

Modulation of Cell Surface Fibronectin Assembly Sites by Lysophosphatidic Acid

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Abstract. Lysophosphatidic acid is a product of activated platelets and has diverse actions on cells. We have characterized the effect of lysophosphatidic acid on cell-mediated binding and assembly of fibronectin, an extracellular matrix protein. Serum made from whole blood, but neither platelet-poor plasma nor serum made from platelet-poor plasma, caused enhanced binding of fibronectin to cultured fibroblastic cells. The ability of whole blood serum to enhance binding of fibronectin was abolished by phospholipase B. These results indicate that lysophosphatidic acid derived from platelets is the principal component in whole blood serum that is active in the fibronectin binding assay. 1-oleoyl lysophosphatidic acid, 20–200 nM, was as active as 0.1–0.2% whole blood serum. The stimulatory effect of lysophosphatidic acid on the binding of fibronectin or the amino-terminal 70-kD fragment of fibronectin was rapid, sustained, and lost

upon removal of lysophosphatidic acid. The stimulatory effect on binding could not be duplicated by bradykinin, platelet-activating factor, bombesin, or a peptide agonist of the thrombin receptor. Enhanced binding of the 70-kD fragment was due to increases in both the number and affinity of binding sites. Enhanced binding and assembly of fibronectin correlated with changes in cell shape and actin-containing cytoskeleton. The binding sites for fibronectin on lysophosphatidic acid-stimulated cells, as assessed by fluorescence, video, and scanning electron microscopy, were on areas of cell membrane containing numerous filopodia that extended between cells or between cells and substratum. These observations suggest that lysophosphatidic acid functions as a powerful and specific modulator of cell shape and early matrix assembly during wound healing.

LYSOPHOSPHATIDIC acid (LPA)¹ is an intermediate for de novo lipid biosynthesis (Bishop and Bell, 1988) and is generated rapidly upon cell activation (Billah et al., 1981; Lapetina et al., 1981; Watson et al., 1985; Gerard and Robinson, 1989; Exton, 1990). As a result of platelet activation during coagulation of whole blood, the concentration of LPA in serum is 1–5 μ M (Eichholtz et al., 1993). Extracellular LPA has many biological effects. These include mobilization of intracellular Ca^{2+} (Moolenaar et al., 1986; Jalink et al., 1990); inhibition of adenylate cyclase, stimulation of phospholipase C, and activation of protein kinase C (van Corven et al., 1989); regulation of p21ras (van Corven et al., 1993); stimulation of mitogen-activated protein kinase (Howe and Marshall, 1993); stimulation of tyrosine phosphorylation of phosphatidylinositol 3-kinase, focal adhesion

kinase and paxillin (Kumagai et al., 1993; Seufferlein and Rozengurt, 1994); stimulation of focal adhesion and actin stress fiber formation (Ridley and Hall, 1992); retraction of neuronal cells (Jalink et al., 1993); induction of smooth muscle cell contraction (Tokumura et al., 1980); activation of platelets (Benton et al., 1982); and induction of mitogenesis (van Corven et al., 1989). The effects of LPA on cell morphology are striking and involve the GTP-binding protein p21rho (Ridley and Hall, 1992; Miura et al., 1993; Kumagai et al., 1993; Jalink et al., 1994). A 55-kD LPA-binding protein has been identified on the surface of cells by covalent crosslinking and conjectured to be a G-protein coupled receptor for LPA (van der Bend et al., 1992). Thus, LPA may stimulate cells by pathways similar to platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycerol-3-phosphocholine), a bioactive lipid with a known G-protein coupled receptor (Prescott et al., 1990; Nakamura et al., 1991). Failure of intracellularly generated or microinjected LPA to mimic the action of extracellular LPA (Watson et al., 1985; Fernhout et al., 1992) also implies the existence of a cell surface LPA receptor.

Fibronectin is a dimeric glycoprotein found at high concentrations in plasma and other body fluids and in an insoluble

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1. *Abbreviations used in this paper:* ACD, 0.8% citric acid, 2.2% trisodium citrate, 2.45% glucose, pH 5; LPA, lysophosphatidic acid.

ble form in connective tissues and basement membranes (Mosher, 1989; Hynes, 1990). Fibronectin is necessary for normal embryogenesis (George et al., 1993). Insoluble tissue fibronectin has several likely biological functions, including promotion of cellular migration during embryogenesis and wound healing (Colvin, 1989; Thiery et al., 1989; Hynes, 1990; Welch et al., 1990). Deposition of plasma or cell-derived fibronectin into extracellular matrix is initiated by the binding of the soluble fibronectin to cell surface sites in a specific and saturable manner (McKeown-Longo and Mosher, 1983; Peters and Mosher, 1987a; Allio and McKeown-Longo, 1988). Reversibly bound fibronectin then is polymerized into high molecular weight multimers (McKeown-Longo and Mosher, 1984; Barry and Mosher, 1988, 1989).

Serum influences elaboration of fibronectin matrix by cultured cells (Mautner and Hynes, 1977; Mosher and Vaheri, 1978; Rennard et al., 1981; Singer, 1982, 1989). We recently identified LPA as a potent enhancer of fibronectin binding and assembly by cultured adherent cells (Checovich and Mosher, 1993). As discussed by Eichholtz et al. (1993), serum LPA has the potential to be an important mediator of wound healing. Therefore, we investigated how much of the stimulatory activity of serum on fibronectin binding to cells can be attributed to LPA. We then explored the mechanism by which LPA stimulates binding of fibronectin to cells. We focused on the specificity of the response to LPA, whether LPA acts upon fibronectin or cells, the time course of response to LPA, alterations in kinetics of binding of the 70-kD amino-terminal region that mediates the reversible interaction of fibronectin with cells, and correlations between state of the actin-containing cytoskeleton and binding of fibronectin to LPA-stimulated cells.

Materials and Methods

Materials

LPAs with different fatty acids at the 1-position were obtained from Avanti Polar Lipids (Birmingham, AL). Bradykinin, bombesin, fatty acid-free bovine albumin, phospholipase A₂ from bee venom, phospholipase B from *Vibrio* species, platelet-activating factor, prostaglandin E₁, and a thrombin receptor peptide agonist (SerPheLeuLeuArgAsnProAsnAspLysTyrGluProPhe) were purchased from Sigma Chem. Co. (St. Louis, MO). Human thrombin was a gift from Dr. J. Fenton (New York State Department of Health, Albany, NY). Rhodamine-phalloidin and FITC were from Molecular Probes (Eugene, OR).

Human plasma fibronectin and the 70-kD amino-terminal, gelatin-binding fragment of fibronectin generated by cathepsin D were isolated, iodinated, and reisolated as previously described (McKeown-Longo and Mosher, 1983, 1985). The labeled proteins were stored in portions at -70°C in TBS (Tris hydrochloride, 20 mM, pH 7.4; sodium chloride, 150 mM) containing 0.1% (wt/vol) fatty acid-free bovine albumin. Purity of labeled proteins was assessed by SDS-PAGE with and without reduction followed by autoradiography. The concentration of ¹²⁵I-70-kD fragment for calculation of specific activity was determined as follows. Dilutions of labeled 70-kD fragment and of known amounts of unlabeled 70-kD fragment, all in 0.1% albumin, were bound in a batch mode to gelatin-agarose. After the affinity matrix was washed, bound protein was eluted with SDS sample buffer, subjected to SDS-PAGE, and quantified by densitometry of the stained gel. Fibronectin and the 70-kD fragment were also labeled with FITC (McKeown-Longo and Mosher, 1983, 1985).

Preparation of Plasma and Serum

Plasma and serum were made on the day of the experiment from a freshly drawn blood sample (Eichholtz et al., 1993). For plasma, six parts of blood

were mixed in a polypropylene tube with one part of ACD (0.8% citric acid, 2.2% trisodium citrate, 2.45% glucose, pH 5) and made 100 nM in prostaglandin E₁. Red cells were removed by centrifugation for 15 min at 120 g. The supernatant was titrated to pH 6.5 with additional ACD, and platelets were removed by centrifugation for 10 min at 1200 g. Remaining cell debris was removed by two additional centrifugations for 5 min at 8000 g. For serum, blood was placed in a glass tube, made 1 U/ml with human α -thrombin, and set at 37°C for 30 min. The retracted clot was removed by centrifugation at 3000 g for 2 min, and serum was further centrifuged twice for 5 min at 8000 g. For plasma-serum, platelet-poor plasma made as above was incubated with 1 U/ml human α -thrombin and 10 mM calcium ion in excess of citrate at 37°C for 30 min. The concentrations of the platelet granule protein, thrombospondin, in these samples were measured by ELISA (Jaffe et al., 1983) and were 0.7 μ g/ml in plasma and plasma-serum and 8.3 μ g/ml in whole blood serum. The clot was removed by centrifugation at 3000 g for 2 min, and plasma-serum was further centrifuged twice for 5 min at 8000 g. Plasma or serum was diluted in four parts of Tyrode's buffer (150 mM sodium chloride, 2.5 mM potassium chloride, 12 mM sodium bicarbonate, 1 mg/ml glucose, 2 mM magnesium chloride, pH 7.4) and treated for 60 min at 37°C with phospholipase A₂ or B, 3 U/ml. Calcium chloride, 2.5 mM in excess of citrate, was added to the samples containing phospholipase A₂. The plasma and serum samples were then diluted further in Tyrode's buffer containing 0.2% fatty acid-free bovine albumin so that the final concentrations in the binding assays were 2% or lower. Controls included plasma or serum incubated without phospholipase and phospholipases incubated without plasma or serum.

Cell Culture

The MG63 human osteosarcoma cell line was obtained from the American Type Culture Collection (Rockville, MD). Human foreskin fibroblasts were a strain derived by Dr. Lynn Allen-Hoffman (University of Wisconsin-Madison). Cells were cultured in DME (GIBCO BRL, Gaithersburg, MD) supplemented with 5% (for MG63 cells) or 10% (for foreskin fibroblasts) FBS (Intergen, Purchase, NY). Cells for experiments were seeded at 40,000 cells/2-cm² well (24-well plates; Costar, Cambridge, MA) and were studied when newly confluent 2-3 d after seeding.

Binding Assays

Cell layers were washed twice with TBS and incubated in Tyrode's buffer and 0.2% fatty acid-free bovine albumin for binding assays of <1-h duration. DME containing 0.2% fatty acid-free bovine albumin, 10 mM Hepes, pH 7.4, was used in the longer time course studies. The binding mixtures contained radiolabeled fibronectin (usually 1 μ g/ml, ~150,000 cpm/well) or 70-kD fragment (usually 0.2 μ g/ml, ~150,000 cpm/well) and various additives in 0.5 ml. After 45 min (except in the case of time-course experiments) at 37°C, cell layers were washed and assayed for the amounts of total cell layer-associated radioactivity (Checovich and Mosher, 1993). Nonspecific binding in the presence of 500 μ g/ml of unlabeled fibronectin or 50 μ g/ml of unlabeled 70-kD fragment (~10-30% of total binding for both intact fibronectin and the 70-kD fragment) was subtracted from total binding to calculate specific binding. Total cell protein was also determined (Lowry et al., 1951). Comparative assays showed that 1 mg of cell protein represented 4.6 and 4.7 \times 10⁶ cells for the MG63 cells and foreskin fibroblasts under the experiment conditions, respectively. The amount of fibronectin or 70-kD fragment bound per mg cell protein varied up to twofold from experiment to experiment. Replicate wells within a given experiment, however, usually varied <5%.

For binding isotherms at equilibrium, confluent cell layers in the absence or presence of LPA were incubated at 37°C for 45 min with increasing concentrations of ¹²⁵I-70-kD fragment without or with an excess of unlabeled 70-kD fragment. Data were analyzed by Scatchard and Hill plots (Limbird, 1986). Association rate constants, *k*₁, were estimated from amounts of ¹²⁵I-70-kD fragment specifically bound after 2 min of incubation at input concentrations of 0.05, 0.1, 0.2, and 0.4 μ g/ml. The concentration of potential binding sites was assumed to be that determined in the 45-min equilibrium-binding studies. Dissociation rate constants, *k*₋₁, of bound 70-kD fragment were estimated by monitoring ligand appearing in the medium after a 45-min binding assay. Rate constants were calculated as described by Limbird (1986). Plots to estimate *k*₁ and *k*₋₁ were linear in all cases (*r*² > 0.92).

Microscopy

Video-enhanced light microscopy was performed as described previously

(Albrecht et al., 1989). Cells cultured on glass coverslips were mounted in a glass perfusion chamber and viewed over a 40-min period at 25°C with a high-magnification, video-enhanced microscope built on a Nikon Diaphot (Garden City, NY). Images were projected to a DAGE-MTI Newvicon camera (Michigan City, IN) modified for manual control of gain and black level. A video-processor and attendant synchronization stripper (Colorado Video 604 and 302-2, Boulder, CO) allowed nonlinear expansion of contrast and brightness. Experiments were recorded with a high-resolution VCR (NV 9240XD; Panasonic, Secaucus, NJ), and selected images were routed to an MS-DOS 80386-based computer equipped with video capture and processing hardware (Imaging Technology, Inc., Woburn, MA).

For fluorescence microscopy of bound fibronectin or 70-kD fragment in cell layers (McKeown-Longo and Mosher, 1983, 1985), cells cultured on glass coverslips were incubated with FITC-labeled fibronectin or 70-kD fragment (20 or 4.5 µg/ml, respectively) in the absence or presence of 200 nM 1-oleoyl LPA for 45 min at 37°C. For actin localization (Ridley and Hall, 1992), cells were fixed in 3% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 5 min, and incubated with 0.1 µg/ml rhodamine-labeled phalloidin for 20 min. Coverslips were mounted with glycerol gel (Sigma Chem. Co.) and cells were viewed on a Nikon epifluorescence microscope. Care was taken to photograph a given fluorochrome at the same settings for all experimental permutations.

For immunoelectron microscopic localization of bound fibronectin, cells cultured on plastic coverslips (Nunc, Inc., Naperville, IL) were incubated with 20 µg/ml FITC-labeled fibronectin in the absence or presence of 200 nM LPA for 1 h at 37°C. Cells were washed, prefixed, and labeled with rabbit anti-fluorescein antiserum (1:100 dilution) followed by secondary donkey anti-rabbit IgG antibodies conjugated to 18-nm gold beads as described (Peters et al., 1990; Dzamba and Peters, 1991). After the immunolabeling, cells were washed with TBS, pH 8.2, containing 20 mM sodium azide and 1% bovine albumin, and post-fixed with 2.5% glutaraldehyde and then 0.1% osmium tetroxide, both in 0.1 M Hepes, pH 7.0. Post-fixed cells were washed, dehydrated with ethanol, and critical point dried (Peters and Mosher, 1987a). The cells were examined on a Hitachi S-900 high resolution, low voltage, scanning electron microscope at the Integrated Microscopy Resource of the University of Wisconsin.

Results

Serum is known to enhance binding of fibronectin to cell layers, as described in the Introduction. To test whether LPA accounts for the enhancing effect of serum on fibronectin binding, whole blood serum and citrated plasma were made the day of experimentation from a freshly drawn blood sample and treated with phospholipases. In addition, plasma-serum was prepared from citrated plasma by addition of thrombin and excess Ca²⁺ after the removal of platelets, white cells, and red cells. Plasma and serum samples were diluted in calcium-free Tyrode's buffer containing 0.2% fatty acid-free bovine albumin so that the final concentrations in the fibronectin binding assay were 2% or lower (Fig. 1 A). 200-fold more plasma or plasma-serum than whole blood serum was required to cause equivalent enhancement of binding of fibronectin to MG63 osteosarcoma cells. Prostaglandin E₁, which was added to the blood that was processed to plasma in order to minimize platelet activation, was present in the assays of plasma or plasma-serum at concentrations up to 2 nM. In control experiments, this concentration of prostaglandin E₁ had no effect on binding of fibronectin (data not shown). When whole blood serum was treated with phospholipase B, which hydrolyzes acyl bonds at the 1- and 2-positions of LPA, the enhancement activity for fibronectin binding was lost (Fig. 1 B). When whole blood serum was treated with phospholipase A₂, which hydrolyzes at the 2-position, activity was not altered. Phospholipase A₂, instead, generated activity in plasma (Fig. 1 B), presumably due to hydrolysis of phosphatidic acid to form LPA. Taken together with the studies of Eichholtz et al. (1993), these

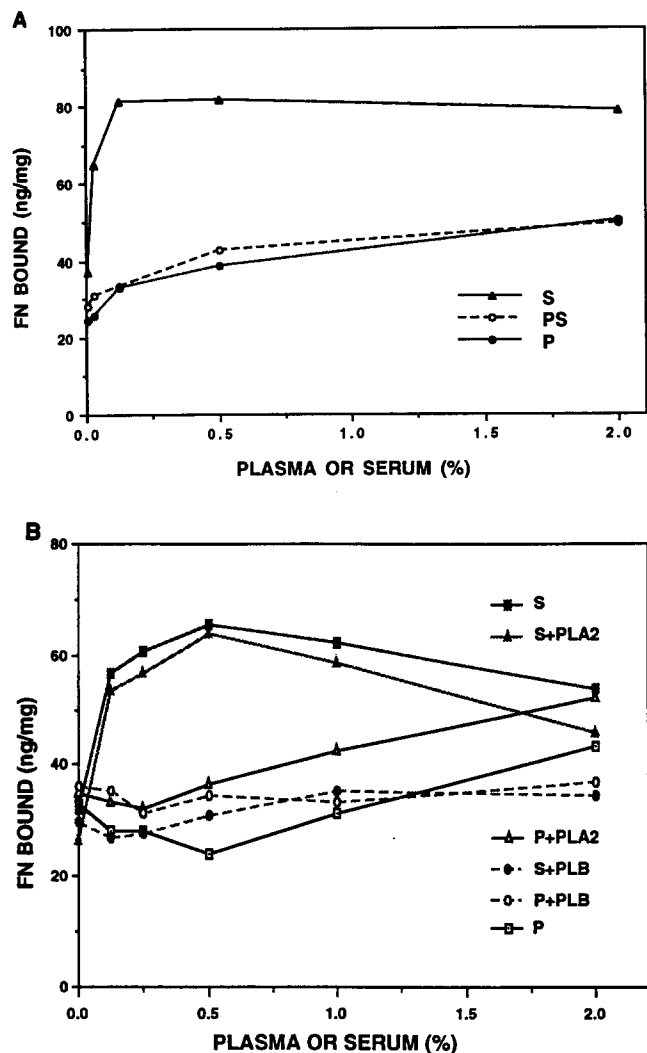


Figure 1. Effects of plasma and serum on binding of fibronectin to MG63 osteosarcoma cells. (A) Fresh human plasma (P), whole blood serum (S), or plasma-serum (PS) was prepared and added to MG63 cells at the designated dilution for 45 min with 1 µg/ml ¹²⁵I-fibronectin (FN). Specific binding was determined as described in the Methods. (B) Fresh human plasma (P) or whole blood serum (S) was prepared and incubated alone, with phospholipase A₂ (+PLA₂), or with phospholipase B (+PLB) as described in Methods and tested in a 45-min binding assay as in A. Points represent averages of duplicate values that varied <5%.

results indicate that the active component in whole blood serum that enhances fibronectin binding to MG63 cells is a product of platelet activation and is LPA.

Binding of ¹²⁵I-fibronectin to human MG63 osteosarcoma cell monolayers was enhanced in the presence of increasing concentrations of LPA containing four different fatty acids (Fig. 2 A). 1-oleoyl LPA was the most potent stimulator followed by the palmitoyl, stearoyl, and myristoyl LPAs. Inasmuch as binding of soluble fibronectin to substrate-attached cells is mediated principally by the 70-kD amino-terminal region of fibronectin (McKeown-Longo and Mosher, 1985, 1989), we tested the effects of the four different LPAs on binding of ¹²⁵I-70-kD fragment to MG63 cells (Fig. 2 B). Enhancement of binding of 70-kD fragment was comparable

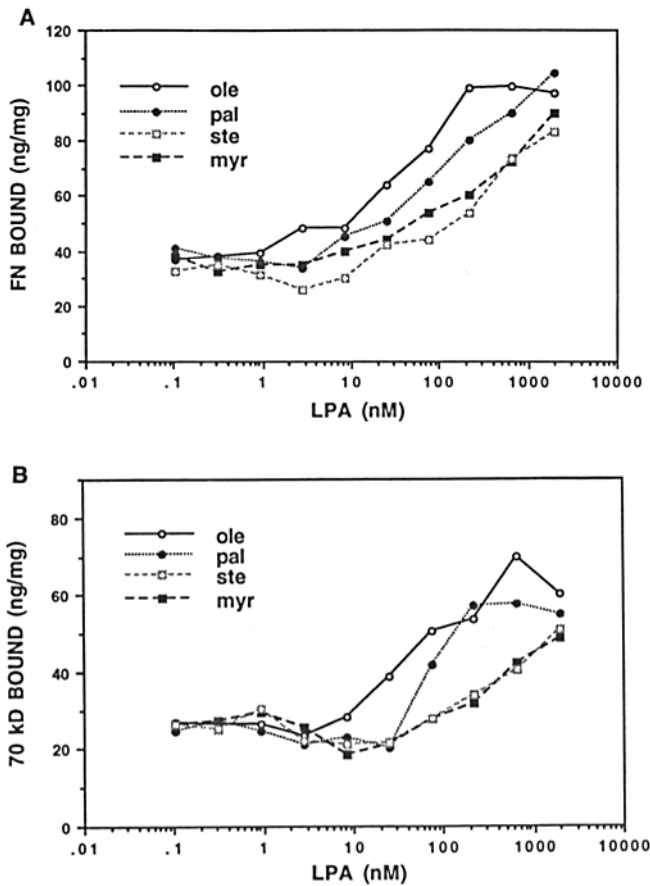


Figure 2. Effects of various LPAs on binding of fibronectin or 70-kD amino-terminal fragment to MG63 osteosarcoma cells. Increasing concentrations of 1-oleoyl (*ole*), 1-myristoyl (*myr*), 1-palmitoyl (*pal*), or 1-stearoyl (*ste*) LPA were incubated for 45 min with (A) 1 μ g/ml radiolabeled fibronectin (*FN*) or (B) 0.2 μ g/ml 70-kD amino-terminal fragment of fibronectin (70 *kD*). Points represent specifically bound fibronectin or 70-kD fragment and are averages of duplicate values that varied <5%.

with that observed with whole fibronectin in terms of both dose responses and relative activities of the LPAs.

LPA at similar doses caused similar enhanced binding over a range of fibronectin concentrations from 0.6 to 200 nM (Fig. 3). These results indicate that LPA acts not by stoichiometric combination with fibronectin but by enhancement of the ability of cells to bind fibronectin.

The increase of fibronectin binding to MG63 cells was significant for up to 24 h of exposure of cells to 200 nM LPA when 125 I-fibronectin was present throughout the treatment period (Fig. 4 A). The difference between control and LPA-treated cell layers, however, diminished during this time (Fig. 4 A). Bound 125 I-fibronectin in both LPA-treated and control cell layers at times >45 min was partly in the form of high molecular weight multimers when analyzed by SDS-PAGE without reduction (data not shown). These results indicate that LPA-enhanced binding of fibronectin to cell layers is coupled to deposition of fibronectin in SDS-insoluble matrix and that the major enhancing effect of LPA occurs in the first 4 h of incubation. When the 70-kD fragment, which does not form multimers and accumulate in the cell layer

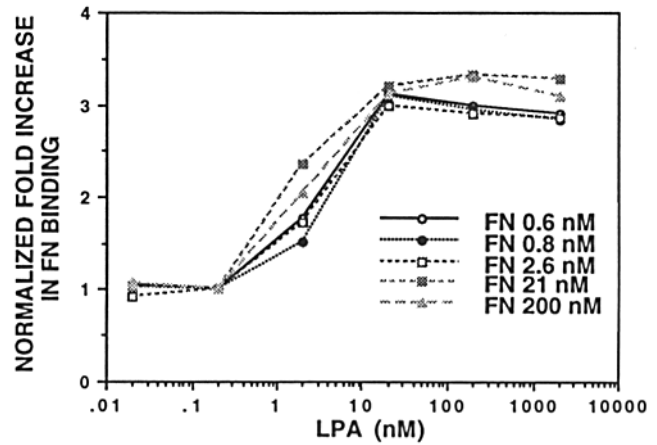


Figure 3. Enhancement of the binding of various concentrations of 125 I-labeled fibronectin (*FN*) to MG63 osteosarcoma cells by increasing concentrations of 1-oleoyl-LPA. Each data set was calculated as the ratio of specific binding measured in the presence and absence of LPA (that is, binding in the absence of LPA = 1). Binding in the presence of an additional 1140 nM (500 μ g/ml) unlabeled fibronectin was considered to be nonspecific and subtracted to calculate specific binding. Control values in the absence of LPA (input fibronectin concentration in nM, specifically bound fibronectin in ng/mg) were: 0.6, 8; 0.8, 9; 2.6, 22; 21, 170; and 200, 1140. Each point represents the average of duplicate values that varied <5%.

(McKeown-Longo and Mosher, 1985), was tested, enhanced binding to cell layers treated with 200 nM LPA was maximal at 1–2 h and gradually decreased to control values at 8 h (Fig. 4 A). A series of experiments were performed to understand why the LPA's effect is lost over time. To test whether cells would become refractory to LPA, cells were incubated with LPA, washed, and then given 125 I-labeled fibronectin or 70-kD fragment along with fresh LPA. Cells incubated with LPA at all time points bound more fibronectin or 70-kD fragment than control cells when incubated with fresh LPA (Fig. 4 B). These results suggest that cells remain responsive to LPA and that the results shown in Fig. 4 A are due to loss of the activity of LPA upon incubation with cell layers. To test stability, LPA was incubated with cells for various lengths of time, then half of the medium was transferred to a new well containing cells that had not been pretreated with LPA and the other half was left with the original cells (Fig. 4 C). Binding assays with 125 I-70-kD fragment showed that LPA-containing medium conditioned by cells lost ability to enhance binding to naive cells in parallel with loss of ability to enhance binding to the original cells. LPA-containing medium incubated at 37°C and not placed over cells, in contrast, retained the ability to enhance binding when subsequently added to cells. The activity of 200 nM LPA decayed to baseline more rapidly than the activity of 2,000 nM LPA (Fig. 4 D). In experiments not shown, LPA was added to conditioned medium from which the activity of a prior addition of LPA had been lost. The second addition of LPA had the expected activity. These results indicate that loss of activity is not due to the presence of an inhibitor in conditioned medium and therefore that an efficient cell-based system exists to destroy LPA or to remove LPA from culture medium.

The enhancement of fibronectin binding to MG63 cells by

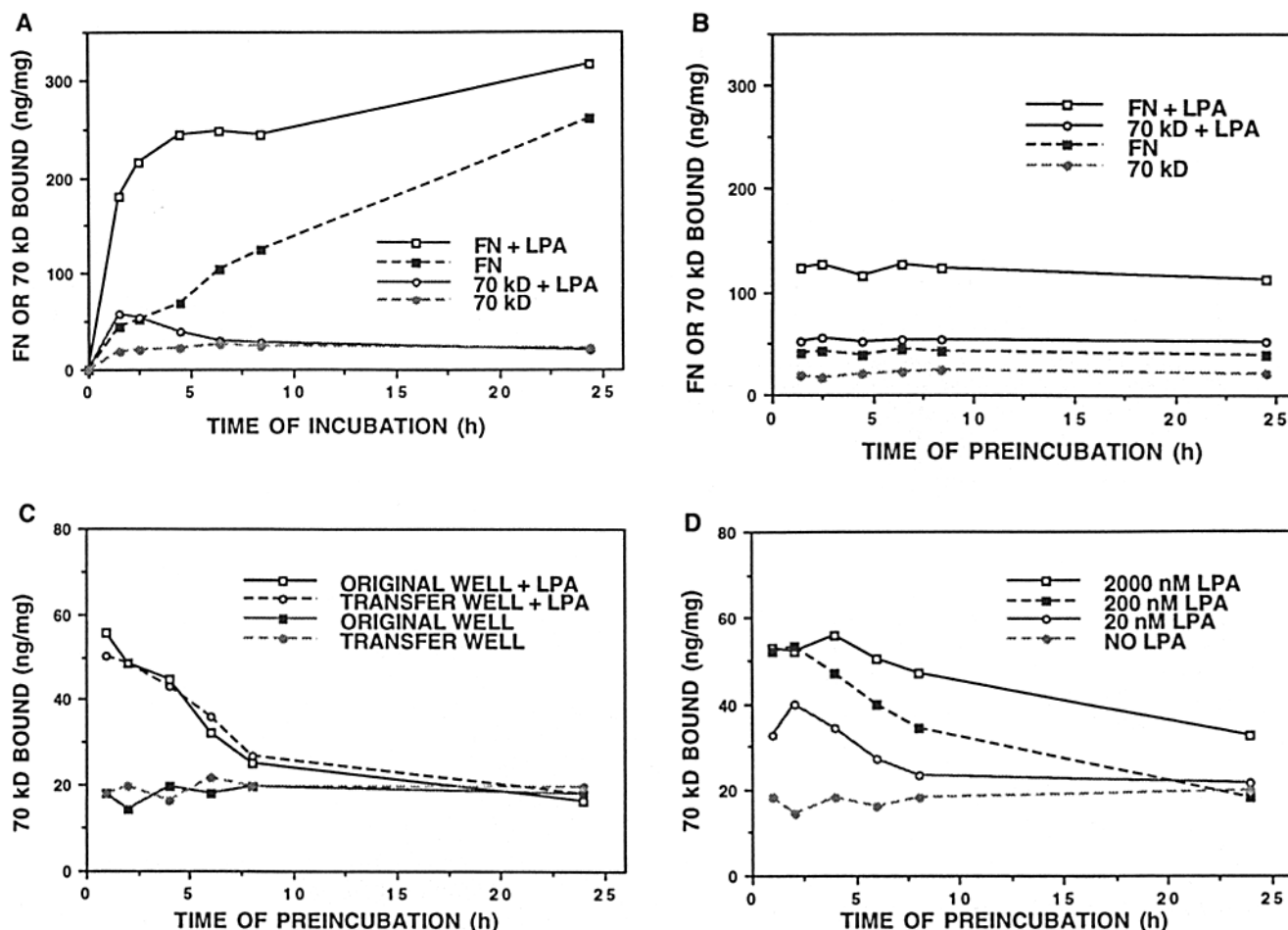


Figure 4. Duration of the LPA effect on binding of fibronectin or the 70-kD amino-terminal fragment to MG63 osteosarcoma cells. (A) Cells were incubated with 1 $\mu\text{g}/\text{ml}$ radiolabeled fibronectin (FN) or 0.2 $\mu\text{g}/\text{ml}$ 70-kD amino-terminal fragment (70 kD) in the presence or absence of 200 nM 1-oleoyl LPA, and radioactivity associated specifically with cell layer was measured at the indicated time points. When data were replotted as mol 70-kD fragment or 240-kD half fibronectin (monomer) per mg cell protein, molar amounts bound at 1.75 h were nearly identical. (B) Cells were preincubated with or without 200 nM LPA for the designated lengths of time. The medium was removed and replaced with a fresh portion of the same medium containing ^{125}I -labeled binding ligand. A 45-min binding assay was performed. (C) Cells were preincubated in medium with or without 200 nM LPA for the designated lengths of time. Half of the medium from each well was transferred to a new well containing cells that had not been treated with LPA. ^{125}I -70-kD fragment in a small volume was then added to both the wells containing cells incubated with LPA (and containing the remaining half of the medium) (ORIGINAL WELL) and the wells to which the medium was transferred (TRANSFER WELL), and 45-min binding assays were performed. The experiment was planned so that medium with or without LPA was kept in the 37°C incubator for the full 24 h and added to cells at intervals to allow the binding assays to be carried out together. Thus, the decay in LPA activity cannot be attributed to time that the LPA-containing medium was in the incubator, because this time was constant. (D) Different concentrations of 1-oleoyl LPA were preincubated with cells. After different lengths of time, half of the medium from each well was transferred to a new well containing cells that had not been treated with LPA, and binding assays of ^{125}I -70-kD fragment were performed as in C. Data represent averages of duplicate values that varied <5%.

LPA was observed within 10 min after the addition of LPA and ligand (Fig. 5). Time course of enhanced binding in response to LPA up to 4 h were the same in the presence or absence of 10 $\mu\text{g}/\text{ml}$ cycloheximide (data not shown), suggesting that no protein synthesis is required for enhancement of binding. The sustained stimulation of ^{125}I -fibronectin binding to MG63 cells by LPA contrasted with the effect of bradykinin, thrombin receptor peptide, or platelet-activating factor (Fig. 5). These agents, like LPA, elevate cytosolic calcium level in suspended fibroblasts (Jalink et al., 1990; Ye et al., 1991; Jalink and Moolenaar, 1992). Bradykinin or thrombin receptor peptide caused only transient and small,

albeit significant ($P < 0.05$), enhanced binding of fibronectin at 10 to 40 min. Platelet-activating factor did not cause any enhancement. Bombesin closely mimics the effects of LPA on cytoskeleton and cell shape (Ridley and Hall, 1992). In separate experiments, bombesin at concentrations of 1 to 100 nM also caused no enhancement of binding of 70 kD (data not shown).

Three independent analyses of the isotherms of binding of ^{125}I -70-kD fragment to LPA-treated and control MG63 cells after 45 min at 37°C indicated that LPA causes changes in both the number of receptor sites per cell ($177,000 \pm 4,000$ for LPA-treated cells and $96,000 \pm 6,000$ for control cells)

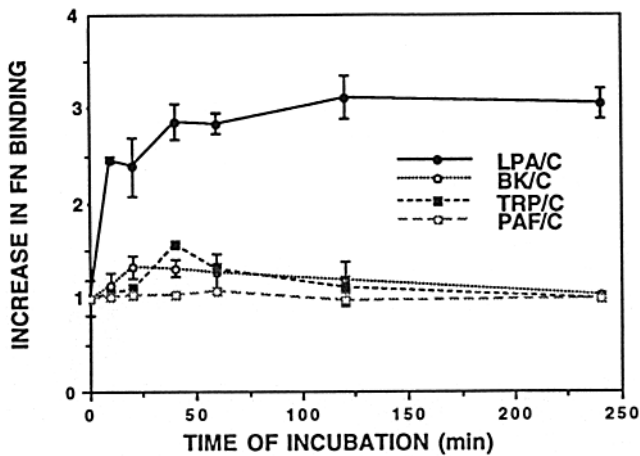
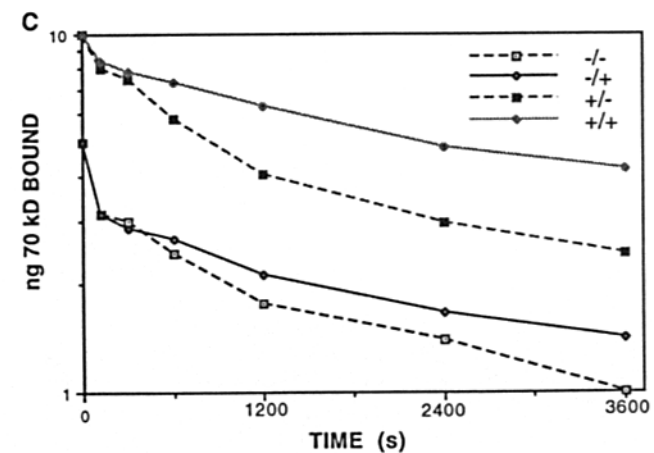
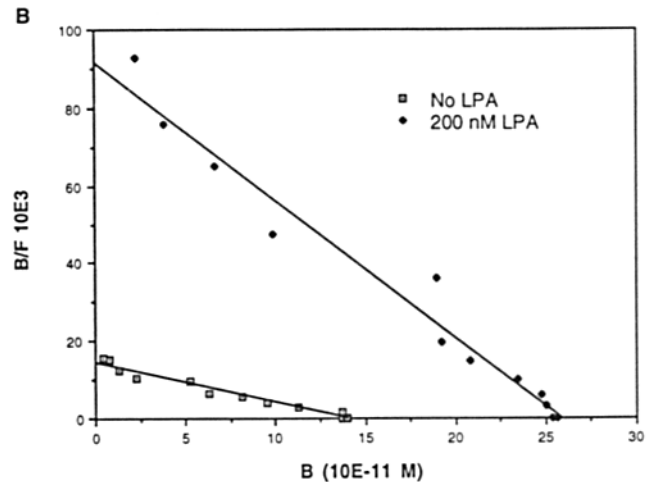
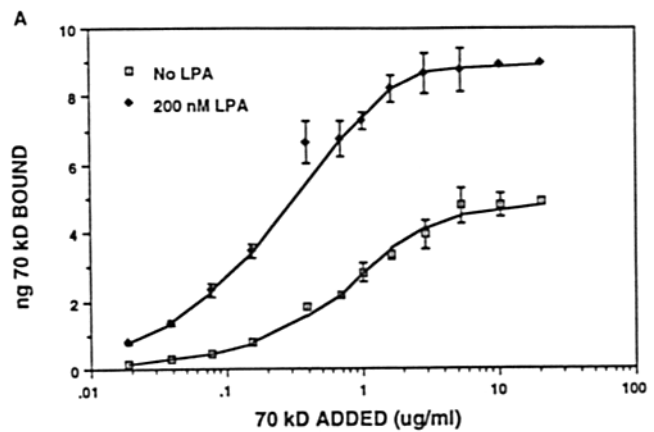


Figure 5. Time course of binding of fibronectin to MG63 osteosarcoma cells in the presence of various agonists. Cells were incubated for the indicated time with 1 $\mu\text{g/ml}$ ^{125}I -fibronectin (FN) and no agonist (C), 200 nM 1-oleoyl LPA (LPA), 1 μM bradykinin (BK), 25 μM thrombin receptor-activating peptide (TRP), or 1 μM platelet-activating factor (PAF). Each data set was calculated as the ratio of specific binding measured in the presence and absence of potential agonist (binding in the absence of agonist = 1). Points represent the mean ratios $\pm\text{SD}$ ($n = 3$).

and K_d (3.0 ± 0.3 nM for LPA-treated cells and 9.1 ± 0.7 nM for control cells) (Fig. 6 A). Scatchard plots were monophasic and compatible with single classes of binding sites on both LPA-treated and control cells (Fig. 6 B). Hill plots revealed no evidence of cooperative binding (not shown). The change in affinity caused by LPA could be attributed to an increased rate of association of ^{125}I -70-kD fragment and a decreased rate of dissociation of bound ^{125}I -70-kD fragment. Observed association rate constants of ^{125}I -70-kD fragment of LPA-treated and control cells were 4.8×10^4 and 3.4×10^4 $\text{M}^{-1} \text{sec}^{-1}$, respectively. The dissociation rate constants were independent of whether ^{125}I -70-kD fragment had been bound to the cells in the presence or absence of LPA (Fig. 6 C). The rate constants of dissociation were 2.2×10^{-4} sec^{-1} in the presence of LPA and 3.8×10^{-4} sec^{-1} in the absence of LPA.

The effects of various sequences of preexposure to 200 nM LPA on LPA-enhanced fibronectin binding to MG63 cells were tested (Fig. 7). Cells preincubated with LPA, with or without an intervening interval, responded to LPA during the binding assay as well as previously untreated cells. Cells from which LPA had been withdrawn at the beginning of the binding assay bound the same low level of fibronectin as control cells that had not been exposed to LPA at all. Similar results were obtained with binding of the 70-kD fragment (not shown).

Figure 6. Kinetics of binding of ^{125}I -70-kD amino-terminal fragment to MG63 osteosarcoma cells. (A) Increasing amounts of ^{125}I -70-kD fragment (70 kD) were incubated in 0.5 ml of modified Tyrode's buffer in the absence or presence of 200 nM 1-oleoyl LPA with cell layer for 45 min. To achieve concentrations <0.5 $\mu\text{g/ml}$, ligand with a specific activity of 700 cpm/ng was added. To achieve concentrations >0.5 $\mu\text{g/ml}$, unlabeled 70-kD fragment was added to ^{125}I -70-kD fragment to yield mixtures with progressively lower



specific activity. Specific binding was calculated by subtraction of nonspecific binding (in the presence of 50 $\mu\text{g/ml}$ or 710 nM unlabeled 70-kD fragment) from total binding and expressed as ng 70-kD fragment bound per well. Points represent mean $\pm\text{SD}$ ($n = 3$). Cultures were in 2-cm² wells each containing approximately 4.3×10^5 cells. Scatchard analyses were performed on these data and are shown in B. (C) Cells were incubated with 1 $\mu\text{g/ml}$ ^{125}I -70-kD fragment for 45 min in the absence (-/-) or presence (+/-) of 200 nM 1-oleoyl LPA. After rapid washing, the binding mixture was replaced with Tyrode's buffer containing 0.2% fatty acid-free bovine albumin with (+) or without (-) 200 nM LPA. At the designated time points, small portions of medium were sampled to estimate dissociation of specifically bound ligand as described in Methods.

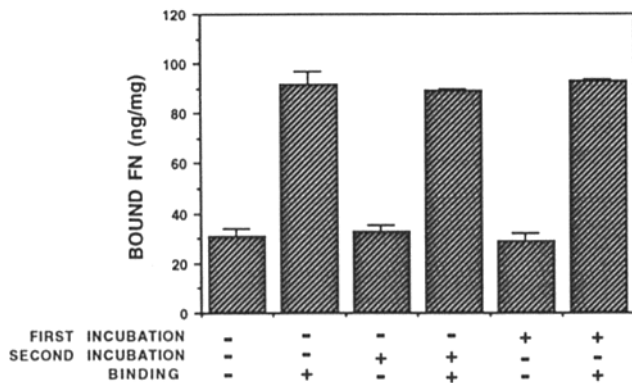


Figure 7. Effect of incubation order on the enhancement by 1-oleoyl LPA of fibronectin binding to MG63 osteosarcoma cells. Cells were incubated twice for 1 h each, with three quick washes with TBS

LPA is known to cause changes in the morphology of neurites (Jalink et al., 1993) and contraction of smooth muscle cells (Tokumura et al., 1980). Therefore, we monitored cells by phase microscopy as we added or washed out LPA in all binding experiments. Binding results correlated with rapid changes in cell shape (not shown). In order to document these shape changes in the same area of the cell layer, we carried out additional studies in which MG63 cells were observed by video-enhanced light microscopy in a perfusion chamber through which LPA could be added or withdrawn (Fig. 8). In the absence of LPA, MG63 cells were plump and

(total wash time of 5 min) between each incubation, and then incubated for 45 min with radiolabeled fibronectin (FN). When present (+), the concentration of LPA during the incubations and/or binding assay was 200 nM. Bars represent mean \pm SD ($n = 3$).

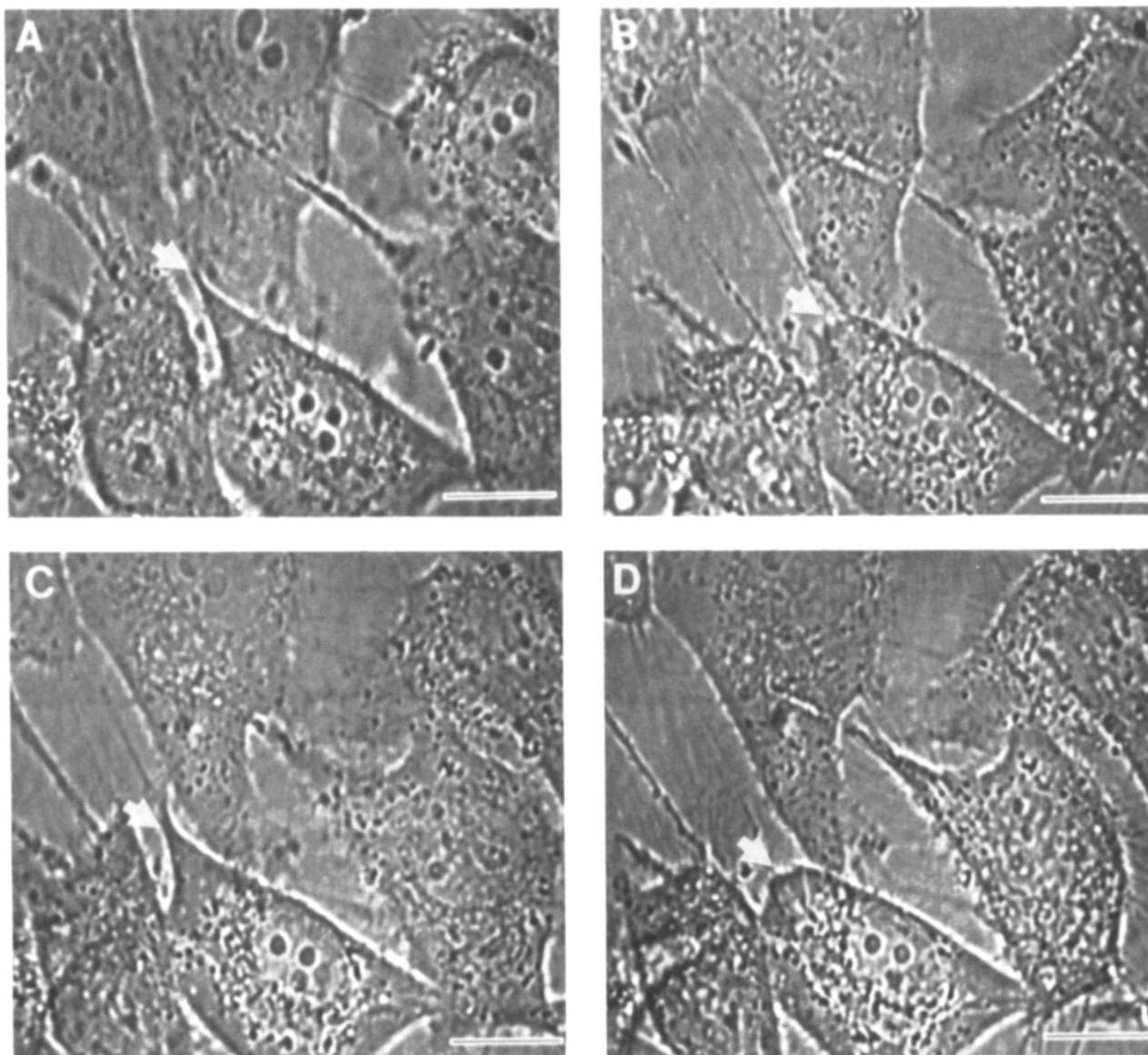


Figure 8. Effect of 1-oleoyl LPA on MG63 osteosarcoma cell morphology. Phase-contrast micrographs of adherent MG63 cells maintained in modified Tyrode's buffer at 22°C. Cells were exposed to 200 nM LPA and photographed at time zero (A) and after 10 min (B). Medium was replaced with the modified Tyrode's buffer alone for 20 min (C), followed by readdition of 200 nM LPA for 4 min (D). Arrows indicate the cell corner described in the text. Bar, 10 μ m.

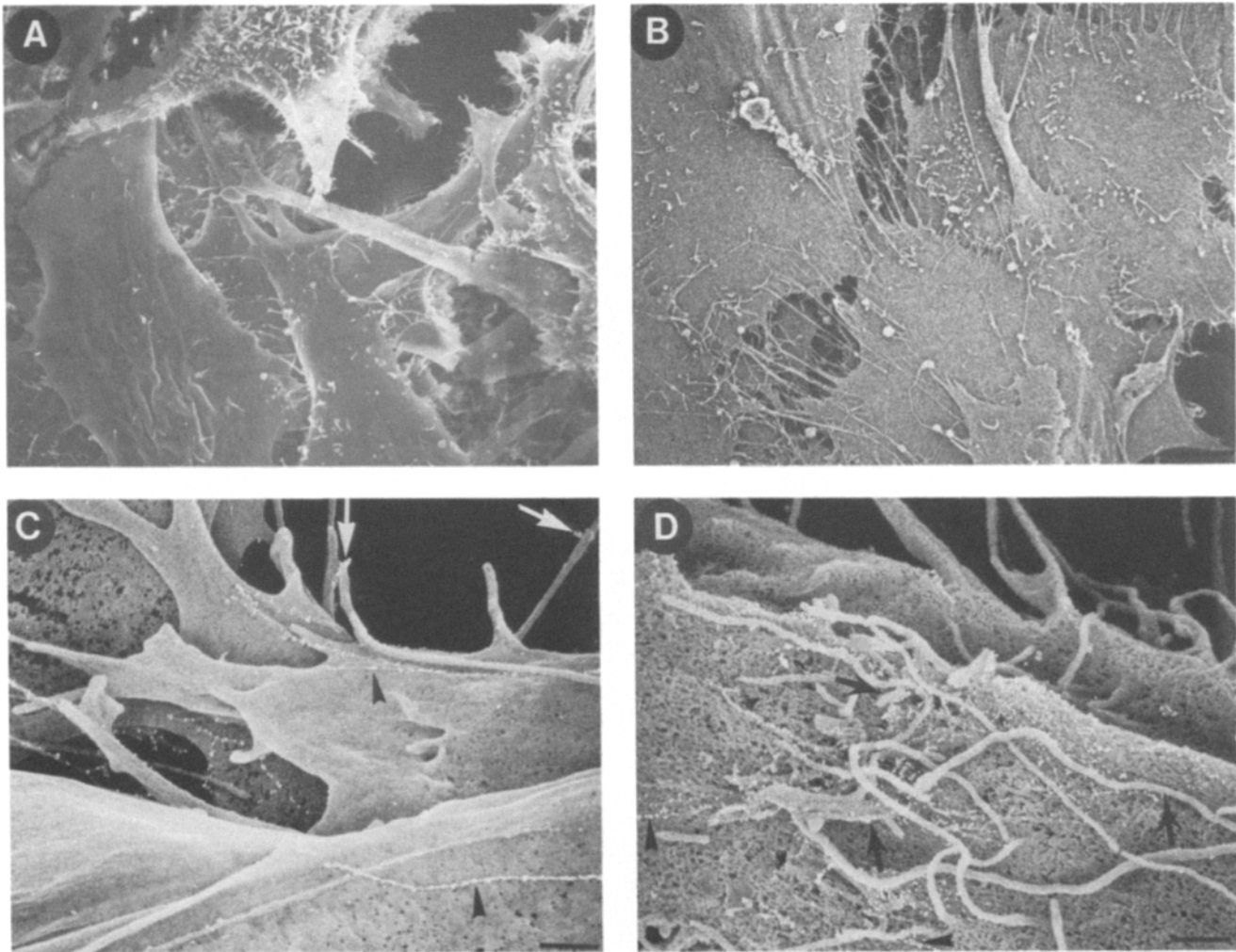


Figure 9. Scanning electron microscopy of control and LPA-treated MG63 osteosarcoma cells. Cells were incubated for 1 h with FITC-fibronectin without (*A* and *C*) or with (*B* and *D*) 200 nM LPA. FITC-fibronectin was localized with anti-fluorescein antibodies and 18-nm colloidal gold beads bound to secondary antibodies as described in Methods. Control cells (*A*) are plump whereas cells treated with LPA (*B*) are flattened and have numerous filopodial extensions. Examination of cells at higher magnification revealed that most (>90%) gold-labeled anti-fluorescein antibodies on both control (*C*) and treated (*D*) cells were localized at the edges of cells or where cells overlapped. These areas were rich in filopodia on LPA-treated cells. The beads were arranged in linear arrays on the dorsal surface or edges of cells (*arrowheads*) where filopodial extensions are found or on the filopodia themselves (*arrows*). Bars: (*A*) 4.8 μm ; (*B*) 3.8 μm ; (*C* and *D*) 0.48 μm .

overlapped one another (Fig. 8 *A*). Cells began to retract as early as 2 min after addition of LPA. Within 10 min, spaces were present between cells that contained numerous cellular protrusions (Fig. 8 *B*). The arrow indicates an area where this retraction is particularly prominent. As with enhanced binding of fibronectin shown in Fig. 7, the morphologic change was reversible. Following removal of LPA, cells relaxed and gradually spread into the vacated areas. The corner marked by the arrow was restored in less than 20 min (Fig. 8 *C*). Upon the readdition of LPA, cells retracted quickly, and within 4 min the marked corner was lost (Fig. 8 *D*).

The changes in cell shape caused by LPA were also dramatic when observed by scanning electron microscopy (Fig. 9, *A* and *B*). LPA-treated cells were flatter than cells not

treated with LPA and had numerous filopodia that extended off the cell surface and onto neighboring cells or substratum.

Changes in actin-containing cytoskeleton induced by LPA (Ridley and Hall, 1992) were correlated with enhanced binding of fibronectin to MG63 cells and foreskin fibroblasts by double label fluorescence microscopy with FITC-labeled fibronectin and rhodamine-phalloidin (Fig. 10). After addition of 200 nM LPA, MG63 cells had a better developed network of phalloidin-stained microfilament bundles when compared to cells not treated with LPA (Fig. 10, *B* and *D*). A network of long, parallel microfilament bundles, which was well developed in control foreskin fibroblasts (Fig. 10 *F*), exhibited increased densities and apparent diameters of bundles after treatment with LPA (Fig. 10 *H*). After a 45-min incubation with either MG63 cells or foreskin fibro-

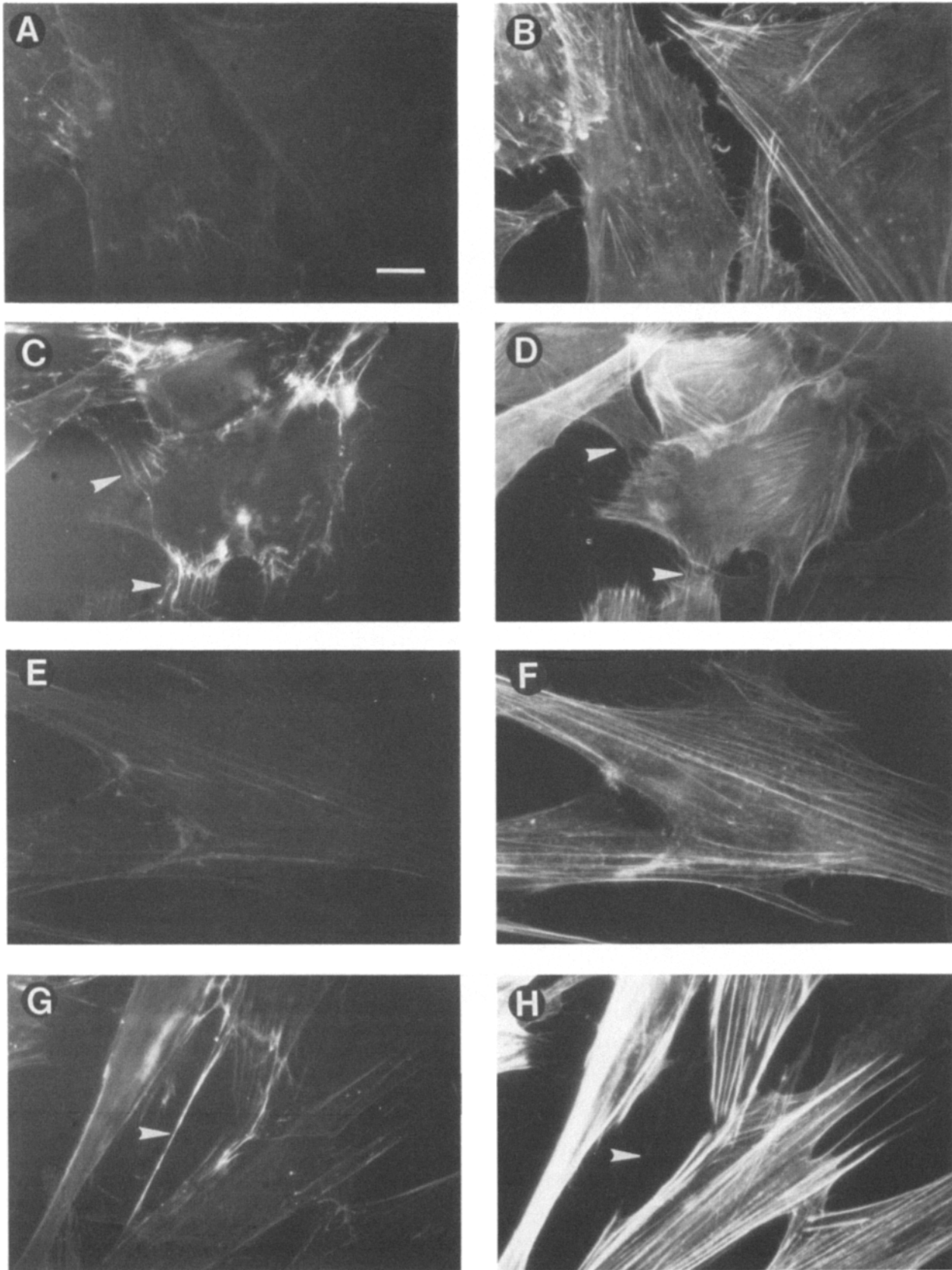


Figure 10. Effect of 1-oleoyl LPA on actin microfilament bundling and binding of exogenous plasma fibronectin to MG63 osteosarcoma cells (A–D) and foreskin fibroblasts (E–H). Cell layers on coverslips were incubated with FITC-labeled fibronectin (A, C, E, and G) in the absence (A, B, E, and F) or presence (C, D, G, and H) of 200 nM LPA in Tyrode's buffer containing 0.2% fatty acid-free bovine albumin at 37°C for 45 min. The coverslips were washed, fixed, permeabilized and incubated with rhodamine-conjugated phalloidin to stain actin microfilament bundles (B, D, F, and H), mounted, and photographed. Fibronectin bound in linear arrays at cell edges or where cells overlapped. Organized actin was detected at these locations as well. However, most organized actin was not associated with bound fibronectin, and fibronectin did localize to areas where there was no organized actin (arrowheads). Bar, 10 μ m.

blasts, FITC-fibronectin was found in linear arrays on the edges of cells and where cells overlapped (Fig. 10, C and G). More labeled protein was present on the LPA-treated cells than on control cells (compare Fig. 10, A, C, and E to G). This pattern represented specific binding inasmuch as little fluorescence was detected when unlabeled fibronectin, 500 $\mu\text{g/ml}$, was included during the incubation with FITC-labeled fibronectin (not shown). Furthermore, FITC-labeled 70-kD fragment bound in a similar distribution (not shown). Along the central bodies of cells, bound fibronectin was invariably associated with underlying organized actin. Actin bundles were also found in areas of cell overlap, but the distribution of actin did not correspond exactly with the arrays of bound fibronectin (compare Fig. 10, C and D). Some arrays of bound fibronectin extended for long distances and were not associated at all with stainable actin bundles (compare Fig. 10, G and H). By low voltage scanning electron microscopy, FITC-labeled fibronectin was found in regions where filopodia were numerous (Fig. 9, C and D). Antibody-coated beads were arranged in lines on nearby dorsal surfaces and edges of cells or on the filopodia themselves.

Discussion

LPA Is the Major Enhancer of Fibronectin Assembly in Whole Blood Serum

We have shown previously that serum, lipoproteins, and purified LPA enhance the binding fibronectin to MG63 osteosarcoma cells and normal fibroblasts (Checovich et al., 1992; Checovich and Mosher, 1993). Whole blood serum has been estimated to contain 1–5 μM LPA whereas no LPA was detected in plasma prepared by methods that minimize platelet activation (Eichholtz et al., 1993). The present results indicate that LPA is the serum enhancement factor by showing that whole blood serum but not plasma or platelet-poor serum enhances the binding of fibronectin. Furthermore, the enhancement activity of whole blood serum is destroyed by treatment with phospholipase B that renders LPA inactive. In our published lipoprotein studies (Checovich et al., 1992), lipoprotein fractions had higher specific enhancement activity than human serum, but substantial enhancement activity was present in serum depleted of lipoproteins. In other studies, the enhancement activity was gradually lost during fractionation of serum on anion-exchange and gel filtration columns; before being lost, the activity was present in albumin-containing fractions (Deetz, D. C., and D. F. Mosher, unpublished data). Similarly, Ridley and Hall (1992) found that serum stress fiber-inducing activity fractionated with albumin on a gel filtration column. Radiolabeled LPA binds to purified albumin (Eichholtz et al., 1993). LPA derivatized with a photoreactive group can be cross-linked in serum to a 68-kD protein, presumably albumin, and to 28- and 15-kD proteins (van der Bend et al., 1992). We feel, therefore, that all of our purification data can be explained in retrospect by LPA.

LPA Likely Synergizes with Other Products of Blood Coagulation to Modulate Wound Healing

The effects of serum on fibronectin binding are probably more complicated than the effect only of LPA. Serum mole-

cules that might synergize with LPA include transforming growth factor- β and platelet-derived growth factor, both of which have been shown to enhance the assembly of fibronectin (Allen-Hoffmann et al., 1988, 1990). In addition, cross-talk between LPA and platelet-derived growth factor on tyrosine phosphorylation of focal adhesion kinase has been demonstrated recently (Seufferlein and Rosengurt, 1994). As with LPA, serum concentrations of transforming growth factor- β and platelet-derived growth factor depend upon activation of platelets (see Mosher et al., 1985, among others). LPA-induced up-regulation of matrix assembly may be an important part of the wound healing process. Healing of gaping wounds occurs in several stages—population of the wound space by fibroblasts, elaboration of a fibronectin-collagen matrix, and alignment of fibroblasts within the matrix, and contraction of the wound (Welch et al., 1990; Grinnell, 1994). LPA and the other products of activated platelets potentially could function in each of the stages.

The Target of LPA Is Cells but Not Fibronectin

The observation that lipoproteins had to be present simultaneously with radiolabeled fibronectin in order to detect enhanced binding led us to speculate that enhancement is due to a direct interaction between fibronectin and lipoproteins (Checovich et al., 1992). Serum also has to present simultaneously to cause enhanced binding (Assadi, F. M., and D. F. Mosher, unpublished data). All experiments with LPA, however, indicate that the phospholipid works by its effects on cells and that the primary effect is to enhance expression of the cell-binding sites for modules in the amino-terminal portion of fibronectin. Binding of fibronectin over a 330-fold concentration range or of the 70-kD amino-terminal fragment was enhanced by the nanomolar concentrations of LPA known to cause perturbations of intracellular signaling pathways and changes in cell shape and cytoskeletal arrangement (see Introduction). There was good agreement between the effects LPA had on cell shape or actin microfilaments and its effect on fibronectin binding in several different experiments. In particular, changes in cell shape induced by addition or withdrawal of LPA were rapid enough to account for the requirement that the enhancer be present during the binding assay.

Several known effectors of fibronectin binding act on pathways known to be influenced by LPA. The inhibition of adenylate cyclase upon treatment with LPA (van Corven et al., 1989) is in accord with the observation that agents which increase intracellular cAMP levels cause reduced numbers of binding sites for fibronectin or the 70-kD amino-terminal fragment (Allen-Hoffmann and Mosher, 1987). Stimulation of phospholipase C and phosphoinositide hydrolysis upon treatment with LPA (van Corven et al., 1989) is in accord with the observation that activation of protein kinase C by phorbol esters causes increased numbers of binding sites (Somers and Mosher, 1993). Treatment of cells with cytochalasin D causes both loss of fibronectin binding sites (Barry and Mosher, 1988) and inhibition of LPA-stimulated tyrosine phosphorylation (Seufferlein and Rosengurt, 1994).

The Effect of LPA on Fibronectin Binding Is Specific and Unique

The concentrations of purified LPA that enhance fibronectin

binding and perturb intracellular signaling pathways are less than the critical micellar concentration of LPA, estimated to be 70 μ M by Das and Hajra (1992) and 1.3 mM by Jalink et al. (1990). Other lysophospholipids are inactive, and phosphatidic acid is >100-fold less active than LPA (Checovich and Mosher, 1993), demonstrating the specificity of the LPA response. Evidence for the existence of a specific cell surface receptor for LPA is presented in the Introduction. Such a receptor, however, has not yet been characterized and remains hypothetical. Bradykinin, a peptide agonist of the thrombin receptor, platelet-activating factor, and LPA all elevate cytosolic calcium level in suspended fibroblasts (Jalink et al., 1990; Ye et al., 1991; Jalink and Moolenaar, 1992). The calcium response to LPA, as with the other agents, is transient and subject to desensitization (Jalink et al., 1990). The enhancement of fibronectin binding to osteosarcoma cells by LPA, however, was sustained and only lost when LPA was removed or the medium became depleted of LPA. Bombesin, which most clearly duplicates the effects of LPA on cytoskeleton (Ridley and Hall, 1992), did not enhance binding of the 70-kD fragment by osteosarcoma cells. A peptide agonist of the thrombin receptor, which mimics the effects of LPA on neuronal cell retractions (Jalink et al., 1994), also did not enhance binding. Our observations indicate, therefore, that LPA's effects are specific and represent a response that is unique among agents that elevate intracellular calcium ion or trigger cytoskeletal organization.

Binding Data Require both the Interconversion of Binding Sites between High and Low Avidity States and the Appearance and Disappearance of Sites

Increased binding of the 70-kD amino-terminal fragment to LPA-treated osteosarcoma cells was due to a doubling of number of binding sites and a threefold increase of avidity of binding. Analyses of equilibrium binding over 1,000-fold concentration range of input ligand were compatible with single, uncooperative classes of binding sites on both LPA-treated and control cells. We found no evidence for low avidity sites on LPA-treated cells or a population of high avidity sites on control cells, even though our experiments would have allowed detection of such sites. When ligand was bound in the presence or absence of LPA and dissociation was measured in medium containing or not containing LPA, the dissociation rates were independent of the conditions of binding but dependent on the conditions of dissociation. Thus, some binding sites on LPA-treated and control cells seem to interconvert between high and low avidity states whereas other high avidity sites appear and disappear completely. The localization of bound FITC-labeled fibronectin shown in Fig. 9 agrees with previous results that localized bound fibronectin-gold bead conjugates to lateral and retracting cell edges and filopodia (Peters and Mosher, 1987a, b). We speculate that the binding kinetics can be explained by the microscopic findings. The interconversion between high and low avidity binding sites upon addition and withdrawal of LPA may be related to polymerization and depolymerization of the actin cytoskeleton whereas the appearance and disappearance of high avidity binding sites may be due to the protrusion and retraction of filopodial processes.

The "Fibronectin Assembly Receptor" Must be Subject to Rapid Up- and Down-regulation

Studies of deletion and mutation sets of the recombinant fragment indicate that the amino-terminal five type I modules act as a unit that mediates binding to cells (Sottile et al., 1991). Proteins of 60–70 kD have been isolated from detergent extracts of macrophages (Blystone and Kaplan, 1992) and myoblasts (Moon et al., 1994) that bind to a subfragment of the 70-kD fragment that contains the five type I modules. Assembly of fibronectin requires fibronectin-fibronectin interactions, raising the possibility that the binding molecule on cell surfaces is fibronectin itself. A fibronectin-fibronectin interaction site had been identified in the most amino-terminal type III module of fibronectin (Chernousov et al., 1991; Morla and Ruoslahti, 1992). Morla et al. (1994) suggested that assembly of fibronectin is controlled by exposure of cryptic binding activity in the III-1 module. Hocking et al. (1994) demonstrated binding of the 70-kD amino-terminal fragment to the III-1 module. However, studies of a dimeric construct of the 70-kD region that has the ability to assemble into extracellular matrix suggest that the determinants for binding to cell surfaces and interaction with other regions of fibronectin are different (Sottile and Wiley, 1994). Our results indicate that if the III-1 module of fibronectin or the 60–70-kD cell surface proteins mediate binding of the 70-kD fragment to cell surfaces, a mechanism to cause rapid conformational change must exist to up- and down-regulate binding activity.

The Role of $\alpha_5\beta_1$ Integrin in Fibronectin Assembly May Be to Facilitate Changes in Cell Shape

The $\alpha_5\beta_1$ fibronectin cell adhesion receptor is subject to such an up- and down-regulation of its ability to bind fibronectin (O'Toole et al., 1994) and therefore is an attractive candidate for a cell surface binding molecule. In CHO cells, expression of $\alpha_5\beta_1$ greatly favors elaboration of fibronectin matrix (Giancotti and Ruoslahti, 1990; Wu et al., 1993). Monoclonal antibodies to $\alpha_5\beta_1$ block fibronectin matrix formation (Akiyama et al., 1989) and binding of exogenous fibronectin or the 70-kD fragment to cell monolayers (Fogerty et al., 1990). Cultured mouse cells and mouse embryos lacking α_5 , however, deposit a normal fibronectin matrix as assessed by immunofluorescence (Yang et al., 1993). Detergent-solubilized $\alpha_5\beta_1$ does not bind to the 70-kD fragment (Fogerty et al., 1990). There is a 10–15-min delay before antibody to $\alpha_5\beta_1$ causes a decrease in fibronectin binding to adherent cells (Fogerty et al., 1990). We have seen similar effects of anti- β_1 on LPA-treated cells; when LPA and antibody were added together, an inhibitory effect of the antibody was only evident after 15–20 min (Zhang and Mosher, unpublished observation). Anti- β_1 also causes changes in cell shape and alterations in the association of cells with preexisting matrix (Fogerty and Mosher, 1990), whereas LPA causes a rapid increase in the number of focal contacts (Ridley and Hall, 1992). Our studies were done on confluent cultures in which the binding sites are on the dorsal (upper and lateral) surfaces of cells rich in filopodia. We have considered two hypotheses by which the effects of LPA on focal contacts and the effects of $\alpha_5\beta_1$ -perturbing agents could be related to our results. First, the same molecules

responsible for focal adhesions could be used in a more dynamic fashion to bind the 70-kD fragment. In favor of this scenario is the observation that the 70-kD fragment localizes to β_1 -containing focal adhesions of newly spread cells (Dzamba et al., 1994). Second, increased focal contacts could allow cells to "grip" the substratum and facilitate shape change, development of filopodia, and display of molecules unrelated to $\alpha_5\beta_1$ that bind the amino-terminal modules of fibronectin. The ability of LPA to regulate cell shape and display of assembly sites rapidly and coordinately provides an experimental system in which to test these hypotheses and identify the molecules at the assembly sites.

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References

- Akiyama, S. K., S. S. Yamada, W.-T. Chen, and K. M. Yamada. 1989. Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization. *J. Cell Biol.* 109:863-875.
- Albrecht, R. M., S. L. Goodman, and S. R. Simmons. 1989. Distribution and movement of membrane-associated platelet glycoproteins: use of colloidal gold with correlative video-enhanced light microscopy, low-voltage high-resolution scanning electron microscopy, and high-voltage transmission electron microscopy. *Am. J. Anat.* 185:149-164.
- Allen-Hoffmann, B. L., C. L. Crankshaw, and D. F. Mosher. 1988. Transforming growth factor β increases cell surface binding and assembly of exogenous (plasma) fibronectin by normal human fibroblasts. *Mol. Cell Biol.* 8:4234-4242.
- Allen-Hoffmann, B. L., and D. F. Mosher. 1987. Matrix assembly sites for exogenous fibronectin are decreased on human fibroblasts after treatment with agents which increase intracellular cAMP. *J. Biol. Chem.* 262:14361-14365.
- Allen-Hoffmann, B. L., S. J. Schlosser, W. H. Brondyk, and W. E. Fahl. 1990. Fibronectin levels are enhanced in human fibroblasts overexpressing the c-*sis* protooncogene. *J. Biol. Chem.* 265:5219-5225.
- Allio, A. E., and P. J. McKeown-Longo. 1988. Extracellular matrix assembly of cell-derived and plasma-derived fibronectins by substrate-attached fibroblasts. *J. Cell. Physiol.* 135:459-466.
- Barry, E. L. R., and D. F. Mosher. 1988. Factor XIII cross-linking of fibronectin at cellular matrix assembly sites. *J. Biol. Chem.* 263:10464-10469.
- Barry, E. L. R., and D. F. Mosher. 1989. Factor XIIIa-mediated cross-linking of fibronectin in fibroblast cell layers. Cross-linking of cellular and plasma fibronectin and of amino-terminal fibronectin fragments. *J. Biol. Chem.* 264:4179-4185.
- Benton, A. M., J. M. Gerrard, T. Michiel, and S. E. Kindom. 1982. Are lysophosphatidic acids or phosphatidic acids involved in stimulus activation coupling in platelets? *Blood.* 60:642-649.
- Billah, M. M., E. G. Lapetina, and P. Cuatrecasas. 1981. Phospholipase A₂ activity specific for phosphatidic acid. A possible mechanism for the production of arachidonic acid in platelets. *J. Biol. Chem.* 256:5399-5403.
- Bishop, W. R., and R. M. Bell. 1988. Assembly of phospholipids into cellular membranes: biosynthesis, transmembrane movement and intracellular translocation. *Annu. Rev. Cell Biol.* 4:579-610.
- Blystone, S. D., and J. E. Kaplan. 1992. Isolation of an amino-terminal fibronectin-binding protein on human U937 cells and rat peritoneal macrophages. *J. Biol. Chem.* 267:3968-3975.
- Checovich, W. J., and D. F. Mosher. 1993. Lysophosphatidic acid enhances fibronectin binding to adherent cells. *Arterioscler. Thromb.* 13:1662-1667.
- Checovich, W. J., R. L. Schultz, and D. F. Mosher. 1992. Lipoproteins enhance fibronectin binding to adherent cells. *Arterioscler. Thromb.* 12:1122-1130.
- Chernousov, M. A., F. J. Fogerty, V. E. Koteliansky, and D. F. Mosher. 1991. Role of the I-9 and III-1 modules of fibronectin in formation of an extracellular fibronectin matrix. *J. Biol. Chem.* 266:10851-10858.
- Colvin, R. B. 1989. Fibronectin in wound healing. In *Fibronectin*. D. F. Mosher, editor. Academic Press, New York. 213-254.
- Das, A. K., and A. K. Hajra. 1992. Critical micellar concentrations of palmitoyl dehydroxyacetone phosphate and 1-palmitoyl-rac-glycerol 3-phosphate. *J. Biol. Chem.* 267:9731.
- Dzamba, B. J., and D. M. Peters. 1991. Arrangement of cellular fibronectin in noncollagenous fibrils in human fibroblast cultures. *J. Cell Sci.* 100:605-612.
- Dzamba, B. J., H. Bultmann, S. K. Akiyama, and D. M. Peters. 1994. Substrate-specific binding of the amino terminus of fibronectin to an integrin complex in focal adhesions. *J. Biol. Chem.* 269:19646-19652.
- Eichholtz, T., K. Jalink, I. Fahrenfort, and W. H. Moolenaar. 1993. The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem. J.* 291:677-680.
- Exton, J. H. 1990. Signaling through phosphatidylcholine breakdown. *J. Biol. Chem.* 265:1-4.
- Fernhout, B. J. H., F. A. Dijkcs, W. H. Moolenaar, and G. S. F. Ruigt. 1992. Lysophosphatidic acid induces inward currents in *Xenopus laevis* oocytes; evidence for an extracellular site of action. *Euro. J. Pharmacol.* 213:313-315.
- Fogerty, F. J., S. K. Akiyama, K. M. Yamada, and D. F. Mosher. 1990. Inhibition of binding of fibronectin to matrix assembly sites by anti-integrin ($\alpha_5\beta_1$) antibodies. *J. Cell Biol.* 111:699-708.
- Fogerty, F. J., and D. F. Mosher. 1990. Mechanisms for organization of fibronectin matrix. *Cell Diff. Dev.* 32:439-450.
- George, E. L., E. N. Georges-Labouesse, R. S. Patel-King, H. Rayburn, and R. O. Hynes. 1993. Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development (Camb.)* 119:1079-1091.
- Gerrard, J. M., and P. Robinson. 1989. Identification of the molecular species of lysophosphatidic acid produced when platelets are stimulated by thrombin. *Biochim. Biophys. Acta.* 1001:282-285.
- Giancotti, F. G., and E. Ruoslahti. 1990. Elevated levels of the $\alpha_5\beta_1$ fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. *Cell.* 60:849-859.
- Grinnell, F. 1994. Fibroblasts, myofibroblasts, and wound contraction. *J. Cell Biol.* 124:401-404.
- Hocking, D. C., J. Sottile, and P. J. McKeown-Longo. 1994. Fibronectin's III-1 module contains a conformation-dependent binding site for the amino-terminal region of fibronectin. *J. Biol. Chem.* 269:19183-19191.
- Howe, L. R., and C. J. Marshall. 1993. Lysophosphatidic acid stimulates mitogen-activated protein kinase activation via a G-protein-coupled pathway requiring p21^{ras} and p74^{rac1}. *J. Biol. Chem.* 268:20717-20720.
- Hynes, R. O. 1990. *Fibronectin*. Springer-Verlag, Inc., New York.
- Jaffe, E. A., J. T. Ruggiero, L. K. Leung, M. J. Doyle, P. J. McKeown-Longo, and D. F. Mosher. 1983. Cultured human fibroblasts synthesize and secrete thrombospondin and incorporate it into extracellular matrix. *Proc. Natl. Acad. Sci. USA.* 80:998-1002.
- Jalink, K., T. Eichholtz, F. R. Postma, E. J. van Corven, and W. H. Moolenaar. 1993. Lysophosphatidic acid induces neuronal shape changes via a novel, receptor-mediated signaling pathway: similarity to thrombin action. *Cell Growth & Differ.* 4:247-255.
- Jalink, K., and W. H. Moolenaar. 1992. Thrombin receptor activation causes rapid neural cell rounding and neurite retraction independent of classic second messengers. *J. Cell Biol.* 118:411-419.
- Jalink, K., E. J. van Corven, T. Hengeveld, N. Morii, S. Narumiya, and W. H. Moolenaar. 1994. Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. *J. Cell Biol.* 126:810-810.
- Jalink, K., E. J. van Corven, and W. H. Moolenaar. 1990. Lysophosphatidic acid, but not phosphatidic acid, is a potent Ca²⁺-mobilizing stimulus for fibroblasts. *J. Biol. Chem.* 265:12232-12239.
- Kumagai, N., N. Morii, K. Fujisawa, Y. Nemoto, and S. Narumiya. 1993. ADP-ribosylation of rho p21 inhibits lysophosphatidic acid-induced protein tyrosine phosphorylation and phosphatidylinositol 3-kinase activation in cultured Swiss 3T3 cells. *J. Biol. Chem.* 268:24535-24538.
- Lapetina, E. G., M. M. Billah, and P. Cuatrecasas. 1981. The phosphatidylinositol cycle and the regulation of arachidonic acid production. *Nature (Lond.)* 292:367-369.
- Limbird, L. 1986. *Cell Surface Receptors: A Short Course on Theory and Methods*. Martinus Nijhoff Publishing, Boston. 51-96.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Mautner, V. M., and R. O. Hynes. 1977. Surface distribution of LETS protein in relation to the cytoskeleton of normal and transformed fibroblasts. *J. Cell Biol.* 75:743-768.
- McKeown-Longo, P. J., and D. F. Mosher. 1983. Binding of plasma fibronectin to cell layers of human skin fibroblasts. *J. Cell Biol.* 97:466-472.
- McKeown-Longo, P. J., and D. F. Mosher. 1984. Mechanism of formation of disulfide-bonded multimers of plasma fibronectin in cell layers of cultured human fibroblasts. *J. Biol. Chem.* 259:12210-12215.
- McKeown-Longo, P. J., and D. F. Mosher. 1985. Interaction of the 70,000-mol-wt amino-terminal fragment of fibronectin with the matrix-assembly receptor of fibroblasts. *J. Cell Biol.* 100:364-374.
- McKeown-Longo, P. J., and D. F. Mosher. 1989. The assembly of the fibronectin matrix in cultured human fibroblast cells. In *Fibronectin*. D. F. Mosher, editor. Academic Press Inc., New York. 163-179.
- Miura, Y., A. Kikuchi, T. Musha, S. Kuroda, H. Yaku, T. Sasaki, and Y. Takai. 1993. Regulation of morphology by rho p21 and its inhibitory GDP/GTP exchange protein (rho GDI) in Swiss 3T3 cells. *J. Biol. Chem.* 268:510-515.
- Moolenaar, W. H., W. Kruijer, B. C. Tilly, I. Verlaan, A. J. Bierman, and

- S. W. de Laat. 1986. Growth factor-like action of phosphatidic acid. *Nature (Lond.)*. 323:171-173.
- Moon, K. Y., K. S. Shin, W. K. Song, C. H. Chung, D. B. Ha, and M. S. Kang. 1994. A candidate molecule for the matrix assembly receptor to the N-terminal 29-kDa fragment of fibronectin in chick myoblasts. *J. Biol. Chem.* 269:7651-7657.
- Morla, A., and E. Ruoslahti. 1992. A fibronectin self-assembly site involved in fibronectin matrix assembly: reconstruction in a synthetic peptide. *J. Cell Biol.* 118:421-429.
- Morla, A., Z. Zhang, and E. Ruoslahti. 1994. Superfibronectin is a functionally distinct form of fibronectin. *Nature (Lond.)*. 367:193-196.
- Mosher, D. F. 1989. Fibronectin. Academic Press, San Diego.
- Mosher, D. F., D. M. Pesciotta, J. C. Loftus, and R. M. Albrecht. 1985. Secreted alpha granule proteins: the race for receptors. In Platelet Membrane Glycoproteins. J. N. George, D. R. Phillips, and A. T. Nurden, editors. Plenum Press, New York. 171-191.
- Mosher, D. F., and A. Vaheri. 1978. Thrombin stimulates the production and release of a major surface-associated glycoprotein (fibronectin) in cultures of human fibroblasts. *Exp. Cell Res.* 112:323-334.
- Nakamura, M., Z. Honda, T. Izumi, C. Sakanaka, H. Mutoh, M. Minami, H. Bito, Y. Seyama, T. Matsumoto, M. Noma, and T. Shimizu. 1991. Molecular cloning and expression of platelet-activating factor receptor from human leukocytes. *J. Biol. Chem.* 266:20400-20405.
- O'Toole, T. E., Y. Katagiri, R. J. Faull, K. Peter, R. Tamura, V. Quaranta, J. C. Loftus, S. J. Shattil, and M. H. Ginsberg. 1994. Integrin cytoplasmic domains mediate inside-out signal transduction. *J. Cell Biol.* 124:1047-1059.
- Peters, D. M., and D. F. Mosher. 1987a. Localization of cell surface sites involved in fibronectin fibrillogenesis. *J. Cell Biol.* 104:121-130.
- Peters, D. M., and D. F. Mosher. 1987b. Assembly and alignment of fibronectin-coated gold beads into fibrils by human skin fibroblasts. *Scan. Microsc.* 1:757-763.
- Peters, D. M., L. M. Portz, J. Fullenwider, and D. F. Mosher. 1990. Co-assembly of plasma and cellular fibronectins into fibrils in human fibroblast cultures. *J. Cell Biol.* 111:249-256.
- Prescott, S. M., G. A. Zimmerman, and T. M. McIntyre. 1990. Platelet-activating factor. *J. Biol. Chem.* 265:17381-17384.
- Rennard, S. I., M. L. Wind, A. T. Hewitt, and H. K. Kleinman. 1981. Effect of collagen and cell shape on binding of fibronectin to cells. *Arch. Biochem. Biophys.* 206:205-212.
- Ridley, A. J., and A. Hall. 1992. The small GTP-binding protein *rho* regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell*. 70:389-399.
- Seufferlein, T., and E. Rozengurt. 1994. Lysophosphatidic acid stimulates tyrosine phosphorylation of focal adhesion kinase, paxillin, and p130. Signaling pathways and cross-talk with platelet-derived growth factor. *J. Biol. Chem.* 269:9345-9351.
- Singer, I. I. 1982. Association of fibronectin and vinculin with focal contacts and stress fibers in stationary hamster fibroblasts. *J. Cell Biol.* 92:398-408.
- Singer, I. I. 1989. Fibronectin-cytoskeleton relationship. In Fibronectin. D. F. Mosher, editor. Academic Press, New York. 139-162.
- Somers, C. E., and D. F. Mosher. 1993. Protein kinase C modulation of fibronectin matrix assembly. *J. Biol. Chem.* 268:22277-22280.
- Sottile, J., J. Schwarzbauer, J. Selegue, and D. F. Mosher. 1991. Five type I modules of fibronectin form a functional unit that binds to fibroblasts and *Staphylococcus aureus*. *J. Biol. Chem.* 266:12840-12843.
- Sottile, J., and S. Wiley. 1994. Assembly of amino-terminal fibronectin dimers into the extracellular matrix. *J. Biol. Chem.* 269:17192-17198.
- Thiery, J. P., J. L. Duband, S. Dufour, P. Savagner, and B. A. Imhof. 1989. Roles of fibronectins in embryogenesis. In Fibronectin. D. F. Mosher, editor. Academic Press, New York. 181-209.
- Tokumura, A., K. Fukuzawa, S. Yamada, and H. Tsukatani. 1980. Stimulatory effect of lysophosphatidic acids on uterine smooth muscles of non-pregnant rats. *Arch. Int. Pharmacodyn.* 245:74-83.
- van Corven, E. J., A. Groenink, K. Jalink, T. Eichholtz, and W. H. Moolenaar. 1989. Lysophosphatidate-induced cell proliferation: identification and dissection of signaling pathways mediated by G proteins. *Cell*. 59:45-54.
- van Corven, E. J., P. L. Hordijk, R. H. Medema, J. L. Bos, and W. H. Moolenaar. 1993. Pertussis toxin-sensitive activation of p21^{ras} by G protein-coupled receptor agonists in fibroblasts. *Proc. Natl. Acad. Sci. USA*. 90:1257-1261.
- van der Bend, R. L., J. Brunner, K. Jalink, E. J. van Corven, W. H. Moolenaar, and W. J. van Blitterswijk. 1992. Identification of a putative membrane receptor for the bioactive phospholipid, lysophosphatidic acid. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:2495-2501.
- Watson, S. P., R. T. McConnell, and E. G. Lapetina. 1985. Decanoyl lysophosphatidic acid induces platelet aggregation through an extracellular action. Evidence against a second messenger role for lysophosphatidic acid. *Biochem. J.* 232:61-66.
- Welch, M. P., G. F. Odland, and R. A. Clark. 1990. Temporal relationships of F-actin bundle formation, collagen and fibronectin matrix assembly, and fibronectin receptor expression to wound contraction. *J. Cell Biol.* 110:133-145.
- Wu, C., J. S. Bauer, R. L. Juliano, and J. A. McDonald. 1993. The $\alpha_5\beta_1$ integrin fibronectin receptor, but not the α_5 cytoplasmic domain, functions in an early and essential step in fibronectin matrix assembly. *J. Biol. Chem.* 268:21883-21888.
- Yang, J. T., H. Rayburn, and R. O. Hynes. 1993. Embryonic mesodermal defects in α_5 integrin-deficient mice. *Development (Camb.)*. 119:1093-1105.
- Ye, R. D., E. R. Prossnitz, A. H. Zou, and C. G. Cochrane. 1991. Characterization of human cDNA that encodes a functional receptor for platelet activating factor. *Biochem. Biophys. Res. Commun.* 180:105-111.