-P-tyr, reprobed with anti-EGFR. (C) Immunoprecipitation with an anti-FAK antibody; blotted with anti-P-tyr, reprobed with anti-FAK. (D) Immunoprecipitation with an anti-paxillin antibody, blotted with anti-P-tyr, reprobed with anti-paxillin.

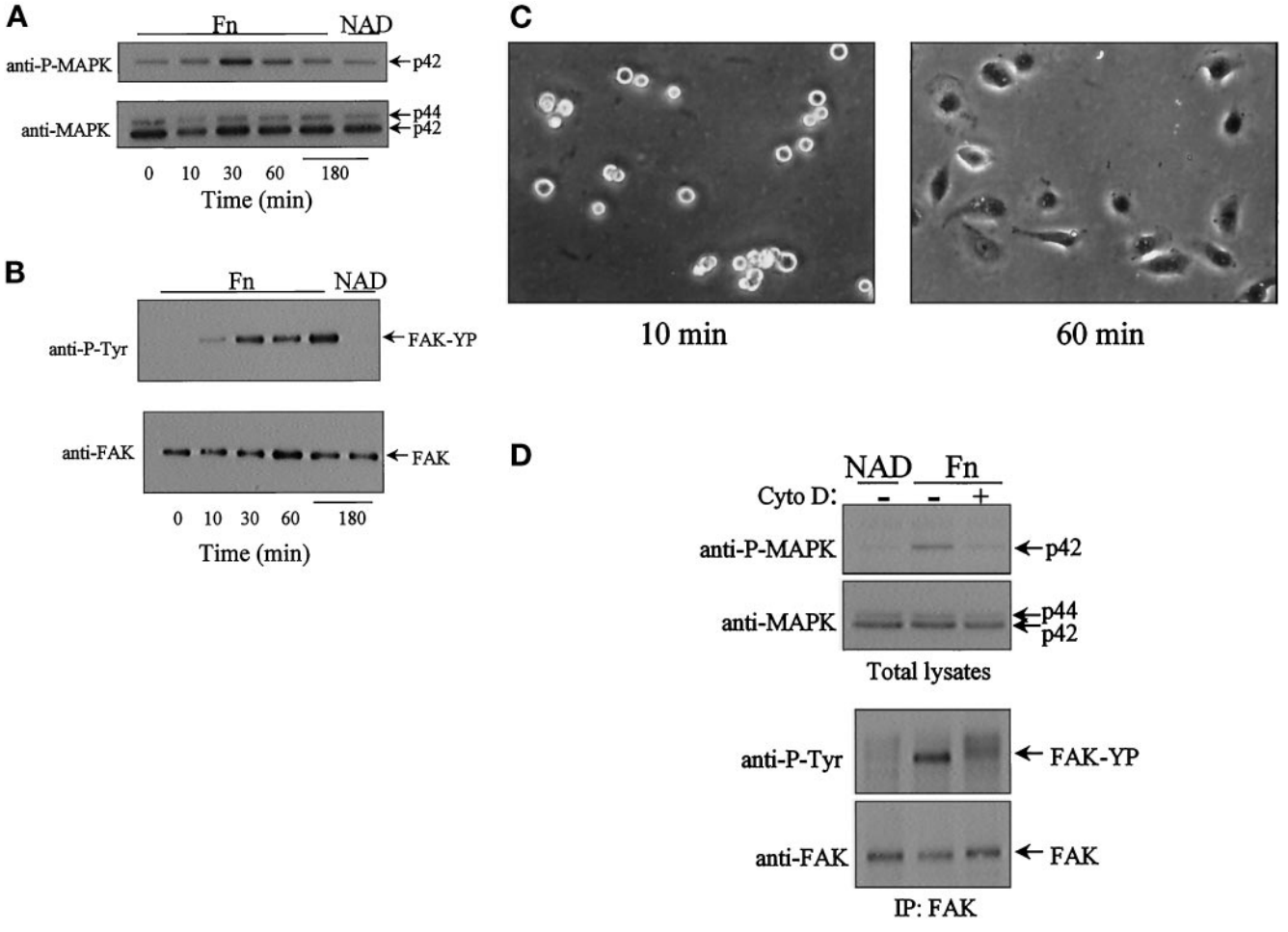

Figure 3. Adhesion to fibronectin activates both MAPK and FAK tyrosine phosphorylation: relationship to cell spreading and cytoskeletal organization. Confluent ECV304 cells were serum starved for 6 to 8 h. Detached cells were suspended in medium containing 2% BSA. Following a 1-h period in suspension, cells were allowed to attach to dishes coated with fibronectin (Fn). For nonadherent conditions, cells were held in suspension (NAD). At the intervals indicated, cells were lysed and analyzed by Western blot analysis for MAPK activation (A) and FAK tyrosine phosphorylation (B) from FAK immunoprecipitates (top panels, A and B). Parallel blots were probed with either a polyclonal anti-MAPK or a polyclonal anti-FAK antibody to show equal loading (bottom panels, A and B). (C) Phase-contrast micrographs of cells after 10 and 60 min on fibronectin show cell attachment at the early time point and attachment and spreading at the later time point. (D) Cells were pretreated with cytochalasin D (Cyto D, 1 μM) for 20 min prior to replating on fibronectin for 30 min and analyzed for both MAPK activation (top panels) and FAK tyrosine phosphorylation (bottom panels).

to the fibronectin substratum, but with no evidence of cell spreading (our unpublished results). This indicates that a degree of organization of the actin cytoskeleton is essential to both FAK and MAPK activation triggered by endothelial cell adherence.

Growth Factor Activation of MAPK Requires Adhesion to Fibronectin

Despite the fact that growth factors and integrins show distinct initial tyrosine phosphorylation patterns, several studies have reported on the convergence of integrin- and growth factor-mediated signaling at the level of MAPK (Miyamoto *et al.*, 1996; Lin *et al.*, 1997b; Renshaw *et al.*, 1997). We also investigated whether polypeptide growth factor activation of MAPK was anchorage dependent in endothelial cells. Western blotting of ECV304 lysates with an antibody specific for the phosphorylated forms of MAPK shows that a 5-min treatment of ECV304 cells with EGF induced rapid and concentration-dependent activation of MAPK (Figure 4A). This activation of MAPK was observed in cells that were plated on fibronectin for 3 h. EGF concentrations as low as 0.2 ng/ml were sufficient to activate MAPK under adherent conditions, with maximal activation observed between 20 and 100 ng/ml. In contrast, cells maintained under

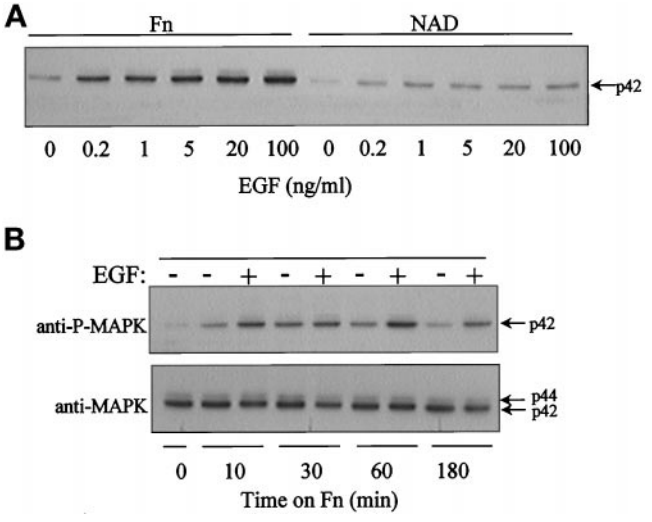

Figure 4. Anchorage dependence of EGF activation of MAPK. Confluent ECV304 cells were serum starved for 6 to 8 h, trypsinized, and suspended in medium containing 2% BSA. Following a 1-h period in suspension, cells were allowed to adhere to dishes coated with fibronectin or were held in suspension. Where indicated, EGF was added to cells 5 min before harvest. Cells were lysed and analyzed by Western blot analysis for MAPK activation. (A) EGF dose response. Cells were treated with EGF (0–100 ng/ml) after attachment to fibronectin (Fn), or after suspension culture (NAD), for 3 h. Lysates were probed with an anti-active MAPK antibody. (B) Time course. Cells were replated on a fibronectin substratum for various periods of time and then treated with 1 ng/ml EGF (+) or not (−). Top panel, anti-active MAPK Western blot. Bottom panel, anti-MAPK Western blot.

nonadherent conditions displayed only a minimal MAPK activation at low doses of EGF, with no significant increases at 100 ng/ml of EGF (Figure 4A). As seen with direct adhesion-mediated activation of MAPK, treatment with EGF in adherent cells predominantly activated the p42 form of MAPK. These results show that integrin engagement is able to modulate EGF-mediated MAPK activation, as evidenced by an increased maximal response.

We next evaluated the time dependence of growth factor-stimulated MAPK activation in endothelial cells plated on a fibronectin substratum. Similar to the results shown in Figure 3A, endothelial cells show a transient adhesion-dependent MAPK activation when plated on a fibronectin substratum (Figure 4B, lanes without EGF). The time course of EGF effects on MAPK activation was also evaluated. At early time points (10–30 min), EGF stimulation had minimal additional effects on the adhesion-induced activation of MAPK. However, as the transient adhesion-mediated MAPK activation decreased to basal levels (between 60 and 180 min), treatment with a low dose of EGF (1 ng/ml) provided a strong MAPK activation (Figure 4B, lanes with EGF). We also examined the effect of longer periods in suspension on the collaboration between cell adhesion and mitogen signaling. Cells held in suspension for 15 min, 3 h, and 6 h and then replated on fibronectin-coated dishes showed similar results to the 1-h point (Figure 4B); treatment with EGF showed adhesion-dependent activation of MAPK (our unpublished results).

Primary endothelial cells were also investigated for anchorage dependence of growth factor activation of MAPK. HUVECs showed a strong, transient activation of MAPK when plated on a fibronectin substratum in the absence of growth factors (Figure 5A). These results are consistent with our results using the endothelial cell line ECV304. However, in HUVECs, the maximal activation of MAPK appears to be more robust, with very low basal levels of activated MAPK (Figure 5A, 0 min and 180 min in suspension). We have noticed that there is relatively little p44 in ECV304s and a somewhat lower amount of p44 than p42 in HUVECs; this is reflected in a weaker signal in the p44 level upon cell adhesion. As with the cell line, HUVECs also display an anchorage dependence of growth factor activation of MAPK (Figure 5B). Thus, both bFGF and VEGF show strong anchorage dependence of MAPK activation (Figure 5B), as does EGF (our unpublished results). Although HUVECs also predominantly activate the p42 phosphorylated form of MAPK, they do show the p44 phosphorylated form when either maximally activated by adhesion (Figure 5A, 10 min) or stimulated with mitogen under adherent conditions (Figure 5B). To validate the results obtained thus far using anti-active MAPK antibodies, we also performed in vitro kinase assays on mitogen-activated HUVECs. A typical experiment is illustrated in Figure 5C using bFGF; similar results were also found for EGF (our unpublished results). Thus, the direct kinase assay parallels results with the anti-active MAPK antibodies and demonstrates a strong anchorage dependence of polypeptide growth factor signaling in HUVECs.

Other Agonists Known to Activate MAPK Pathways also Require Adhesion to Fibronectin

We next decided to test whether other agonists known to activate MAPK pathways might do so in an anchorage-dependent manner. In HUVECs, the neuropeptide bombesin, the bioactive lipid lysophosphatidic acid (LPA), and the cytokine TNFα all activated MAPK in an adhesion-dependent manner (Figure 6). Although working by diverse mechanisms (Liu *et al.*, 1996; Della Roca *et al.*, 1997), these signaling paths were all modulated by integrin-mediated cell adherence. Since TNF is thought to act more directly on the JNK pathway rather than the "classic" MAPK pathway, we decided to examine JNK activity to further investigate the relationship between TNF signaling and cell anchorage. Thus, a time course of JNK acti-

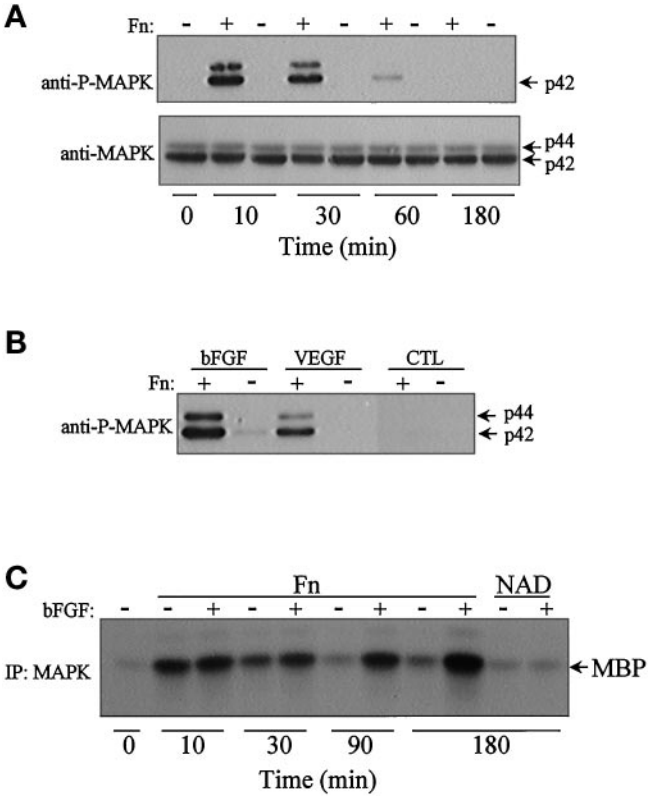

Figure 5. Mitogen-mediated activation of MAPK is anchorage dependent in primary human endothelial cells (HUVECs). Confluent HUVECs were serum starved for 8 h, trypsinized, and suspended in medium containing 2% BSA. Following a 1-h period in suspension, cells were allowed to adhere to dishes coated with fibronectin (Fn) or held in suspension (NAD). In some cases, the cells were treated for 5 min with bFGF or VEGF. Cells were washed and lysed, and lysates were analyzed for MAPK activation. (A) Cells were maintained in suspension (−) or attached to fibronectin (+) for various intervals, in the absence of growth factors, and the cell lysates were probed with an anti-active MAPK antibody. (B) Cells were replated on fibronectin (+) or held in suspension (−) for 3 h, and then stimulated with either VEGF (20 ng/ml) or bFGF (10 ng/ml) or not stimulated (CTL); cell lysates were probed with an anti-active MAPK antibody. (C) Cells were replated on fibronectin (Fn) or held in suspension (NAD) for 3 h, and then stimulated (+) with bFGF (10 ng/ml) or not stimulated (−); the cell lysates were analyzed using an in vitro MAPK assay, as described in MATERIALS AND METHODS. The autoradiogram represents the ^{32}P phosphorylation of MBP.

vation is shown in Figure 7A, while Figure 7B shows the effects of increasing concentrations of TNFα. Equal loading of JNK was demonstrated in both cases (our unpublished results). As seen in Figure 7B, there was very little activation of JNK in suspended cells treated with TNFα, whereas even low concentrations of the ligand produced robust activation in cells anchored on fibronectin. A time course of JNK activation is shown in Figure 7A. Taken together, these data suggest that there might be a generalized influence of integrin-mediated anchorage on MAPK pathways, including

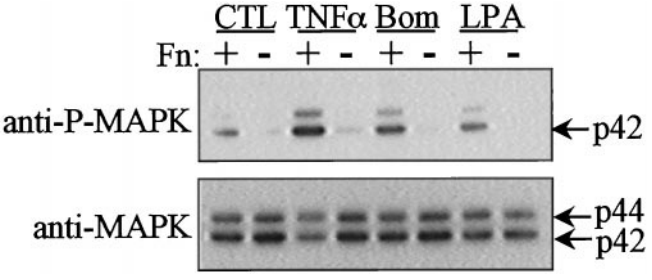

Figure 6. G protein-coupled receptor agonists and TNF-α show anchorage-dependent activation of MAPK in HUVECs. Confluent HUVECs were serum starved, trypsinized, and suspended in medium containing 2% BSA. Following a 1-h period in suspension, cells were allowed to adhere to dishes coated with fibronectin (Fn) or held in suspension (NAD) for 3 h prior to agonist stimulation. Cells were stimulated with either bombesin (Bom, 5 min, 10 nM), LPA (5 min, 2 μM), or TNF-α (10 min, 10 ng/ml), and lysates were analyzed by Western blotting. The top panel shows the activated forms of MAPK using an anti-active MAPK antibody; the bottom panel shows loading using an anti-MAPK antibody.

the classic MAPK (or ERK) pathway, as well as the related JNK pathway.

DISCUSSION

In this study, we examined whether cell anchorage regulates growth factor signaling in endothelial cells by comparing tyrosine phosphorylation events and MAPK activation in adhesion- and growth factor-stimulated cells. In addition to HUVECs, we used a relatively recently described immortalized human endothelial cell line, EVC304, as a model. Unlike primary endothelial cells, ECV304 cells do not require special growth factors, can be maintained in culture indefinitely, and display markers characteristic for endothelial cells (Takahashi *et al.*, 1990). Both HUVECs and ECV304 cells displayed typical "cobblestone" endothelial morphologies and had well-developed focal contacts and actin stress fibers. In addition, both displayed a complex repertoire of integrin subunits, including substantial amounts of α5β1, a key fibronectin receptor. Thus, both types of cells should be useful in various aspects of studying integrin-mediated signaling in endothelial-like cells.

As an initial approach to identifying signals activated by adhesion, we chose to examine tyrosine phosphorylation events stimulated by adhesion in endothelial cells and compared this to stimulation by growth factors. In recent years, numerous studies have reported on the tyrosine phosphorylation of cellular components as a key transducer of integrin-generated signaling pathways (Burridge *et al.*, 1992; Lin *et al.*, 1995; Rosales *et al.*, 1995). Consistent with a recent report using NIH3T3 cells (Chen *et al.*, 1996), cell adhesion and growth factor stimulation

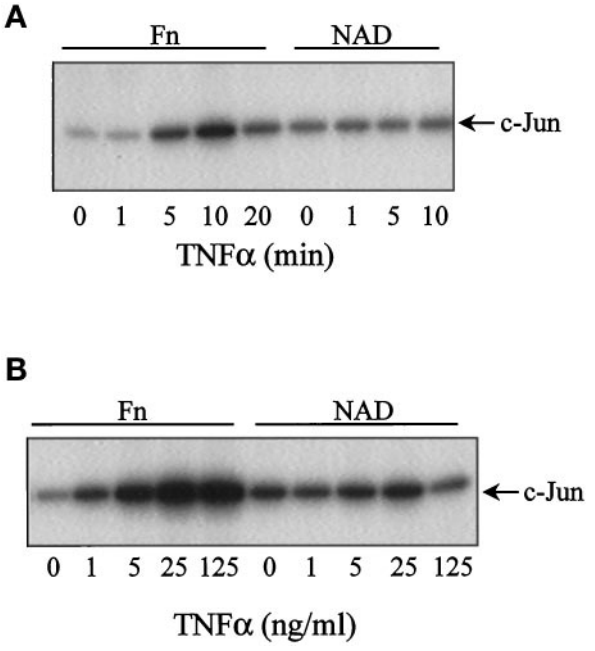

Figure 7. Activation of JNK is anchorage dependent in HUVECs. Confluent HUVECs were serum starved, trypsinized, and suspended in medium containing 2% BSA. Following a 1-h period in suspension, cells were allowed to adhere to dishes coated with either fibronectin (Fn) or held in suspension (NAD) for 3 h prior to agonist stimulation. (A) TNFα time course. Following a 3-h period of cells replated on fibronectin or held in suspension, cells were treated with TNFα for various times (0–20 min). (B) TNFα dose response. Cells were stimulated with TNFα (0–125 ng/ml) for 10 min. Cells were lysed, and cell lysates were analyzed using an in vitro JNK assay, as described in MATERIALS AND METHODS. The autoradiograms represent the ^{32}P phosphorylation of the substrate c-Jun.

triggered distinct initial tyrosine phosphorylation events in endothelial cells. Tyrosine phosphorylation of FAK and paxillin require anchorage to a matrix substratum. Studies in fibroblasts show that phosphorylation of FAK on tyrosine 397 generates a site for binding of the SH2 domain of src family kinases which leads to further phosphorylation and recruitment of SH2 proteins. It has also been reported that when endothelial cells migrate into a wounded area on tissue culture plastic, FAK tyrosine phosphorylation is induced (Romer *et al.*, 1994), suggesting an important role for FAK in endothelial cell migration. In contrast, treatment of endothelial cells with EGF caused receptor autophosphorylation, independent of anchorage, and stimulation with EGF did not enhance the adhesion-mediated phosphorylation of either FAK or paxillin. We also show that treatment of cells with either bFGF or VEGF induced receptor phosphorylation in an anchorage-independent manner. Interestingly, another group has found that VEGF caused tyrosine phosphorylation of both FAK and paxillin in endothelial cells (Abedi and Zachary, 1997). At present we cannot account for this difference. In our studies, we have consistently found that cell adhesion and growth factor stimulation induce distinct upstream tyrosine phosphorylation events.

MAPKs are key components of signaling pathways downstream of a variety of cell surface receptors including tyrosine kinase receptors, integrins, G protein-coupled receptors, and perhaps by as yet not identified shear stress receptors (Miyasaka *et al.*, 1990; Duff *et al.*, 1992; Chen *et al.*, 1994; Karin, 1994; Schlaepfer *et al.*, 1994; Morino *et al.*, 1995; Zhu and Assoian, 1995; Arai and Escobedo, 1996; Jo *et al.*, 1997). MAPKs phosphorylate and regulate key intracellular enzymes and transcription factors required for cell cycle progression and cell proliferation. The pathways leading from growth factor receptor activation via receptor tyrosine kinases to MAPK have been relatively well characterized (Khosravi-Far and Der, 1994). MAPK has also been shown to be stimulated by integrin activation, although key signaling events upstream of MAPK are still unclear, and differences in signaling cascades may be cell-type specific (Chen *et al.*, 1994; Schlaepfer *et al.*, 1994; Morino *et al.*, 1995; Zhu and Assoian, 1995; Chen *et al.*, 1996). Consistent with studies using fibroblasts, we show a transient anchorage-dependent activation of MAPK in endothelial cells. We also see a more rapid and robust activation of MAPK in HUVECs compared with ECV304s. However, this may not be surprising, since it is often observed that primary cells yield stronger signals than established cell lines. Although we do not know why anchorage-dependent MAPK activation is transient, it is plausible that the kinetics of MAPK may be regulated by MAPK phosphatases (Cook *et al.*, 1997). Although activation of MAPK and FAK both seem to be key early signaling events upon integrin ligation, our data are consistent with studies using other cell lines indicating that MAPK activation and FAK phosphorylation, although both dependent on adhesion, are nonetheless independent events (Lin *et al.*, 1997a). This is evidenced by their distinct time courses of activation. MAPK activation is rapid and transient and is simultaneous with initial anchorage of cells to fibronectin prior to cell spreading, whereas FAK shows a somewhat more delayed activation, is much more persistent, and its activation is coincident with cell spreading.

Recent studies in fibroblasts indicate that integrins can cooperate with growth factor receptors to contribute to efficient intracellular signal transduction events, including the activation of MAPKs (Miyamoto *et al.*, 1995, 1996; Lin *et al.*, 1997b; Renshaw *et al.*, 1997). Our results suggest that integrin-mediated anchorage to fibronectin also regulates growth factor signaling in endothelial cells, as shown by enhanced activation of MAPK by EGF and other peptide mitogens in anchored cells as compared with suspension culture cells. Interestingly, the diminished signaling to MAPK

seen in suspension cells cannot be overcome by increasing levels of growth factor, suggesting a reduced maximal response rather than a shift in the dose-response curve. Since initial activation and autophosphorylation of the EGF receptor is anchorage independent, our observations suggest that anchorage permits efficient coupling between upstream and downstream components of the signaling cascade. In fibroblasts, there are conflicting observations concerning the locus in the receptor tyrosine kinase to MAPK signaling pathway that is regulated by anchorage (Lin *et al.*, 1997b; Renshaw *et al.*, 1997). In this study, we have not attempted a full dissection of the signaling process but report only on MAPK activation.

As cells go from suspension culture to an anchored situation, two sets of events occur that affect MAPK. First, as discussed above, there is a rapid direct adhesion-mediated MAPK activation that peaks at 15–30 min and then declines. Second, the cells go from a state of being refractory to growth factor activation of MAPK to a state where growth factor activation of MAPK is very efficient. This change begins at about 30 min after cell attachment and continues until well after the initial burst of adhesion-dependent MAPK activity is past. Thus, events relatively late in the cell adhesion process seem to set the stage for efficient signaling from receptor tyrosine kinases to MAPK.

It should be noted that anchorage to fibronectin regulates growth factor signaling in HUVECs as well as in the ECV304 endothelial cell line. Indeed, the effects of anchorage seem stronger in the primary cell type, perhaps because of more stringent control of signaling events. Anchorage dependence is characteristic not only of EGF signaling to MAPK, but also for other growth factors that operate through receptor tyrosine kinases, including VEGF and FGF. Thus, the effects first noted with EGF in ECV304 cells seem to have some generality for other polypeptide growth factors.

Interestingly, mitogens that operate through receptors other than receptor tyrosine kinases also seem to have their signaling cascades modulated by cell anchorage. Tyrosine phosphorylation of several signaling proteins has been linked to the action of mitogens that operate through G protein-coupled receptors, including the neuropeptide bombesin and the lipid mediator LPA (Jalink *et al.*, 1994; Casamassima and Rozengurt, 1997). In fibroblasts, tyrosine phosphorylation of FAK and paxillin has been shown to be induced by LPA and bombesin (Seufferlein and Rozengurt, 1994; Rankin *et al.*, 1996), whereas LPA has been shown to activate MAPK in a number of cell types (Dikic *et al.*, 1996; Cook *et al.*, 1997; Della Roca *et al.*, 1997; Renshaw *et al.*, 1997). At present, relatively little is known about the signaling pathways that link either the bombesin or LPA receptors to phosphorylation of MAPK. One possibility is that the known anchorage regulation of phosphoinositol lipid formation may impact on such G protein-coupled signaling pathways (McNamee *et al.*, 1993); however, other possibilities also exist. Here, we present evidence that, in endothelial cells, integrins may be involved in modulating the pathway between these G protein-coupled receptors and key downstream kinases associated with cell proliferation and differentiation. We also report that TNFα shows anchorage-dependent activation of MAPK. In vivo, TNF is a mediator of systemic inflammation and immune responses (Vilcek and Lee, 1991). A major site of action of TNF for these effects is the vascular endothelium, where it induces inflammatory responses by enhancing adhesion molecule expression and cytokine secretion (Pober and Cotran, 1990; Grau and Lou, 1993). TNF can activate the ERK MAPK cascade in some cells, but it more usually triggers a parallel pathway that activates the p38 and JNK kinases (Liu *et al.*, 1996). Our data suggest that TNF cascades are also influenced by integrin-mediated cell adhesion. The finding that several different types of agonists, each triggering distinct upstream biochemical events, all display anchorage dependence of MAPK pathways, suggests that anchorage regulates events at loci where these diverse pathways converge.

Current observations indicate that integrin-mediated cell anchorage plays an important role in the regulation of signal transduction processes in endothelial cells. In the case of growth factors that act through receptor tyrosine kinases, integrin-mediated anchorage does not affect the initial autophosphorylation of the receptor, but it does seem to regulate the efficiency of signaling to downstream effectors such as MAPK. Cell anchorage also modulates the activation of MAPK by certain G protein-coupled receptors and by TNF, suggesting a very general role for anchorage in the regulation of cell signaling. It remains to be seen whether specific integrins are involved in this process and what the precise role of anchorage modulation of signaling might be in the complex biology of endothelial cells.

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