Substratum-Growth Factor Collaborations Are Required for the Mitogenic Activities of Activin and FGF on Embryonal Carcinoma Cells

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Abstract. When P19 mouse embryonal carcinoma cells are grown in a serum-free N_2 medium on surfaces of tissue culture plastic, they die within two days. The death of these P19 cells is prevented by activin A and basic FGF (bFGF). The cells do not divide under these conditions. However, when P19 cells are cultured on substrata of extracellular matrix proteins such as laminin and fibronectin, activin A and bFGF are potent mitogens. These data show that the substratum to which cells are exposed can regulate their mitogenic response to growth factors.

UNDER normal growth conditions for cultured cells, serum supplies the growth factors required for cell division, but serum alone may not be sufficient to cause cell division unless the cells are adhered to a surface. For example, cell cycling can be blocked by placing cells on nonadhesive surfaces (Folkman and Moscona, 1978), and conversely the adhesion of cells to a surface per se is sufficient to turn on growth-associated genes such as *fos* (Dike and Farmer, 1988). The mitotic frequency of some nontransformed cell lines is dependent upon their adhesion to a solid surface, a process termed anchorage dependence for growth (Stoker et al., 1968). This apparent requirement for cell adhesion becomes less stringent as the cells become more transformed, and this loss of substratum dependence for growth has become a criterion for transformation (Wittelsberger et al., 1981; Tucker et al., 1981).

In contrast to the suspension versus attached culture paradigms described above, less is known about the effect of biological substrata on the mitogenic responses of cells to individual growth factors. Activin A is a multifunctional growth factor that promotes the survival, but not the division, of a neurogenic embryonal carcinoma cell line when the cells are grown on tissue culture plastic (Schubert et al., 1990). The P19 embryonal carcinoma cells (Edwards and McBurney, 1983) live for over a week in N_2 medium (Bottenstein and Sato, 1979) supplemented with 10 pM activin A, but the cells die within 2 d in its absence. We observed, however, that when the P19 cells are plated on other surfaces, their responses to activin A were quite different. In addition, we found that basic FGF (bFGF) has properties similar to, but not identical with, activin A in this system. The following paragraphs show that activin A and bFGF are mitogens for P19 cells when they are grown on substrata of extracellular matrix proteins, but that they only support P19 survival on tissue culture plastic.

Materials and Methods

Materials

Bovine fibronectin and mouse type IV collagen were obtained from Sigma Chemical Co. (St. Louis, MO), and human laminin from Calbiochem Behring Corp. (La Jolla, CA). Falcon plastic tissue culture dishes (Falcon Labware, Becton, Dickson and Co., Oxnard, CA) were used exclusively. Recombinant bovine bFGF was a generous gift from Dr. Andrew Baird (Whittier Institute, La Jolla, CA) and porcine activin A was obtained from Joan Vaughan and Wylie Vale (Salk Institute; see Vaughan et al., 1988 for purification procedures).

Cell Culture

P19 embryonal carcinoma cells (Edwards and McBurney, 1983) were obtained from Dr. J. Levine (SUNY, Stonybrook, NY). The P19 clone P19S1801A1 used in these experiments was not subcloned. The exponentially dividing cells were dissociated with 10% pancreatin (Gibco Laboratories, Grand Island, NY), washed once with 10% FCS in DME, twice with N_2 medium, and plated in 2 ml of N_2 medium at 5×10^4 cells per 35-mm uncoated tissue culture dish or on tissue culture dishes coated with 10 μg of laminin, type IV collagen, or fibronectin. N_2 is serum-free DME medium containing insulin, transferrin, progesterone, sodium selenite, and putrescine (Bottenstein and Sato, 1979). The tissue culture dishes were coated with the indicated proteins by incubation in serum-free DME overnight at 37°C. The dissociation of cells with pancreatin is critical to the success of these experiments. Survival was determined visually by trypan blue exclusion or with the metabolic dye, MTT (Pettman et al., 1989). Direct cell counts were made by dissociating the cells with 10% pancreatin for 30 min at 37°C in serum-free DME and counting the cells on a Coulter Counter.

Adhesion Assays

Exponentially dividing, low density cultures ($1\text{–}2 \times 10^5$ cells/100-mm culture dish) were labeled overnight with [^{3}H]leucine in complete medium, washed, and 5×10^4 cells pipetted into 35-mm tissue culture dishes containing 2 ml of Hepes-buffered DME culture medium containing the N_2 supplements. In some cases, the surfaces of the dishes were coated by incubation with the indicated amount of protein in 2 ml of serum-free DME overnight at 37°C. The percentage of input cells that adhere was determined by swirling the medium 10 times, aspirating the medium, and counting the

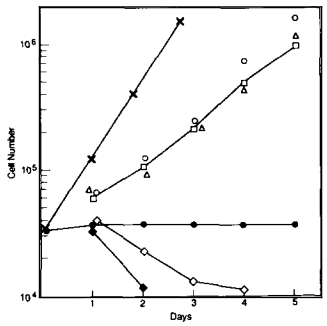

Figure 1. Substratum modulation of cell growth with activin A. P19 cells were dissociated as described in Materials and Methods, and plated into 35-mm tissue culture dishes containing 2 ml of N_2 medium and either coated with nothing or with 10 μg per dish of the indicated extracellular matrix proteins. At the indicated times cells were dissociated and viable cell number was determined as in Materials and Methods. Each point represents the average of duplicate cultures and the variation between duplicates was <10%. (●—●) Tissue culture plastic plus activin A; (x—x) 10% serum; (△—△) type IV collagen plus 100 pm activin A: (○—○) fibronectin plus activin A; (□—□) laminin plus activin A; (◇—◇) laminin alone; (◆—◆) tissue culture plastic alone. Fibronectin alone and collagen alone were similar to laminin alone.

isotope associated with the attached cells after the cells were lysed with 3% triton X-100. The data are plotted as the percent of input cells that remained attached to the dish as a function of time and are the average of duplicate dishes. The cells did not aggregate during the assays, which were done at low cell densities and in the absence of agitation. The variation between duplicates was <10%.

Results

P19 is an embryonal carcinoma cell line of undifferentiated stem cells that becomes nerve-like in the presence of serum and 5×10^{-7} M retinoic acid (Edwards and McBurney, 1983; Levine and Flynn, 1986). When P19 cells are placed in plastic tissue culture dishes in N_2 medium (Bottenstein and Sato, 1979), the cells die within 2 d. However, in the presence of activin A cells survive for several additional days (Schubert et al., 1990; see also Fig. 1). There is no cell division under these conditions as defined by time-lapse cinematography. The dose response curve for activin A on P19 cell survival is shown in Fig. 2. Half maximum survival is at 4 pM activin A. The effect of activin A on P19 cells was discovered because of its presence in the growth-conditioned medium of a clonal cell line from the eye (Schubert et al., 1990). Since bFGF has neurotrophic effects and is also found in the eye (Baird and Bohlen, 1990), bFGF was assayed for its ability to promote the survival of P19 cells. Like activin A, bFGF causes P19 cell survival (Fig. 3). bFGF requires, however, a higher concentration than activin A to stimulate half-maximal survival (Fig. 2). These data show that activin A and bFGF have similar effects on the survival of P19 embryonal carcinoma cells, but that neither causes net cell division of P19 cells in N_2 medium on tissue culture plastic. The phenotypes of the surviving cell populations are not known, but are under investigation.

Since bFGF was isolated on the basis of its mitogenicity (Baird and Bohlen, 1990), it was asked if bFGF and activin A are mitogenic for P19 cells when they are grown on substrata other than tissue culture plastic. P19 cells were plated on tissue culture dishes coated with the basement membrane components laminin, type IV collagen, or fibronectin in N_2

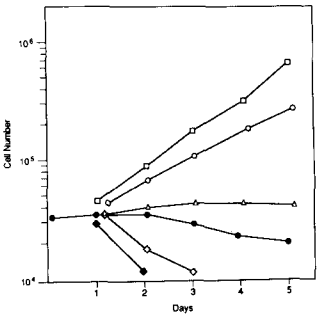

Figure 3. Substratum regulation of cell growth with bFGF. P19 cells were dissociated and plated on the various precoated surfaces in N_2 medium with 100 pM bFGF and the cell number was determined as a function of time exactly as described in Fig. 1. (□—□) Laminin plus bFGF; (○—○) fibronectin plus bFGF; (△—△) type IV collagen plus bFGF; (●—●) tissue culture plastic plus bFGF; (◇—◇) laminin alone (fibronectin and collagen alone gave similar results); (◆—◆) tissue culture plastic alone.

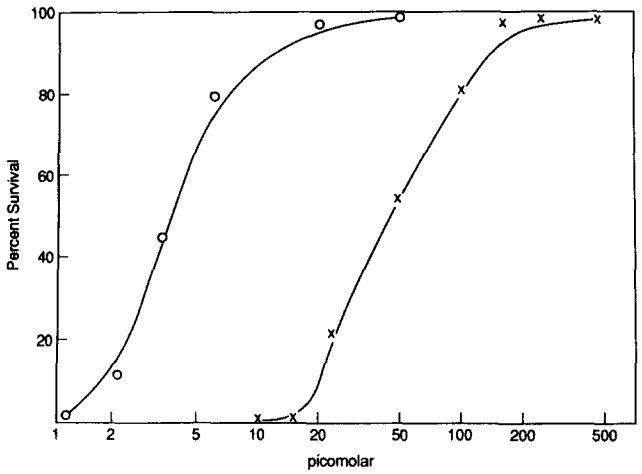

Figure 2. Concentration dependence of survival. Cells were dissociated, plated on uncoated tissue culture dishes in N_2 medium containing bFGF or activin A, as described in Fig. 1. Three days later the viable cell number was determined as described in Materials and Methods and the percent of surviving cells relative to day 0 plotted as a function of growth factor molarity. (x—x) bFGF; (○—○) activin A.

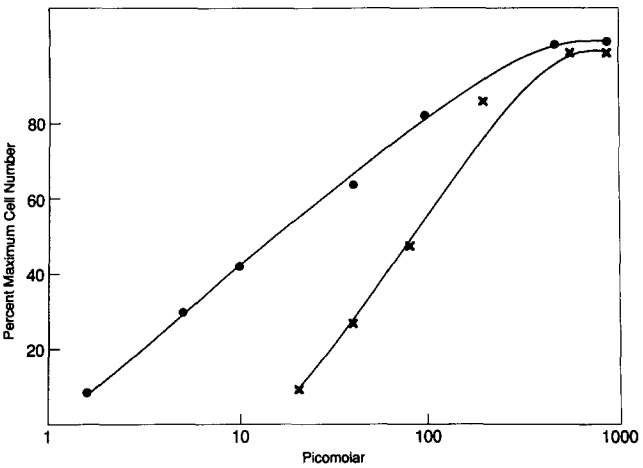

Figure 4. Growth factor concentration dependence for cell division. Exponentially dividing P19 cells were plated out at 1×10^4 cells per 35-mm tissue culture dish precoated with 10 μg of laminin. Increasing amounts of bFGF or activin A were added and the cell number was determined in duplicate cultures after 4 d. The data are plotted as the percent maximum (saturating growth factor) cell number for each concentration. (●—●) Activin A; (x—x) bFGF.

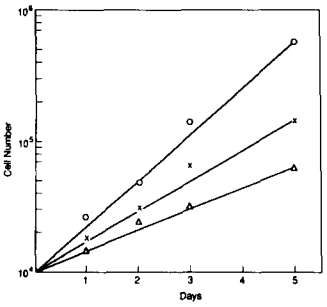

Figure 5. Synergistic effect of bFGF and activin A. Saturating amounts of bFGF, activin A, or both together were added to low density exponentially dividing P19 cells plated on laminin and the cell number per dish was determined as described in Fig. 1. (Δ—Δ) bFGF (1 nanomolar); (×—×) activin A (1 nanomolar); (○—○) activin A plus bFGF.

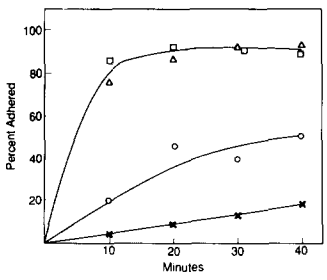

Figure 6. Adhesion of P19 cells to various substrata. Exponentially dividing P19 cells labeled with ^{3}H-leucine and their adhesion to 35-mm tissue culture dishes coated with 10 μg each of the indicated substrates was determined in Materials and Methods. (□—□) Tissue culture plastic alone; (Δ—Δ) fibronectin; (○—○) collagen IV; (×—×) laminin.

serum-free medium. Fig. 1 shows that in the presence of laminin, fibronectin, or collagen IV, activin A causes the cells to divide, although at a slower rate than in the presence of 10% FBS. The mitogenic responses to activin A on the three surfaces are about equal. In contrast to the response of P19 cells to activin A, when cells growing on these three substrata are exposed to bFGF, they only divide on laminin and fibronectin. Cells placed upon collagen IV survive slightly better than on tissue culture plastic, but they do not divide (Fig. 3). The rates of cell division on laminin and fibronectin are approximately the same for activin A and bFGF; the dose-response relationship between bFGF, activin A, and division rate is shown in Fig. 4. The half-maximal mitogenic responses to activin A and bFGF are at 12 pM and 100 pM, respectively; both about twofold higher than required for survival on tissue culture plastic (Fig. 2). The amount of substrate protein was saturating for cell division, since increasing the amount of laminin, collagen, or fibronectin over 10 μg per 35-mm dish does not enhance the rate of cell division (data not shown). PDGF (A chain homodimer), EGF, TGFβ, and TGFα are inactive in promoting P19 cell survival on any of the four surfaces.

Since there is a synergistic interaction between each of the growth factors and the extracellular matrix proteins, it was asked if bFGF and activin A were applied together on a laminin substrate, there is also an additive effect of the two growth factors on cell division. Fig. 5 shows that when cells are exposed to both bFGF and activin A at concentrations of each that stimulate maximal cell division on laminin (Fig. 4), there is an increase in cell number which is greater than additive. For example, at day 5 there are 6×10^4 cells per dish with bFGF, 1.5×10^5 with activin A, but 6×10^5 cells when the maximum effective concentrations of both growth factors are added to cultures at the same time. These results show that there is a synergistic interaction between the two proteins which leads to a greater rate of cell division than when the cells are exposed to either growth factor alone.

There are four classes of alternatives that could explain the above results. (*a*) Differences in cellular adhesiveness to the different surfaces are directly reflected in the mitogenic response. (*b*) The different surfaces allow differential cell spreading which modifies cellular responsiveness. (*c*) The surfaces change receptor function such that the cells become more mitogenically responsive to activin A and bFGF. (*d*) The substrata generate specific intracellular signals that cooperate with the soluble growth factors to produce a mitogenic response.

The first alternative was tested by examining the initial rates of adhesion of P19 cells to laminin, collagen IV, fibronectin, and tissue culture plastic under the conditions used in the mitogenic assays. Fig. 6 shows that cells adhere rapidly to tissue culture plastic and to fibronectin-coated dishes, but less rapidly to substrata containing collagen and laminin. Thus, there is no direct correlation between initial adhesion rates, substrata, and mitogenic responses.

The second alternative was examined by determining the extent of cell spreading after 24 h on the four surfaces. Fig. 7 shows that the cellular morphology on all four surfaces is similar, although there are some differences in the thickness of their processes (Fig. 7). The quantitative data in the legend show that the relative areas of the cells are indistinguishable on all surfaces.

The third possibility is that the substratum alters the number or function of growth factor receptors. The receptors for activin A and bFGF are present on cells plated on tissue culture plastic, for both growth factors potentiate cell viability on this surface. In addition, the half-maximal response for survival on plastic (Fig. 2) and mitogenicity on laminin (Fig. 4) are within a factor of 2 of each other, suggesting that the efficiency of coupling receptor occupancy to the biological response did not change to a great extent with different surfaces. These data do not rule out the possibility that the different surfaces change the intracellular coupling between the receptors and response elements.

The fourth alternative is that the surfaces themselves generate a cytoplasmic response that interacts with that of bFGF or activin A to produce a mitogenic response. For example, laminin contains EGF repeats that may interact with EGF receptors (Davis, 1990). It was therefore asked if EGF would act in conjunction with bFGF or activin A to stimulate mitosis when the cells are grown on tissue culture plastic. The use of 1 ng per ml of bFGF or activin A and varying concentrations of EGF between 0.1 and 50 ng/ml did not produce a mitogenic response (data not presented). A related possibility that collagen IV, laminin, and fibronectin preparations contain a bound growth factor as an impurity which is active in these assays can not be formally ruled out. This is unlikely, however, since the sources of fibronectin (bovine plasma), collagen IV (Swarm mouse sarcoma), and laminin (human placenta) are distinct.

Discussion

The above data show that the substratum on which P19 cells are growing can regulate their mitotic response to bFGF and activin. Although bFGF was isolated as a mitogen (Baird and Bohlen, 1990), activin A was initially purified on the ba-

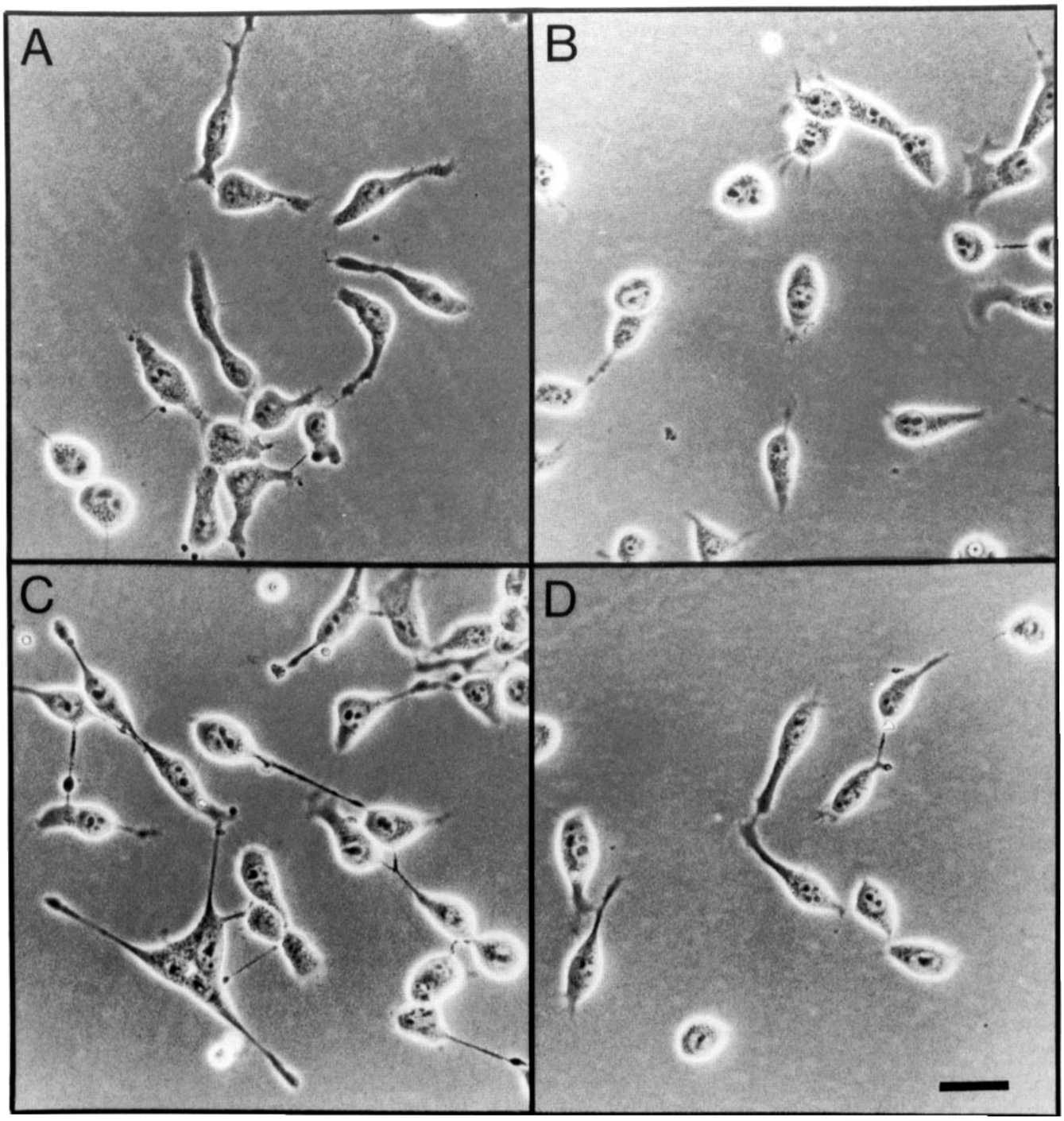

Figure 7. Phase-contrast photographs of cells on different substrata. P19 cells were plated on different surfaces as described in Fig. 1 and their morphology was determined 24 h later. (*A*) Tissue culture plastic; (*B*) type IV collagen; (*C*) laminin; (*D*) fibronectin. The relative areas of 10 cells on each substratum were determined by tracing enlarged photomicrographs with a Macintosh digitizer; the data are presented as the mean area (in undefined units) plus or minus the standard error. (*A*) 0.67 ± 0.09; (*B*) 0.56 ± 0.06; (*C*) 0.64 ± 0.07; (*D*) 0.57 ± 0.09. Bar, 50 μm.

sis of its ability to stimulate FSH secretion (Vaughan et al., 1988). The only report that activin A can act as a mitogen is with BALB/c 3T3 cells (Kojima and Ogata, 1989). Because activin A is growth inhibitory for rat thymocytes (Hedger et al., 1989), it follows that the biological activity of activin A is dependent upon the phenotype of the responsive cells. The above data show that it is also dependent upon the substratum.

The requirement for substratum adhesion in order to divide usually becomes less stringent as cells become transformed (Wittelsberger et al., 1981; Tucker et al., 1981). Although the P19 cell line is derived from a tumor, it still shows a strong substrate dependence for growth in the presence of defined growth factors. This dependence is not due to the strength of adhesion per se, for the initial rates of adhesion of P19 cells to collagen IV, fibronectin, and laminin are

different (Fig. 6), yet their mitotic response to activin A on these surfaces is about the same (Fig. 1). Although nonspecific cell-substratum adhesive interactions can lead directly to the induction of growth-associated genes in suspension arrested fibroblasts (Dike and Farmer, 1988), the above data suggest that growth factor regulation of the P19 cell cycle is quantitatively dependent upon the biochemical nature of the cell-substratum interaction.

It is well documented that the substratum on which cells are grown can alter their phenotype. For example, retinal pigmented epithelial cells are induced to transdifferentiate into neurons by growth on laminin (Reh et al., 1987), and laminin facilitates the differentiation of skeletal myoblasts much better than either fibronectin or collagen (von der Mark and Ocalan, 1989). Laminin and fibronectin also have different effects on melanogenesis in avian neural crest cultures (Rogers et al., 1990). Earlier studies also have shown that the interactions of liver (Spray et al., 1987), mammary (Li et al., 1987), and mesenchymal (Tomasek and Hay, 1984) cells with components of the extracellular matrix can lead to the expression of tissue-specific genes. Therefore, different biological substrates may lead to cellular differentiation such that the growth factor receptor or its coupling to the mitotic pathway within the cell are different.

An alternative explanation for the above data is that substrate-induced physical changes in cellular or cytoskeletal organization may directly alter the responsiveness of cells to growth factors without changes in protein synthesis, thus, distinguishing this process from differentiation. For example, diffusible signals, such as cyclic nucleotides and divalent ions generated by cell-matrix interactions, may alter the polymerization of cytoskeletal proteins (Inber and Folkman, 1989*a*). Cell spreading on fibronectin activates phosphatidylinositol turnover in BHK cells (Breuer and Wagener, 1989) and changes in intracellular pH in fibroblasts (Schwartz et al., 1989). Many of these changes, including those involved in mitosis, may involve changes in the substratum-induced tension within the cytoskeleton (Inber and Folkman, 1989*b*). The transmission of tensile forces through cytoskeletal interconnections can trigger stretch-activated membrane channels which generate intracellular signals (Lansman et al., 1987). These are capable of interacting with other signal transduction mechanisms, such as those generated by a growth factor. This alternative is unlikely in the case of P19 cells, since the growth of different substrata does not significantly alter cell spreading (Fig. 7).

A final alternative is that two signals are required to produce a mitogenic response, and that the extracellular matrix component is responsible for the generation of one signal. There is increasing evidence that cellular interactions with matrix proteins can activate specific second message systems in much the same way as growth factors generate internal signals. For example, the binding of fibrinogen to the platelet integrin glycoprotein IIb/IIIa causes both protein tyrosine phosphorylation (Ferrell and Martin, 1989) and modulates Na^+/H^+ exchange (Banga et al., 1986). The interaction with matrix molecules also appears to be required for ciliary neurotrophic factor to induce certain types of astrocyte differentiation (Lillien et al., 1990), and in experiments similar to those reported here, it was shown that a laminin substratum is required for the response of Schwann cells to an undefined mitogen (Porter et al., 1987). Extracellular matrix proteins such as laminin contain many EGF precursor repeats that have been implicated in developmental processes as well as the stimulation of cell division (Panayotou et al., 1989; Davis, 1990). The P19 response to activin A and bFGF in the presence of laminin does not, however, appear to be simply related to EGF receptor occupancy, for EGF is not synergistic with bFGF or activin A when P19 cells are grown on tissue culture plastic.

It can be concluded that the collaborative interaction between bFGF or activin A and extracellular matrix proteins is due either to a matrix-mediated alteration in receptor coupling such that the cell becomes mitogenically competent or that the matrix proteins directly generate a "second message" that interacts with those derived from activin A and bFGF receptors to produce a mitogenic response. Similar combinatorial interactions of cells with extracellular matrix and growth factors may be involved in the regulation of cell division during embryogenesis.

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