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Integrin-dependent induction of early growth response genes in capillary endothelial cells

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SUMMARY

Studies were carried out to explore how extracellular matrix molecules, such as fibronectin (FN), promote capillary endothelial (CE) cell growth. When G_0-synchronized cells were plated on FN-coated dishes, expression of the immediate-early mRNAs, c-*fos*, c-*myc* and c-*jun*, was rapidly induced, even in the absence of serum or soluble growth factors. Moreover, plating cells on different FN densities (5-200 μg/150 mm dish), resulted in a dose-dependent increase in the steady state levels of these mRNAs. Addition of FGF potentiated gene activation and was required for maximal DNA synthesis, however, the overall steady-state level of gene induction was dictated primarily by the density of immobilized FN. Expression of *junB* also was induced when suspended cells bound RGD-peptide coated microbeads that promote integrin clustering, but not when the suspended cells bound beads coated with other receptor ligands (e.g. acetylated low density protein) or when they were stimulated by soluble FN or FGF in the absence of substrate adhesion. c-*Jun* exhibited a similar requirement for gene induction except that it also was partially induced by binding to soluble FN alone. In contrast, c-*fos* expression was induced by all stimuli tested. Interestingly, inhibition of Na^+/H^+ exchange using hexamethylene-amiloride prevented most of the FN-induced increase in c-*jun* expression whereas it was relatively ineffective when cells were simultaneously stimulated by both FN and FGF. These data demonstrate that cell adhesion to extracellular matrix and associated integrin binding can directly activate signaling cascades in quiescent CE cells that lead to induction of immediate-early genes associated with the G_0/G_1 transition and thereby, stimulate these cells to reenter the growth cycle.

Key words: Fibronectin, c-*myc*, c-*jun*, *junB*, c-*fos*, Na^+/H^+ antiporter, Angiogenesis

INTRODUCTION

Angiogenesis, the growth of new blood vessels, is stimulated by soluble mitogens such as basic fibroblast growth factor (FGF). The capillary endothelial (CE) cell response to mitogens, however, appears to be governed by extracellular matrix (ECM) in the local tissue microenvironment (Ingber et al., 1987, 1989a,b; Ingber, 1990). This regulatory mechanism is critical since capillary morphogenesis requires the establishment of local growth differentials in which one cell responds to growth factor but not its neighboring cells (Ausprunk and Folkman, 1977; Folkman, 1982). The mechanism by which ECM regulates cell sensitivity to soluble mitogens and switches cells between quiescence and growth remains unclear.

ECM molecules, such as fibronectin (FN), modulate cell behavior by binding transmembrane receptors, such as integrins. Integrins comprise a large family of dimeric receptors that differ in their ligand binding specificity due to combinations of different α and β subunits (Hynes, 1992). Integrins bind specific ligand recognition sequences, such as arg-gly-asp (RGD), that are found in many ECM proteins including FN. Unlike many growth factor receptors, integrin receptors do not have any known endogenous protein kinase activity (Hynes, 1992). Integrin binding, however, has been shown to activate many signal transducing molecules that are normally associated with growth factor stimulation, including protein kinases (Guan et al., 1991, 1992; Chen et al., 1992, 1994; Kornberg et al., 1992; Chen and Guan, 1994; Lin et al., 1994), inositol lipid kinases (McNamee et al., 1993), G proteins (Kapron-Bras et al., 1993; Schlaepfer et al., 1994) and the Na^+/H^+ antiporter (Ingber et al., 1990; Schwartz et al., 1991). Integrin binding also has been shown to regulate gene expression in many cell types. However, this work focused primarily on the regulation of differentiation-specific genes (Adams and Watt, 1989, 1990; Schmidhauser et al., 1990; Chen et al., 1992; Damsky and Werb, 1992) or on the expression of growth response genes in mitogen-stimulated cells (Guadagno and Assoian, 1991; Guadagno et al., 1994; Hansen et al., 1994). Cell adhesion to FN has been shown to activate c-*fos* transcription in fibroblasts in the absence of growth factor (Dike and Farmer, 1988), however, the roles of integrins and associated chemical signaling events were not explored.

In the present study, we asked whether integrin-mediated signals can propagate to the nucleus, activate immediate-early response genes and promote reentry into the cell cycle, independently of growth factors. We first characterized the kinetics of cell cycle progression and analyzed expression of immediate-early growth response genes in CE cells stimulated by adhesion to FN in the presence or absence of soluble growth

factors. We then examined how cell adhesion to FN induces gene activation, with specific emphasis on the role of integrin binding. In addition, we explored whether the Na^+/H^+ antiporter, a cell surface signaling molecule that is activated by integrin clustering and required for growth in CE cells (Schwartz et al., 1991), may mediate adhesion-dependent activation of immediate-early gene expression. These studies demonstrate that cell binding to ECM and associated integrin clustering can directly stimulate quiescent CE cells to pass through the G_0/G_1 transition, in addition to regulating growth factor responsiveness. They also suggest a possible connection between integrin-mediated chemical signaling events at the cell surface and gene activation within the nucleus.

MATERIALS AND METHODS

Cell culture

CE cells were isolated from bovine adrenal cortex as previously described (Folkman et al., 1979) and serially passaged on gelatin-coated tissue culture dishes in DMEM supplemented with 10% calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 20 mM Hepes buffer, pH 7.4 (Gibco Laboratory, Grand Island, NY), and 10 μg/ml endothelial mitogen (Biomedical Technologies Inc., Stoughton, MA). To induce a state of growth arrest, CE cells were grown to confluence and serum starved with DMEM containing 0.4% calf serum, 48 hours prior to the experiment. Quiescent cells were dissociated with trypsin or 2 mM EDTA in PBS (Ca^{2+}, Mg^{2+} free), rinsed with DMEM containing 1% BSA (Fraction V; Armour Pharmaceutical Co., Tarrytown, NY), plated in defined medium (DM): DMEM supplemented with 5 μg/ml transferrin (Collaborative Research, Lexington, MA), 20 μg/ml high density lipoprotein (HDL) (Bionetics Research, Rockville, MD), 1% BSA and 5 ng/ml recombinant basic FGF (Takeda Chemical Industries Limited, Osaka Japan), onto FN-coated bacteriological plates, as described previously (Ingber, 1990; Ingber et al., 1990). Human serum FN (Boehringer Mannheim or Organon Teknika-Cappel, Melvern, PA) was coated onto non-adhesive bacteriological plastic dishes at the designated coating densities by applying the protein overnight at 4°C in pH 9.4 carbonate buffer (2 ml/35 mm Petri dish, 25 ml/150 mm dish, and 0.1 ml/well in 96-well plates), as previously described (Ingber, 1990). In experiments using 5-(*N*,*N*-hexamethylene)-amiloride (HMA; Research Biochemical Inc, Natick, MA), cells were transferred into low sodium medium consisting of 25 mM $NaHCO_3$, 135 mM choline chloride, 1.8 mM calcium chloride, 1 mM $MgCl_2$, 4 mM KCl, 1× MEM amino acids (Mediatech, Washington, DC) and 10 mM glucose (Boyarsky et al., 1988; Ingber et al., 1990). HMA was solubilized in DMSO and stored at –20°C. Control cells were treated with equivalent amounts of DMSO.

In experiments analyzing the role of integrin clustering in gene activation, tosyl-activated magnetic beads (4.5 μm diameter; Dynal, Robbins Scientific) were coated with RGD-containing synthetic peptide (Peptite 2000, Telios), conconavalin A (Con A) or acetylated low density lipoprotein (AcLDL) at approximately 2 mg/8×10^8 beads in carbonate buffer, as previously described (Plopper and Ingber, 1993). Non-specific sites on beads were quenched with 1% BSA in DMEM for 1 hour at 37°C prior to their addition to cells at a ratio of 5 beads per cell. Cells were incubated with the beads at 37°C for 45 minutes with mild agitation. Cells bound to beads were separated from unbound cells using a magnetic separator (Advanced Magnetics), rinsed with ice-cold PBS, and RNA was extracted as described below.

Determination of S phase entry

Entry into S-phase was measured by pulsing CE cells with [^{3}H]thymidine (5 μCi/ml, 84 Ci/mmole; New England Nuclear, Boston, MA) for 2 hour intervals over a 24 hour period, as previously described (Ingber et al., 1987). Briefly, incorporation of [^{3}H]thymidine into TCA precipitable material was measured directly within adherent cells on FN-coated 96-well plates. To normalize DNA synthesis on a per cell basis, the numbers of attached CE cells were quantified using an acid phosphatase reaction.

Northern analysis

Poly(A)$^+$ RNA was isolated from tissue culture cells at different times after plating, as described by Gonda et al. (1982). Briefly, cells were lysed with Proteinase K (200 μg/ml; Boehringer Mannheim) and transferred to a 50 cm^3 tube. DNA was sheared through a 22 Gauge needle and the lysate incubated for 2 hours at 37°C. The solution was adjusted to 0.5 M NaCl final concentration, a slurry of oligo dT beads (Boehringer Mannheim) was added and the mixture was incubated for 1 hour, rotating at room temperature. The oligo dT was rinsed 3 times with a high salt buffer to remove non-bound material. Poly(A)$^+$ RNA was eluted by sequential addition of low salt buffer pre-heated to 65°C. The combined fractions were extracted with phenol:chloroform and precipitated with ethanol. RNA was quantitated by measuring absorbance at 260 and 280 nm. Equal amounts of RNA (2 μg) were fractionated on formaldehyde denaturing gel and transferred to nitrocellulose. The nitrocellulose filters were hybridized to ^{32}P-labeled cDNA clones at $0.5\text{-}1\times10^6$ cpms/lane. The various cDNA probes used were as follows: murine c-*myc* (Dean et al., 1983), bovine c-*fos* (from H. Liley, Boston Children's Hospital, Boston, MA), murine c-*jun* and *junB* (kindly provided by J. Darnell, Rocke-feller Institute, NY, NY), and rat β-actin (provided by S. Farmer, Boston University School of Medicine, Boston, MA). Radiolabeled nitrocellulose blots were subjected to autoradiography and quantitated by phosphorimage analysis. The blots included in the paper represent results from a single representative experiment; the phosphorimage data presented in graph form were pooled from multiple experiments. DNA synthesis controls were run in parallel with each experiment to assure that growth synchrony was maintained by culturing cells on FN-coated dishes under similar conditions in the presence of [^{3}H]thymidine (2 μCi/ml). After 24 hours at 37°C, cells were fixed with methanol and processed for autoradiography as described (Dike and Farmer, 1988).

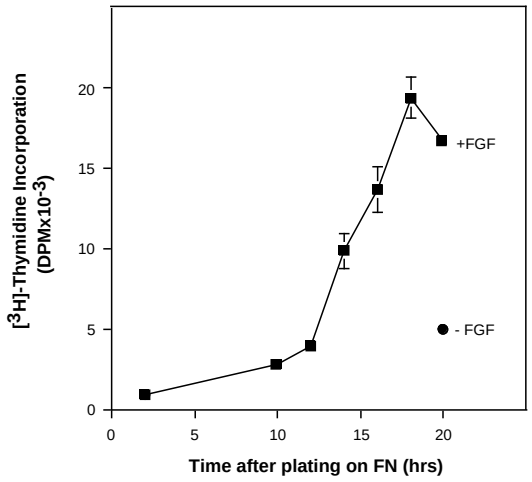


Fig. 1. The kinetics of cell cycle progression in capillary cells. Quiescent CE cells were trypsinized and plated onto a high (saturating) density of FN in defined medium in the presence (squares) or absence (circles) of FGF (5 ng/ml). Cells were pulsed with [^{3}H]thymidine for 2 hour intervals over a 24 hour period and S phase entry was quantitated by measuring its incorporation into DNA, as described in Materials and Methods. Data presented represent mean + s.e.m.

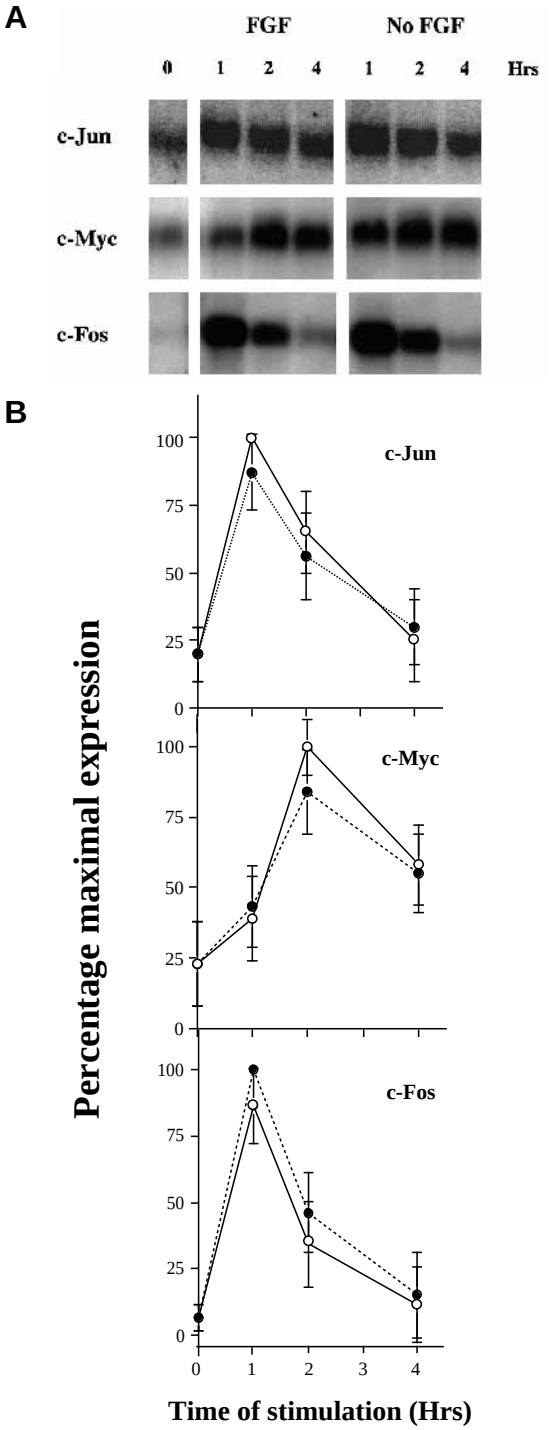


Fig. 2. Induction of immediate-early growth response genes associated with the G_0/G_1 transition by cell attachment to FN. Quiescent CE cells (t=0) were plated on high FN in defined medium in the presence or absence of FGF (5 ng/ml). Equal amounts of poly(A)$^+$ RNA isolated at 0, 1, 2, and 4 hours post-stimulation were analyzed by northern blot analysis. (A) A representative northern blot that was hybridized to ^{32}P-labeled cDNAs corresponding to c-*jun*, c-*myc* and c-*fos* and subjected to autoradiography. (B) Quantitation of northern blot data pooled from three different experiments, as determined by phosphorimage analysis. Data are presented as a percentage of maximal expression for each mRNA (open circles, cells on FN in the presence of FGF; closed circles, cells on FN without FGF).

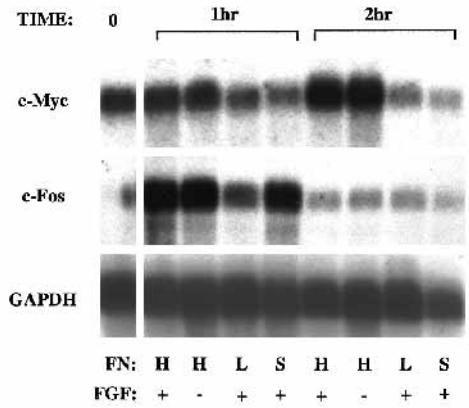


Fig. 3. Control of gene induction by FGF and adhesion to FN. Quiescent cells (t=0) were stimulated with FGF (FGF+) when plated on dishes coated with high (H) or low (L) FN densities (200 and 10 μg/150 mm dish, respectively) or maintained in suspension in the absence of FN contact (S). Cells were also plated on high FN (H) in the absence of FGF (FGF–). Poly(A)$^+$ RNA was isolated at 1 and 2 hours post-stimulation and analyzed by northern hybridization and autoradiography for the expression of c-*myc* and c-*fos*. The same filters were washed and reprobed with ^{32}P-labeled GAPDH cDNAs. Quantitation was carried out using phosphorimage analysis.

RESULTS

Cell cycle progression in CE cells

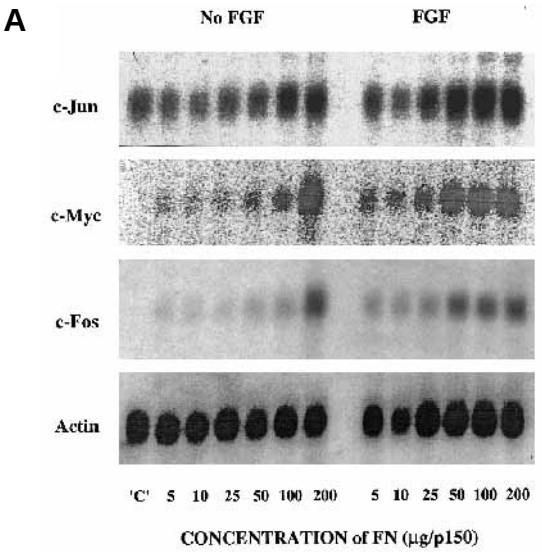
We established a state of growth arrest in CE cells, as defined by a G_1 DNA content and a [^{3}H]thymidine nuclear labeling index of less than 5%, by growth to confluence and serum deprivation for 48 hours. Quiescent cells were dissociated by EDTA or trypsin and stimulated to reenter the cell cycle by plating on bacteriological dishes coated with a saturating density of FN in defined medium supplemented with basic FGF (5 ng/ml) from the time of plating, as described previously (Ingber, 1990). Under these conditions, CE cells progressed in a synchronous manner from G_0 through G_1 and into S phase (Fig. 1). Entry into S phase began at 12-14 hours after attachment and peaked between 16-18 hours, a result consistent with the kinetics of G_0 exit and progression to S phase reported in other cell types stimulated by adhesion (Dike and Farmer, 1988) and/or growth factors (Rozengurt, 1986). CE cells showed a small but significant increase in DNA synthesis in response to adhesion to FN alone (without FGF), as previously described (Ingber et al., 1990).

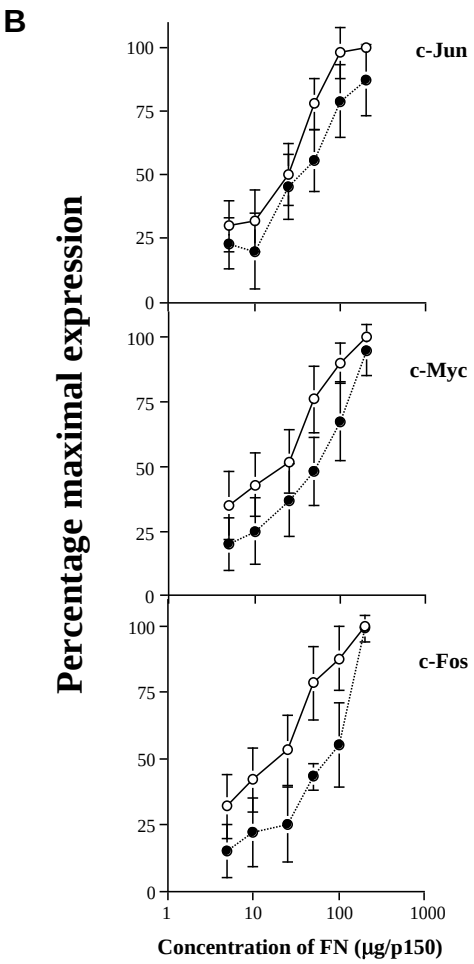
Analysis of changes in gene expression in cells plated on FN in FGF-containing medium confirmed that adhesion induced these quiescent cells to progress through the G_0/G_1 transition, as characterized by the induction of immediate-early mRNAs (Fig. 2A,B). c-*Jun* and c-*fos* mRNAs were transiently induced by 4- to 5-fold and 40-fold, respectively, at 1 hour after plating on FN. c-*Myc* mRNAs increased by 4 fold at 2 hours and by 4 hours returned to quiescent levels that remained constant for the remainder of G_1 (not shown). Because quiescent CE cells exhibited a higher baseline level of c-*myc* and c-*jun* mRNAs, their induction (4- to 5-fold) was low relative to that previously observed in other cell types (Rozengurt, 1986) although clearly detectable. Importantly, adhesion to FN induced a similar time course of gene induction when soluble growth factors were absent (Fig. 2A,B), even though most of these cells did not enter S phase (Fig. 1).

FN-dependent activation of immediate-early growth response genes

To discriminate between the effects of FN and FGF on gene expression, quiescent CE cells were plated on dishes coated with high or low FN densities (200 or 10 μg/150 mm dish, respectively) or cultured in suspension in the absence of any matrix attachment. Cell adhesion to high FN increased the levels of c-*fos* at 1 hour and c-*myc* at 2 hour by approximately 40- and 5-fold, respectively, independently of FGF (Fig. 3). In contrast, c-myc was not induced in cells when cultured on low FN or in suspension, even when saturating amounts of soluble FGF were present (Fig. 3). In fact, c-*myc* expression appeared to actually decrease relative to quiescent levels in certain studies (Fig. 3), however, the extent of this response varied depending on the initial baseline level of expression exhibited by quiescent cells. Although c-*fos* expression was induced by FGF in cells on low FN or in suspension, the level of induction was again considerably lower than that observed in cells on high FN (10- and 20-fold lower, respectively) (Fig. 3). In contrast, expression of a control gene, GAPDH, did not significantly change under these conditions.

If the binding of FN to specific cell surface receptors activates gene expression directly, then we would expect to observe a dose-dependent and saturable response. To determine whether immediate-early gene expression was dependent on the density of immobilized matrix ligand, quiescent CE cells were plated on dishes pre-adsorbed with increasing FN coating concentrations (5 μg to 200 μg/150 mm dish). Northern blot analysis demonstrated a dose-dependent increase in the expression of all three immediate-early genes in response to increasing the density of immobilized FN, with maximal levels of gene induction being observed on the highest FN densities (Fig. 4A). Moreover, FN-dependent induction of immediate-early gene expression was observed in the presence or absence of FGF whereas the expression of actin mRNAs remained unchanged (Fig. 4A). Analysis of results from 3 different experiments quantitated by phosphorimage analysis (Fig. 4B), revealed that addition of FGF slightly enhanced the degree of c-*jun*, c-*myc* and c-*fos* gene expression in cells cultured on low to moderate FN densities whereas it had no additive effect in cells on the highest matrix density.





Integrin clustering is required for gene induction

These results suggested that cell binding to immobilized FN induced the expression of growth-associated genes independently of FGF and thus, implicated an important role for cell surface integrin receptors in CE cell growth activation. However, the FN densities that exhibited the greatest ability to induce gene activation also promoted extensive cell spreading. To confirm that integrin binding, rather than cell spreading, was the primary stimulus leading to gene activation, suspended G_0-synchronized CE cells were bound to microbeads that were precoated with a synthetic RGD-containing peptide (Hynes,

Fig. 4. Dose-dependent induction of growth-associated mRNAs in response to cell attachment to different densities of immobilized FN. Quiescent cells ('C') were plated in defined medium on dishes pre-coated with increasing amounts of FN (5 to 200 μg/150 mm dish), in the presence (open circles) or absence (closed circles) of FGF (5 ng/ml). (A) A representative northern blot showing steady-state mRNA levels for c-*jun*, c-*myc*, and c-*fos* in CE cells cultured on different FN densities. Poly(A)$^+$ RNAs were isolated at 1.5 hours after plating so that the effects on expression of these three different genes could be compared in a single experiment. Filters were washed and reprobed with ^{32}P-labeled actin cDNAs. (B) Quantitation of the northern data from three different experiments, as determined by phosphorimage analysis. Data are expressed as a percentage of maximal gene expression in cells on the highest FN density in the presence of FGF. Values were corrected for small differences in RNA loading by normalizing for β-actin mRNA levels.

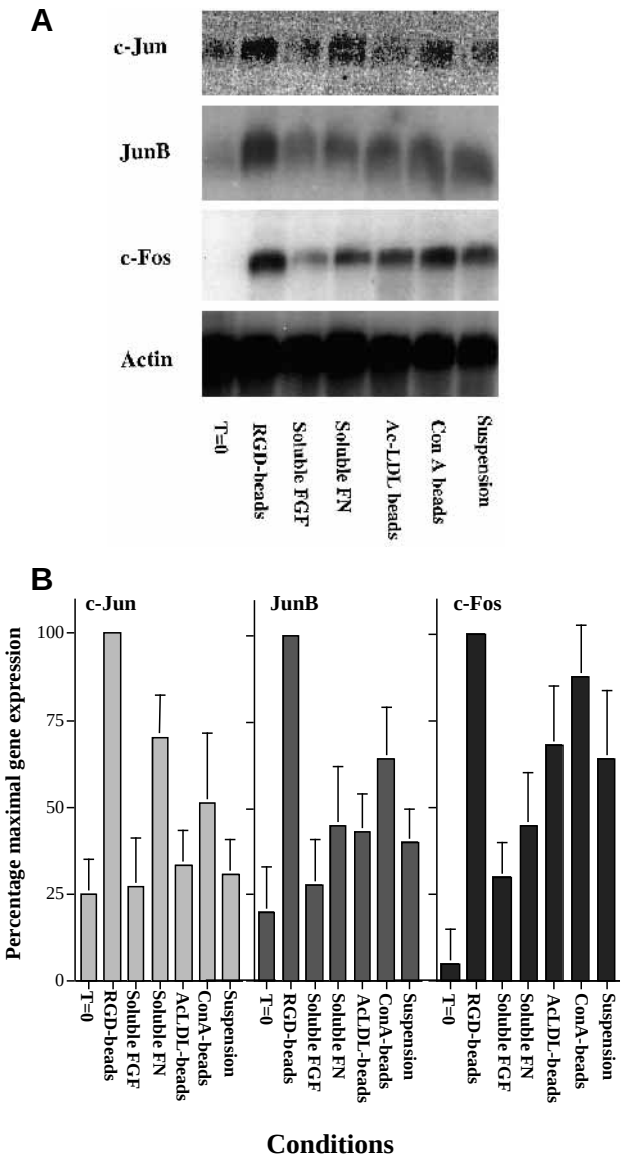


Fig. 5. Analysis of the role of integrin clustering in gene activation. Quiescent CE cells (*t*=0) were suspended for 45 minutes at 37°C alone (suspension) or in the presence of soluble FGF (5 ng/ml), soluble FN (80 μg/ml), or microbeads (5 beads/cell) coated with RGD-peptide (RGD-beads), AcLDL, (AcLDL-beads), or concanavalin A (ConA-beads). (A) A northern blot showing steady-state mRNA levels under the different culture conditions in a representative experiment. Poly(A)$^+$ RNAs were isolated and analyzed by northern hybridization and autoradiography for the expression of c-*jun*, *junB*, and c-*fos*. Filters were washed and rehybridized to [^{32}P]β-actin cDNAs. (B) Quantitation of the northern data pooled from three separate experiments, as determined by phosphorimage analysis. Data are expressed as a percentage of maximal expression for each gene; error bars indicate standard error of the mean. Values were corrected for small differences in loading by normalizing for actin mRNA levels.

1992). We have previously shown that these beads induce local integrin clustering, focal adhesion formation, and Na^+/H^+ antiporter activation within minutes after they bind to the CE cell surface whereas beads coated with acetylated-low density lipoprotein (AcLDL), a ligand for a transmembrane metabolic receptor (the scavenger receptor; Kodama et al., 1990), do not (Plopper and Ingber, 1993; Schwartz et al., 1991).

We analyzed changes in gene expression induced 45 minutes after CE cells bound to the RGD-beads to focus on the earliest effects of integrin binding on growth-associated genes. We found that expression of the immediate-early genes, c-*jun*, and *junB*, increased by approximately 3- to 5-fold, relative to quiescent cells at time zero or to cells cultured in suspension (Fig. 5A,B). c-*Jun*, but not *junB*, also was activated when suspended cells were stimulated with soluble FN (2- to 3-fold relative to suspended cells alone) whereas neither gene was significantly induced when soluble FGF was added. Increases in the expression of both c-*jun* and *junB* (approximately 2- to 3-fold) were observed when cells bound to beads coated with conconavalin A, a lectin that binds to a variety of different cell surface glycoproteins (Wang and Edelman, 1978). In contrast, binding of AcLDL-coated beads that interact with specific transmembrane scavenger receptors on the surfaces of endothelial cells had little effect on the expression of these genes (Fig. 5A,B). On the other hand, c-*fos* was induced by all stimuli, including a 17-fold induction in response to suspension alone (Fig. 5A,B). c-*Fos*, however, is known to be promiscuous in that it is activated by a wide variety of stimuli, both specific and non-specific, in many cell types (Chang et al., 1988; Curran and Morgan, 1986; Pine et al., 1988); this appears to be true in CE cells as well. c-*Myc* could not be analyzed under similar conditions because it is not maximally induced until approximately 2 hours after adhesion (Fig. 2).

A potential role for the Na^+/H^+ antiporter

We have previously shown that the Na^+/H^+ antiporter is activated within minutes by RGD-bead binding and hence, integrin receptor clustering in CE cells (Schwartz et al., 1991). To determine whether Na^+/H^+ exchange was involved in the induction of immediate-early gene expression by FN, quiescent CE cells were plated on a high FN density in defined medium containing low Na^+ (10 mM) and HMA. HMA is an amiloride analogue that preferentially inhibits the Na^+/H^+ antiporter by competing for Na^+ binding (Kapus et al., 1988; Schwartz et al., 1991; Simchowitz and Cragoe, 1986). In cells simultaneously stimulated by attachment to FN-coated dishes and soluble FGF, 20 μM HMA only inhibited c-*jun* induction by approximately 25% and this effect appeared to be maximal since similar effects were produced even when higher concentrations of HMA (40 μM) were utilized (Fig. 6). In contrast, the same dose of HMA (20 μM) reduced c-*jun* mRNA levels by more than 50% in cells that were stimulated only by adhesion to FN (i.e. in the absence of FGF) (Figs 6 and 7). Importantly, the inhibitory effects of HMA on c-*jun* expression were almost completely suppressed when the concentration of Na^+ in the medium was raised from 10 to 140 mM (Fig. 7), confirming that the effects of HMA on gene expression were due to direct inhibition of Na^+ transport. Inhibition of c-*jun* gene expression by HMA did not result from a generalized block in mRNA expression since actin mRNAs levels remained relatively constant in these cells (Fig. 6).

DISCUSSION

Characterization of the genes and regulatory proteins that mediate CE cell cycle control is critical for the understanding

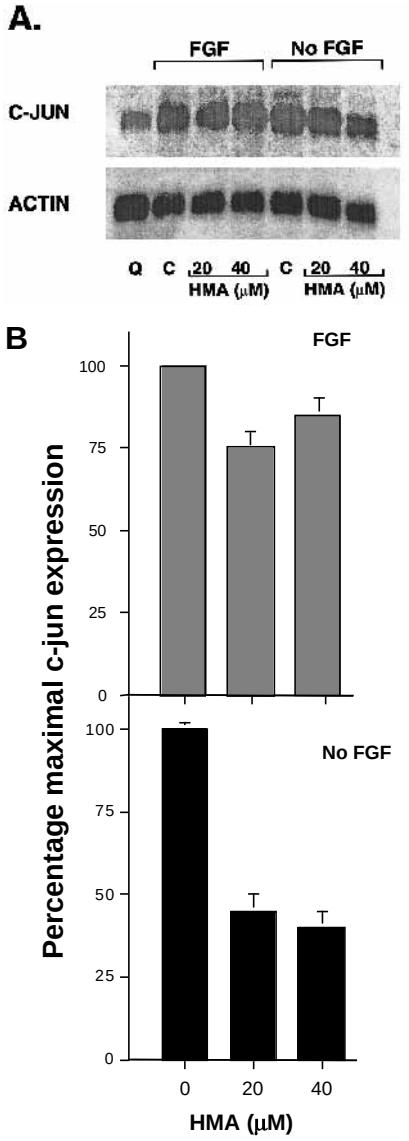


Fig. 6. Inhibition of the Na^+/H^+ antiporter by hexamethylene amiloride (HMA) preferentially inhibited the induction of c-*jun* expression by FN. Quiescent CE cells were plated onto high FN and exposed to 0, 20, or 40 μM HMA in defined medium containing low sodium (10 mM), with or without FGF. (A) Poly(A)$^+$ RNAs were isolated 2 hours post-stimulation and analyzed for c-*jun* and *actin* mRNAs by northern hybridization in conjunction with phosphorimage analysis. (B) Phosphorimage data obtained from analysis of northern blots expressed as a percentage of maximal expression induced by cell attachment to FN in the presence or absence of FGF; error bars represent standard error of the mean. Data were corrected for small differences in loading by normalizing against actin mRNA levels. Control levels of gene expression in low sodium medium were approximately 60% of those measured in CE cells grown in normal culture medium; these effects directly paralleled those observed on DNA synthesis under similar culture conditions (Ingber et al., 1990).

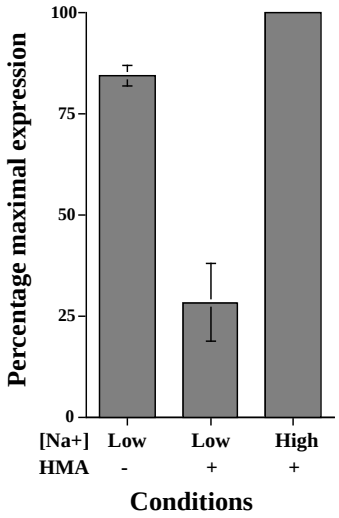


Fig. 7. The effect of salt concentration in the medium on the inhibition of c-*jun* expression by HMA (20 μM). Quiescent CE cells were plated in defined medium containing either 10 mM (low) or 140 mM (high) sodium. Expression of c-*jun* was quantitated as describe in Fig. 6. Data are presented as a percentage of maximal induction of c-*jun* expression relative to quiescent cells. Data were corrected for differences in loading by normalizing against actin mRNA levels.

of angiogenesis and hence, tumor growth control. While the kinetics of gene expression have been well characterized in response to stimulation by growth factors in many cell types, little is known about how these genes are regulated in CE cells. Even less is known about the sensitivity of these genes to control by ECM. Thus, in the present study, we set out to characterize the time course of expression of previously described growth-associated genes in CE cells and to analyze how cell binding to ECM induces gene activation.

Our data show that CE cells enter and progress through the cell cycle in response to FGF and adhesion to FN with kinetics that are similar to those in other non-transformed cell types (Rozengurt, 1986). However, S phase entry can be prevented in FGF-stimulated CE cells by inhibiting cell binding to ECM, as previously demonstrated (Ingber and Folkman, 1981b; Ingber et al., 1987; Ingber, 1990). Data presented here reveal that restricting cell-ECM contact formation suppresses growth factor responsiveness and inhibits cell cycle progression by preventing quiescent CE cells from passing through the G_0/G_1 transition. Specifically, the ability of soluble FGF to stimulate immediate-early gene expression was greatly inhibited in CE cells that were cultured on low FN or in suspension in the absence of ECM contact, even though FGF can activate other signaling mechanisms (e.g. Na^+/H^+ exchange) in these cells (Ingber et al., 1990). In contrast, previous studies showed that growth factors can induce the expression of immediate-early genes (Dhawan and Farmer, 1990) and promote progression to the G_1/S border in the absence of cell contact with ECM in certain established cell lines (Guadagno and Assoian, 1991; Han et al., 1993; Guadagno et al., 1994). Many of these lines, such as NRK cells, have undergone partial transformation and exhibit a decreased anchorage requirement for growth. Our CE cells, which are highly anchorage-dependent and represent a cell 'strain' rather than an established cell line, apparently retain additional adhesion-sensitive restriction points early in G_1, as indicated by the lack of induction of immediate-early genes (c-*jun*, *junB*, c-*myc*) in suspended cells when stimulated by FGF alone (Figs 3 and 5). Normal NIH-3T3 fibroblasts also

have been found to exhibit an early adhesion-sensitive restriction point relative to NRK cells that is required for activation of cyclin D (Bohmer et al., 1996). However, immediate-early genes (e.g. c-*myc*) could still be activated by mitogens in the absence of adhesion in these cells, suggesting that this integrin-dependent restriction point may fall earlier in the cell cycle in CE cells.

Importantly, our results show that ECM can act directly (i.e. independently of growth factors) to induce quiescent CE cells to reenter the growth cycle. This point was most clearly demonstrated by the finding of a dose-dependent relationship between the density of immobilized ECM ligand and the level of expression of multiple immediate-early mRNAs (c-*myc*, c-*jun*, c-*fos*) in the absence of soluble mitogen. It is unlikely that this observed gene induction can be attributed to FGF released by CE cells during attachment and spreading (Shaw et al., 1990) since large amounts of exogenous soluble FGF were unable to induce gene expression in cells that lacked contact with ECM (Ingber, 1990; and Figs 3 and 5). Also, DNA synthesis induced by adhesion to FN is only minimally suppressed by addition of blocking antibodies against FGF (unpublished data). Gene induction also could not be explained by contaminating growth factor in the commercial FN preparations we utilized since FN did not stimulate CE cell growth when added in a soluble form. Furthermore, the same adhesion-dependent effects on DNA synthesis and CE cell cycle progression can be obtained using surfaces coated with an entirely synthetic RGD-peptide (Ingber et al., 1995).

While CE cells increased their gene expression in a dose-dependent manner on dishes coated with increasing FN densities, we could not distinguish whether these effects were due to direct integrin receptor binding events or to cell spreading. Using the microbead system, however, we were able to demonstrate that integrin clustering was sufficient to induce expression of c-*jun* and *junB*, independent of cell shape changes. Importantly, this effect was specific for integrins since c-*jun* and *junB* mRNA levels did not increase when cells bound to AcLDL-coated beads that interact with other specific transmembrane receptors. Small increases in these mRNAs were seen when cells bound ConA-beads, however, this lectin is known to bind to a variety of cell surface glycoproteins and, thus, it may cause indiscriminate activation of a small number of different types of membrane signaling receptors (e.g. growth factor receptors, hormone receptors, certain integrin subtypes, etc).

Integrin occupancy has been previously shown to convey distinct signals from integrin clustering in certain cell types (Adams and Watt, 1989; Miyamoto et al., 1995). The addition of soluble FN, which ligates integrins without inducing receptor clustering in CE cells (Schwartz et al., 1991), did not induce *junB* expression. However, soluble FN did produce an increase in c-*jun* mRNAs, although not to the same level that was induced by integrin clustering using RGD-beads. Thus, integrin occupancy and clustering also may convey distinct signals in CE cells. While c-*fos* was induced by integrin clustering in suspended cells, it was activated by all other stimuli tested as well. c-*Fos* is known to be sensitive to a wide range of stimuli, including many that are not known to be involved in growth activation (Curran and Morgan, 1986; Pine et al., 1988; Chang et al., 1988).

Taken together, these findings suggest that the gene induction we observed in response to CE cell adhesion to FN resulted from direct ECM-integrin binding interactions. Based on our results, we cannot determine whether the observed increase in growth-associated mRNAs was due to transcriptional activation or an increase in message stability. However, these genes have been previously shown to be regulated at the transcriptional level by both growth factors and ECM in other cell types (Rozengurt, 1986; Dike and Farmer, 1988). It is also important to emphasize that while ECM acted directly to activate gene expression in CE cells, exogenous FGF augmented the FN-induced increase in gene expression at low to moderate ECM densities and both stimuli were required for optimal DNA synthesis. Similar separate, but additive, signaling mechanisms have been observed in past studies analyzing activation of the Na^+/H^+ antiporter in CE cells by FN and FGF (Ingber et al., 1990; Schwartz et al., 1991). We have previously shown that the effects of FN on signal pathways and growth is mediated, at least in part, through binding of integrin β1 in CE cells (Schwartz et al., 1991; McNamee et al., 1996). However, future studies will be necessary to define the relative roles of different integrin subtypes in this response.

While binding to a high ECM density directly induced expression of immediate-early growth response genes and promoted CE cell passage through the G_0/G_1 transition, it did not stimulate DNA synthesis in these cells unless growth factor was present. Conversely, growth factor binding was not sufficient for growth in the absence of adhesion to ECM. Past studies with CE cells and other anchorage-dependent cell types (e.g. hepatocytes), similarly have shown that growth factor binding will not stimulate S phase entry, even in the presence of adhesion and integrin signaling, if cell spreading is not promoted (Ingber, 1990; Hansen et al., 1994; Singhvi et al., 1994; Ingber et al., 1995; Bohmer et al., 1996). Thus, while ECM, growth factors, and cell spreading can independently transmit growth signals to the cell, all three stimuli appear to be required for progression through the entire cell cycle. Nevertheless, the present results demonstrate that many of the effects of ECM on cell behavior are exerted through integrin signaling and activation of gene expression early in the cell cycle, independently of growth factors or cell shape.

Although many signaling molecules have been shown to be activated by integrin binding, few have been shown to have functional significance in relation to growth activation (Guan et al., 1991; Guan and Shalloway, 1992; Chen and Guan, 1994). Activation of the Na^+/H^+ antiporter, on the other hand, appears to be required for growth in many cell types including CE cells (Ingber et al., 1990). Increased Na^+/H^+ exchange is also required for activation of immediate-early growth response genes in some cell types (Grinstein et al., 1988; Church et al., 1989; Panet et al., 1989; Hultgardh et al., 1990; Vairo et al., 1992). For these reasons and because the Na^+/H^+ antiporter requires integrin clustering for activation in CE cells (Schwartz et al., 1991), we explored whether the antiporter might be involved in the integrin signaling response that leads to gene activation. In fact, we found that the induction of c-*jun* expression by FN was inhibited by more than 50% when Na^+/H^+ exchange was prevented using the amiloride analogue, HMA. Thus, these results suggest that the induction of c-*jun* expression by adhesion to FN and integrin binding is mediated, at least in part, through activation of the Na^+/H^+ antiporter in

CE cells. Interestingly, the inhibition of c-*jun* expression produced by HMA could be largely reversed by FGF addition, suggesting that FGF may utilize different signaling pathways to induce the expression of this gene. However, given that FGF had little effect on c-*jun* expression in cells that lacked ECM-contacts, integrin signaling apparently must be switched on before FGF can activate these alternative pathways.

In summary, these data show that binding to FN is sufficient to induce expression of multiple immediate-early growth response genes in CE cells and to promote passage of these cells through the G_0/G_1 transition. Furthermore, cell adhesion to FN induced gene activation directly, that is, by promoting integrin ligation and clustering, independently of exogenous growth factors or large-scale changes in cell shape. Soluble mitogens, however, augmented the level of gene activation and all three stimuli (growth factors, integrin clustering and cell spreading) were required for efficient entry into S phase. Our results also implicate a potential role for the Na^+/H^+ antiporter in the signaling cascade that is triggered by FN binding and results in activation of at least one early-immediate growth response gene, c-*jun*. These results may explain, in part, why non-transformed cells, such as CE cells, require both anchorage and soluble growth factors in order to proliferate. Moreover, the finding that ECM can act directly to stimulate CE cell entry into the growth cycle may also explain how modulators of ECM turnover (e.g. plasminogen activator) can directly trigger angiogenesis in vivo (Berman et al., 1982).

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