Rho-stimulated Contractility Drives the Formation of Stress Fibers and Focal Adhesions

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Abstract. Activated rhoA, a ras-related GTP-binding protein, stimulates the appearance of stress fibers, focal adhesions, and tyrosine phosphorylation in quiescent cells (Ridley, A.J., and A. Hall, 1992. Cell. 70:389-399). The pathway by which rho triggers these events has not been elucidated. Many of the agents that activate rho (e.g., vasopressin, endothelin, lysophosphatidic acid) stimulate the contractility of smooth muscle and other cells. We have investigated whether rho's induction of stress fibers, focal adhesions, and tyrosine phosphorylation is the result of its stimulation of contractility. We demonstrate that stimulation of fibroblasts with lysophosphatidic acid, which activates rho, induces myosin light chain phosphorylation. This precedes the formation of stress fibers and focal adhesions and is accompanied by increased contractility. Inhibition of contractility by several different mechanisms leads to inhibition of rho-induced stress fibers, focal adhesions, and tyrosine phosphorylation. In addition, when contractility is inhibited, integrins disperse from focal adhesions as stress fibers and focal adhesions disassemble. Conversely, upon stimulation of contractility, diffusely distributed integrins are aggregated into focal adhesions. These results suggest that activated rho stimulates contractility, driving the formation of stress fibers and focal adhesions and elevating tyrosine phosphorylation. A model is proposed to account for how contractility could promote these events.

Focal adhesions are sites where cells in culture adhere strongly to the underlying extracellular matrix (ECM) via specific members of the integrin family of ECM receptors (Burr ridge et al., 1988; Jockusch et al., 1995). At their cytoplasmic face, focal adhesions provide attachment for bundles of actin filaments (stress fibers). More than just sites of structural linkage between the ECM on the outside and the cytoskeleton on the inside, focal adhesions are regions of signal transduction. Components involved in multiple signal transduction pathways have been identified in focal adhesions (Hynes, 1992; Juliano and Haskill, 1993; Clark and Brugge, 1995; Richardson and Parsons, 1995; Schwartz et al., 1995) with most attention being directed toward tyrosine phosphorylation at these sites. Indeed, these are the most prominent sites of tyrosine phosphorylation within fibroblasts as well as many other cells in culture and, typically, the most prominently tyrosine-phosphorylated proteins in whole cell lysates are focal adhesion components, such as paxillin and the tyrosine kinase, FAK. Tyrosine phosphorylation is stimulated under conditions of focal adhesion assembly when cells are plated on ECM substrata (Guan et al., 1991; Burridge et al., 1992; Hanks et al., 1992; Kornberg et al., 1992). In addition, tyrosine phosphorylation is necessary for focal adhesion assembly, although whether this involves focal adhesion components or upstream steps in the pathway has not been determined (Burridge et al., 1992).

Several agents that induce the formation of focal adhesions and stress fibers in quiescent cells lacking these structures act via the low molecular GTP-binding protein, rhoA (Paterson et al., 1990; Ridley and Hall, 1992). Direct microinjection of activated rho into quiescent fibroblasts stimulates focal adhesions and stress fibers to assemble rapidly. Conversely, inactivation of rho via ADP-ribosylation catalyzed by the C3 exotransferase, blocks the assembly of these structures. How rho stimulates these cytoskeletal events has not been resolved. Because there is elevated pp125 focal adhesion kinase (FAK) activity in rho-stimulated cells (Zachary et al., 1992; Kumagai et al., 1993; Barry and Critchley, 1994; Chrzanowska-Wodnicka and Burridge, 1994; Hordijk et al., 1994; Ridley and Hall,
and because tyrosine kinase inhibitors decrease the tyrosine phosphorylation of FAK and prevent the assembly of focal adhesions and stress fibers (Burrage et al., 1992; Chrzanowska-Wodnicka and Burridge, 1994; Ridley and Hall, 1994; Romer et al., 1994), the general assumption in the field has been that rho promotes focal adhesion and stress fiber formation by stimulating FAK activity.

Many of the agents that stimulate focal adhesion and stress fiber formation via rho also induce cells to contract. For example, in smooth muscle, contractility is induced by lysophosphatidic acid (LPA), thrombin, vasopressin, endothelin, and bombesin (Said, 1983; Schmid et al., 1983; Liard, 1984; Yanagisawa et al., 1988; for review see Moolenaar, 1994). Some of these compounds also have been shown to stimulate contraction in other cell types. LPA causes chicken embryo fibroblasts and human endothelial cells to contract (Kolodny and Elson, 1993) and induces neurite retraction (Jalink et al., 1994). Similarly, thrombin stimulates fibroblast and endothelial cell contraction (Giuliano and Taylor, 1990; Goekeler and Wysolmerski, 1995), and also induces neurite retraction (Jalink and Moolenaar, 1992; Suidan et al., 1992). With neurite retraction, this effect of LPA and thrombin has been shown to be mediated by rho, because it is inhibited by the C3 exotransferase (Jalink et al., 1994). In addition, activated rho has been shown to affect directly the contractility of permeabilized smooth muscle by decreasing the calcium requirement for contractility (Hirata et al., 1992).

The stimulation of contractility by rho suggests a mechanism by which rho may induce the formation of stress fibers. Previously it was suggested that stress fibers arise as a result of isometric tension generated in cells (Heath and Dunn, 1978; Burridge, 1981). Much evidence has accumulated supporting this idea and that contractility/tension also leads to focal adhesion assembly. For example, microinjection into fibroblasts of antibodies that prevent myosin filament assembly and a functional contractile complex disrupt stress fibers (Honer et al., 1988). Contractility in nonmuscle cells is regulated by the phosphorylation of the 20-kD myosin light chain (MLC) catalyzed by a myosin light chain kinase (MLCK) (Citi and Kendrick-Jones, 1987). Diverse agents acting directly or indirectly to inhibit the MLCK, thereby inhibiting contractility, promote disassembly of stress fibers and focal adhesions (Lamb et al., 1988; Turner et al., 1989; Fernandez et al., 1990; Volberg et al., 1994). Conversely, when contractility is induced in cultured fibroblasts by depolymerizing microtubules, this is accompanied by increased assembly of stress fibers (Danowski, 1989) and focal adhesions (our unpublished observations).

In this paper, we have asked whether rho induces the assembly of focal adhesions and stress fibers by stimulating contractility. We have examined MLC phosphorylation and fibroblast contractility in response to rho activation. In addition, we have investigated the effects of agents that block contractility, either by inhibiting the MLCK or by inhibiting actin–myosin interaction, on rho-induced stress fibers, focal adhesions, and tyrosine phosphorylation. Our results indicate that rho-stimulated contractility drives the formation of stress fibers and focal adhesions, and elevates the tyrosine phosphorylation of focal adhesion components.

Materials and Methods

Cell Culture and Inhibitors of Contractility

Balb/c 3T3 Clone A31 (Balb/c) fibroblasts (American Type Culture Collection, Rockville, MD), were grown in DME (Life Technologies, Inc., Grand Island, NY), with 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B as fungizone, supplemented with 10% bovine calf serum (BCS; JRH Biosciences, Lenexa, KS). To obtain quiescence, cells were seeded at identical density in tissue culture dishes and maintained in the culture medium with serum until they reached confluence. The cells were then extensively washed and starved in serum-free culture medium for 2–4 h. Quiescent, serum-starved cells were stimulated with culture medium containing 2 µg/ml LPA (1-oleoyl-2-hydroxy-sn-glycero-3-phosphate, monosodium salt in H2O; Avanti Polar Lipids, Alabaster, AL).

In some experiments inhibitors were used: an inhibitor of MLCK, KT5926 (Calbiochem Corp., La Jolla, CA) was used at 15 µM; 1-(5-isouquinolinesulfonyl)-2-methylpiperazine (H7; Sigma Chemical Co., St. Louis, MO) was used at 300 µM final concentration; an inhibitor of actin–myosin interactions, 2,3-butanedione 2-monoxime (BDM; Sigma Chemical Co.) was used at 20 nM. These compounds were added to the culture media during stimulation with LPA. To determine the effect of BDM on cellular ATP levels, ATP was measured using a luciferin/luciferase assay kit (Enlighten; Promega Corp., Madison, WI).

Silicone Rubber Substrates

To visualize cell contractility, flexible rubber substrates have been generated by a modification of the protocol of Harris et al. (1980). Silicone rubber (dimethyl polysiloxane, viscosity: 10,000–60,000 centistokes; Sigma Chemical Co.) was aliquoted into tissue culture dishes and allowed to spread for 1–2 h. The top of the silicone was then coated with a thin layer of gold-palladium coating using a cold sputter coater. The UV glow discharge that occurred during the gold-palladium coating served to polymerize the silicone rubber. The thin layer of gold-palladium decreased the hydrophobic nature of the rubber surface and enhanced the adhesion and spreading of cells plated onto this surface. Cells were plated on the gold-coated, polymerized flexible rubber and processed as described above for cells plated on tissue culture plastic.

Immunofluorescence Microscopy

Immunofluorescence was essentially performed as described before (Chrzanowska-Wodnicka and Burridge, 1994). Actin was stained with 400 mU/ml TRITC-phalloidin (Molecular Probes, Inc., Eugene, OR), myosin was stained with a polyclonal anti-platelet myosin antibody (Kawamoto and Adelstein, 1987; a kind gift from Dr. Robert Adelstein, NIH) followed by FITC-labeled goat anti-mouse IgG (Chemicon International, Inc., Temecula, CA). Phosphotyrosine was visualized using antiphosphotyrosine antibody20 (Transduction Laboratories, Lexington, KY), vinculin was stained with an mAb (7f9), kindly provided by Dr. Alexey Belkin, University of North Carolina, Chapel Hill (Glukhova et al., 1990) followed by TRITC- or FITC-labeled goat anti-mouse IgG. Integrins were detected either with an affinity-purified rabbit antibody directed against the β1 cytoplasmic domain, kindly provided by Dr. Crispin Taylor, University of North Carolina, Chapel Hill, or with a goat antibody directed against the α5β1 complex, kindly provided by Dr. Rudy Juliano, University of North Carolina, Chapel Hill, followed by TRITC- or FITC-labeled donkey anti-rabbit or rabbit anti-goat IgG. Cells microinjected with recombinant proteins were visualized by staining with rabbit anti-GST IgG (Molecular Probes, Inc.) followed by an FITC-labeled goat anti-rabbit IgG (Chemicon International, Inc.).

Analysis of Tyrosine Phosphorylation of Focal Adhesion Proteins

For analysis of tyrosine phosphorylation in focal adhesion proteins, immunoprecipitates from whole cell lysates were analyzed by Western blotting as described before (Chrzanowska-Wodnicka and Burridge, 1994). Briefly, cells were lysed on ice with cold lysis buffer consisting of TBS (150 mM NaCl, 50 mM Tris-Cl, pH 7.6) with 0.1% sodium azide, 0.1% Triton X-100, 0.1% sodium deoxycholate, 2 mM EDTA, 2 mM EGTA, 1 mM sodium orthovanadate, and 25 µg/ml leupeptin. The lysates were clarified by a 10-min centrifugation at 13,000 g. Supernatants were either incubated with anti-paxillin or anti-FAK monocular IgG for 2–4 h at 4°C, followed
by rabbit anti-mouse-conjugated protein A-Sepharose (Chemicon International, Inc.). Proteins bound to the sepharose were dissolved in SDS-sample buffer, separated by electrophoresis on 7.5% polyacrylamide gels (Laemmli, 1970), and transferred electrophoretically to nitrocellulose. Nitrocellulose was blocked with 2% cold water fish gelatin (Sigma Chemical Co.) in TBSTB (TBS with 0.1% BSA and 0.05% Tween 20) for 20–30 min. The nitrocellulose was then incubated for 45 min with anti-phosphotyrosine IgG conjugated to HRP, py20-HRP (ICN Biochemicals Inc., Irvine, CA) and extensively washed with TBSTB and TBS. Enhanced chemiluminescence was developed in 0.1 M Tris, 1% nonfat milk, 0.05%Tween-20, and 0.01% H2O2. Immunoblots were stripped in 62.5 mM Tris-HCl, pH 6.75, 2% sodium dodecyl sulfate, and 100 mM 13-mercaptoethanol at 55°C for 45 min. Stripped blots were washed in TBS and reprobed with 2A7, anti-FAK monoclonal IgG (Upstate Biotechnology, Inc., Lake Placid, NY), or with anti-paxillin antibody in TBSTB. After washing in TBSTB the blots were incubated with HRP-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA).

**Purification and Microinjection of Recombinant Proteins**

Recombinant Val4rhoA (a gift from Dr. A. Hall, University College, London, UK) was expressed as a glutathione-S-transferase (GST) fusion protein in *Escherichia coli*. GST was also expressed in *E. coli*. As a control, some cells were injected with GST alone expressed in *E. coli*. Proteins were purified on glutathione agarose beads as described before (Ridley et al., 1992). Proteins were released from beads by triple elution with 25 mM reduced glutathione in a buffer (50 mM Tris-Cl, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 1 mM DTT, 1 mM PMSE), dialyzed against microinjection buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 0.1 mM DTT) and concentrated to 2–7 mg/ml using a Centricon concentrator (Amicon Inc., Beverly, MA). Purified proteins were loaded by capillarity into needles pulled on a Brown-Fleming micropipette puller (Sutter Instrument Co.) and injected into cells as described previously (Burridge and Feraamico, 1980). Cells were injected for 15–30 min and then incubated at 37°C in an atmosphere of 10% CO2 for varying time intervals according to the experiment. In some experiments inhibitors were present during the injection and after incubation.

**Cell Labeling, Myosin Immunoprecipitation, and Quantitation of Myosin Light Chain Phosphorylation**

For analysis of MLC phosphorylation a procedure was adapted from the work of Goekeler and Wysolmerski (1995). Cells were labeled with trans-35S-label (70% L-methionine [35S] + L-cysteine [35S]) (ICN Biochemicals, Inc.) and 32P for 2 hours. Cells were then labeled with 100 iCi/ml of 35S-label (3 activity) with a filter present. A second exposure was obtained in which a filter was present. The amount of 35S was calculated by subtracting the number of counts for each myosin band obtained with the filter present (which represents 97% of 32p), adjusted to 100% 32p, from the number of counts obtained without the filter (total 32P + 35S). The number of 32p counts from the corresponding MHC. This procedure allowed us to calculate MLC phosphorylation relative to the amount of myosin in each immunoprecipitate.

**Results**

**Inhibitors of Actin–Myosin Interaction Block LPA-stimulated Contractility**

LPA induces the formation of stress fibers and focal adhesions in quiescent fibroblasts by a mechanism dependent on rho (Ridley and Hall, 1992). Previous studies have indicated that LPA can stimulate contractility of chicken embryonic fibroblasts (Kolodney and Elson, 1993). To explore whether LPA induces contractility in quiescent Balb/c 3T3 cells, we have used a modification of the flexible rubber substrate assay of Harris et al. (1980). Confluent cells in the presence of serum wrinkle the substrate extensively (Fig. 1). Starvation of cells in serum-free media for 2 h leads to a relaxation of the wrinkles generated by cells. Stimulation of these quiescent cells with LPA rapidly restores wrinkles in the substrate. The time course of the reappearance of the wrinkles in the substrate correlates well with the time course of reappearance of stress fibers and focal adhesions and increased tyrosine phosphorylation in focal adhesions. Increased wrinkling can be seen within 5 min of stimulation with LPA. At this time stress fibers and focal adhesions become visible in cells grown on plastic (Ridley and Hall, 1992; see below) and LPA-induced tyrosine phosphorylation of focal adhesion components peaks (Seufferlein and Rozengurt, 1994).

We have used several inhibitors of actin–myosin interaction with distinct modes of action to block LPA-induced contractility of Balb/c 3T3 cells, using the flexible rubber substrates as an assay. KT5926 is an inhibitor of MLCK, a key enzyme in the regulation of smooth muscle and non-muscle cell contractility (Nakanishi et al., 1990). KT5926 inhibits MLCK phosphorylation of the myosin regulatory light chain (MLC), which in turn inhibits actomyosin contractility. Butanedione-2-monoxime (BDM) has been used by several groups to inhibit muscle contractility. BDM inhibits muscle myosin ATPase activity (Higuchi and Takemori, 1989) by slowing the release of phosphate from myosin after ATP hydrolysis (McKillop et al., 1994). Cramer and Mitchison (1995) have shown that BDM also inhibits the ATPase activity of nonmuscle myosin II, as well as several of the unconventional myosins. Compound H7 (1-(5-isquinolinolylsulfonyl)-2-methylpipеразине), a broad specificity kinase inhibitor, often used to inhibit protein kinase C (PKC), has been shown to inhibit actomyosin contractility in fibroblasts and epithelial cells in a way that is independent of its action on PKC (Volberg et al., 1994). BDM, H7, or KT5926 were added with LPA to serum-starved Balb/c 3T3 cells plated on the flexible rubber substrates. In each
LPA stimulates fibroblast contractility. Balb/c 3T3 cells are plated on silicon rubber substrates. In the presence of serum, cells generate tension on the underlying substrate that is revealed by wrinkles in the rubber. Quiescence, induced by serum starvation of the cells for 2 h, leads to relaxation of tension and loss of wrinkles (top). Treatment of quiescent cells with LPA stimulates reappearance of wrinkles in the substrate, here shown at 1 h after stimulation. This behavior of cells in response to LPA is blocked by BDM, KT5926 (middle and bottom panels, respectively), and H7 (data not shown). Upon removal of the inhibitors (here shown after 1 h), wrinkles in the substrate reappear, indicating reversibility. Each panel in a row represents the same field of view. Bar, 100 μm.

Figure 1. LPA stimulates fibroblast contractility. Balb/c 3T3 cells are plated on silicon rubber substrates. In the presence of serum, cells generate tension on the underlying substrate that is revealed by wrinkles in the rubber. Quiescence, induced by serum starvation of the cells for 2 h, leads to relaxation of tension and loss of wrinkles (top). Treatment of quiescent cells with LPA stimulates reappearance of wrinkles in the substrate, here shown at 1 h after stimulation. This behavior of cells in response to LPA is blocked by BDM, KT5926 (middle and bottom panels, respectively), and H7 (data not shown). Upon removal of the inhibitors (here shown after 1 h), wrinkles in the substrate reappear, indicating reversibility. Each panel in a row represents the same field of view. Bar, 100 μm.

case, these drugs inhibited the development of wrinkles in the substrate induced by LPA treatment of quiescent cells (Fig. 1, and data not shown), indicating that they blocked LPA-stimulated contractility. After 1 h, the medium containing the drugs was replaced with normal medium and 10% serum. This led to extensive wrinkling of the underlying substrate by the cells within 5 min.

LPA-stimulated Myosin Light Chain Phosphorylation Is Blocked by Inhibitors of Contractility

We analyzed the level of MLC phosphorylation in quiescent cells and cells stimulated with LPA in the absence or presence of the inhibitors, BDM, H7, and KT5926. Myosin was immunoprecipitated from cells labeled simultaneously with [32P]orthophosphate and [35S]methionine/[35S]cysteine. The level of 32P incorporation into MLCs was calculated relative to the amount of 35S incorporation into the MHC in the same immunoprecipitated samples, to account for variable protein content in the immunoprecipitates, as described in Materials and Methods. This double-labeling protocol improves quantitation of the level of phosphate incorporation into MLCs occurring during stimulation or inhibition (Goekeler and Wysolmerski, 1995). The results are presented in Fig. 2. LPA stimulates an increase in MLC phosphorylation that is blocked by BDM, H7, and KT5926. Although BDM is an inhibitor of myosin ATPase activity, previous studies have also noted decreased MLC phosphorylation (Osterman et al., 1993; Siegman et al., 1994). With BDM and H7, the level of MLC phosphorylation was depressed even below that found in the quiescent cells. For most experiments, MLC phosphorylation was measured at 10 min after LPA stimulation. When a time course of MLC phosphorylation was investigated, LPA treatment for 1 min was sufficient to stimulate nearly peak phosphorylation (data not shown). This was more rapid than the appearance of stress fibers and focal adhesions, which were visible after 5 min, but were usually most prominent after 20 min of treatment with LPA (see below).

Myosin Relocalizes to Stress Fibers upon LPA Stimulation

Phosphorylation of MLCs leads to a conformational changes in myosin enabling it to assemble into filaments and promotes its productive interaction with F-actin (Citi and Kendrick-Jones, 1987; Tan et al., 1992). We studied the
distribution of myosin in quiescent cells and after stimulation with LPA (Fig. 3). In quiescent cells myosin is dispersed throughout the cytoplasm and reveals very little detectable organization at the level of light microscopy (Fig. 3 A). Stimulation with LPA induces rapid reorganization of myosin into stress fibers and generates a characteristic periodic staining pattern that is not detectable in quiescent cells (Fig. 3 B). The time course of this rearrangement of myosin upon LPA treatment paralleled that of actin, with prominent stress fiber staining being seen within 20 min of stimulation.

Inhibitors of Contractility Block LPA-stimulated Stress Fibers, Focal Adhesions, and Tyrosine Phosphorylation

To investigate the possible role of contractility in the formation of stress fibers and focal adhesions we have examined the effects of the inhibitors of actin-myosin contractility on the formation of these structures induced by LPA. BDM, H7, and KT5926 all inhibit LPA-induced formation of stress fibers and focal adhesions in Balb/c 3T3 cells (Fig. 4). Essentially identical results were obtained with Swiss 3T3 cells (data not shown). These three inhibitors, however, result in different morphologies, as seen in Fig. 4, G, J, and M. The differences may reflect additional effects of these inhibitors on other kinases or enzymes.

Tyrosine phosphorylation of several focal adhesion proteins, including FAK, paxillin, tensin, and p130, occurs during the formation of focal adhesions when quiescent cells are stimulated with LPA (Kumagai et al., 1993; Barry and Critchley, 1994; Chrzanowska-Wodnicka and Burridge, 1994; Hordijk et al., 1994; Ridley and Hall, 1994; Seufferlein and Rozengurt, 1994) and when cells are plated on fibronectin (Guan et al., 1991; Burridge et al., 1992; Guan and Shalloway, 1992; Hanks et al., 1992; Bockholt and Burridge, 1993; Petch et al., 1995). LPA induces increased phosphotyrosine staining in focal adhesions (Fig. 4 F), compared with quiescent cells (Fig. 4 C) (Barry and Critchley, 1994; Chrzanowska-Wodnicka and Burridge, 1994; Seufferlein and Rozengurt, 1994). When BDM, H7, and KT5926 are present during LPA stimulation, phosphotyrosine staining in focal adhesions is diminished (Fig. 4, I, L, and O). This immunofluorescence data correlate with biochemical analysis of tyrosine-phosphorylated proteins in cells under different conditions of stimulation and inhibition. As reported before, LPA induces increased tyrosine phosphorylation of several focal adhesion components. Here we have specifically examined two of the most prominent tyrosine-phosphorylated focal adhesion proteins, FAK and paxillin (Fig. 5, A and B). BDM, KT5926, and H7 all inhibit the LPA-induced increase in tyrosine phosphorylation of these proteins. With BDM and H7 the level of tyrosine phosphorylation is decreased to below that found in quiescent cells. KT5926 had the least effect on tyrosine phosphorylation. Of the three inhibitors at the respective concentrations used, KT5926 was generally the least effective at inhibiting tyrosine phosphorylation. At the concentration of tyrosine phosphorylation more in paxillin than FAK. This may be due in part to paxillin being downstream of FAK, with changes in the signal being amplified in downstream targets. Consistent with this, LPA induces a greater change in the tyrosine phosphorylation of paxillin than FAK. FAK is phosphorylated on multiple tyrosines (Schwartz et al., 1995), some of which may be less affected or unaffected by LPA stimulation. This would also tend to lessen the apparent change in tyrosine phosphorylation of FAK in response to LPA or inhibition of LPA action.

Rho-induced Stress Fibers, Focal Adhesions, and Tyrosine Phosphorylation Are Blocked by Inhibitors of Contractility

The effects of LPA on the cytoskeleton have been shown to be mediated by rho, but the steps between LPA and rho have not been fully elucidated. We wished to determine whether the inhibitors of contractility that block the actions of LPA act upstream or downstream of rho. To investigate this question, we explored the effects of these inhibitors of actomyosin contractility on cells microinjected with recombinant rho (Val14rho). As described previously (Ridley and Hall, 1992), activated rho induced the rapid...
formation of stress fibers and focal adhesions in injected cells (Fig. 6, A and D). However, when BDM or H7 were present during injections, the formation of stress fibers (Fig. 6, B and C) and focal adhesions (Fig. 6 H) was inhibited. Similar results were found with Swiss 3T3 cells (data not shown). In some cells, there appeared to be an increase in actin staining in the presence of these inhibitors without the formation of large stress fibers, suggesting that actin polymerization induced by rho had not been inhibited, but that the assembly of the filaments into stress fibers had. BDM and H7 greatly decreased the phosphotyrosine staining in focal adhesions in rho-injected cells (Fig. 6, E and F, compared with Fig. 6 D). These results suggest that rho-induced assembly of stress fibers and focal adhesions involves stimulation of contractility.

Contractility Modulates the Distribution of Integrins

We wanted to follow the behavior and distribution of integrins during the transition of cells from quiescence, when cytoplasmic focal adhesion proteins are dispersed, to the stimulated state, when focal adhesions are prominent. However, mouse integrins have been difficult to immunolocalize due to the lack of good anti-mouse integrin antibodies. Hence, we used another system to look at the behavior of integrins under conditions of relaxation and contraction. Rat embryo fibroblasts were allowed to spread on fibronectin in serum-free media and were stained for actin (Fig. 7 A) and β1 integrin (Fig. 7 D). To mimic the quiescent state and inhibit contractility, cells were incubated in the presence of H7. The cells remained spread but the stress fibers (Fig. 7 B) and focal adhesions disassembled. Notably, the distribution of integrins became diffuse and no focal adhesion staining was detected (Fig. 7 E). Removal of H7 followed by stimulation with LPA induced the reformation of stress fibers (Fig. 7 C) and relocation of integrins into focal adhesions (Fig. 7 F). This behavior of integrins is similar to that of other focal adhesion proteins upon rho stimulation and indicates that integrins mediating adhesion to the underlying ECM can be rapidly reorganized into focal adhesions while maintaining adhesion to the substrate.

Discussion

In this paper we have demonstrated that rho-induced formation of stress fibers, focal adhesions, and tyrosine phosphorylation depends on contractility. Agents that inhibit contractility, either by inhibiting the MLCK or actin–myosin interaction, inhibit all three events. At the outset of this work we picked the three inhibitors used here because of their reported different modes of inhibiting actin–myosin force generation. KTs926 is a well-characterized inhibitor of MLCK with relatively high specificity for this enzyme in vitro (Nakanishi et al., 1990). BDM is an inhibitor of skeletal muscle myosin ATPase (Fryer et al., 1988; Horiuti et al., 1988; Higuchi and Takemori, 1989) which acts by slowing the release of phosphate from the myosin head following ATP hydrolysis (Herrmann et al., 1993; McKillop et al., 1994). BDM also inhibits the ATPase activity of nonmuscle myosins (Cramer and Mitchison, 1995). It was demonstrated, however, that BDM does not affect the ATPase activity of kinesin, nor does it affect several myosin-independent motile phenomena, such as actin polymerization, mitosis, and the intracellular movement of Listeria monocytogenes (Cramer and Mitchison, 1995). Effects of BDM on energy metabolism and ATP synthesis have been described (Hebisch et al., 1993). However, we did not find significant differences in the levels of ATP in cells treated with BDM for the times used in our experiments (data not shown). BDM has been reported to decrease MLC phosphorylation in smooth muscle (Osteman et al., 1993) and depresses MLCK activity (Siegmam et al., 1994). In our work we also have found that it inhibits MLC phosphorylation (Fig. 2). At present, we cannot say whether the inhibition of nonmuscle contractility by BDM is due to a direct action on the nonmuscle myosin ATPase, to inhibition of MLCK, or to a combination of both.

The third inhibitor in this study, H7, was picked because of its inhibition of fibroblast and epithelial contractility (Volberg et al., 1994). H7 has often been used to inhibit PKC but Volberg and colleagues demonstrated that more specific PKC inhibitors had no effect on contractility. Similarly, we have found that the more specific PKC inhibitor, GF109203X has little effect on stress fibers, focal adhesions, or tyrosine phosphorylation (data not shown). In addition, previous work demonstrated that the effects of LPA on the cytoskeleton did not involve a PKC pathway (Ridley and Hall, 1994; Mooelenaar, 1995) and that LPA- and bombesin-induced tyrosine phosphorylation of FAK and paxillin are independent of PKC (Zachary et al., 1993; Seufferlein and Rozengurt, 1994). Further evidence that
Figure 4. LPA-stimulated stress fiber and focal adhesion assembly is blocked by inhibitors of contractility. Quiescent, serum-starved cells (A–C) were stimulated with LPA which led to increased stress fibers (D), focal adhesions (E), and tyrosine phosphorylation (F) in focal adhesions. These effects of LPA were abolished when BDM (G–I), H7 (J–L), or KT5926 (M–O) were present during LPA stimulation. Actin was stained with phalloidin in A, D, G, J, and M; vinculin staining is shown in B, E, H, K, and N, and phosphotyrosine is shown in C, F, I, L, and O. Bar, 20 µm.
Inhibitors of contractility block LPA-induced tyrosine phosphorylation. FAK (A) or paxillin (B) were immunoprecipitated from confluent, quiescent Balb/c 3T3 fibroblasts starved in the absence of serum for 2-4 h (lanes 1) and stimulated with 2 μg/ml LPA for 10 min (lanes 2), or with LPA and BDM (lanes 3), or with LPA and H7 (lanes 4), or with LPA and KT5926 (lanes 5). LPA elevated FAK and paxillin tyrosine phosphorylation. This is inhibited by BDM, H7, and KT5926. Blots were stripped and re-probed for FAK or paxillin to ensure equal protein loading (data not shown).

Within 1 min of LPA stimulation we detect significant elevation of MLC phosphorylation. In our hands this precedes the detection of stress fibers, focal adhesions, and tyrosine phosphorylation induced by rho injection or LPA treatment. Similarly, Kolodney and Elson (1993) found that LPA led to a rapid stimulation of contractility and light chain phosphorylation in fibroblasts. Likewise, Goekeler and Wysolmerski (1995) demonstrated in thrombin-stimulated endothelial cells that MLC phosphorylation and the development of tension preceded the reorganization of actin and myosin into stress fibers. Together these results suggest that, in the pathway from rho, light chain phosphorylation and the resultant onset of contractility are upstream of stress fiber and focal adhesion assembly, as well as upstream of the tyrosine phosphorylation of focal adhesion components.

the absence of serum for 2-4 h (lanes 1) and stimulated with 2 μg/ml LPA for 10 min (lanes 2), or with LPA and BDM (lanes 3), or with LPA and KT5926 (lanes 5).

The elevation of MLC phosphorylation could result from increased MLCK activity or from decreased phosphatase activity. In permeabilized smooth muscle, C3 exo- transferase promotes dephosphorylation of MLCs, suggesting that rho inhibits the MLC phosphatase (Noda et al., 1995). Whether the elevation of MLC phosphorylation in nonmuscle cells is due to enhanced MLCK activity or depressed phosphatase activity, we suspect that, in either case, a kinase cascade is involved. The rho-related proteins, rac and cdc42, activate kinase cascades that resemble the ras/raf/MAP kinase pathway (Coso et al., 1995; Hill et al., 1995; Minden et al., 1995). Rho has been shown recently to bind and activate protein kinase N (PKN) (Amano et al., 1996; Watanabe et al., 1996) and to bind RhoA-binding kinase (RokA) (Leung et al., 1995). Both may be initial steps in rho-activated cascades.

How could contractility lead to the formation of stress fibers, focal adhesions, and elevated tyrosine phosphorylation? One scenario that we can envision is outlined in Fig. 8. In a quiescent cell, the integrins responsible for adhesion to the underlying ECM are not clustered in focal adhesions but are dispersed over the ventral surface. At their cytoplasmic face, these integrins are linked to the actin cytoskeleton, via proteins such as α-actinin or talin, but instead of being bundled the filaments are organized as a loose network. Stimulation by rho would result in MLC phosphorylation, converting the myosin from a relaxed conformation to an elongated conformation that can assemble into bipolar filaments (Citi and Kendrick-Jones, 1987; Tan et al., 1992). Light chain phosphorylation would also promote the productive interaction of myosin heads with actin filaments, generating contractility and isometric tension. The actin filaments would become aligned both because of the tension generated and because myosin filaments are very effective at bundling F-actin. At the membrane, the bundled actin filaments would aggregate the integrins to which they are attached. Associated with the integrins is FAK (Schaller et al., 1995). Clustering of integrins from the outside with antibodies has been shown to stimulate FAK activity (Kornberg et al., 1992). Indeed, clustering rather than ligand occupancy appears to be critical for FAK activation (Miyamoto et al., 1995). Clustering from the inside by bundling of the associated filaments would also be expected to stimulate FAK activity. This would account for the observed increase in tyrosine phosphorylation of focal adhesion components that occurs in rho-stimulated cells.

Tyrosine kinase inhibitors block the assembly of focal adhesions and stress fibers induced either by adhesion to ECM or by stimulation with activated rho (Burridge et al., 1992; Romer et al., 1994; Chrzanowska-Wodnicka and
Figure 7. Contractility modulates the distribution of integrins. REF 52 fibroblasts were plated on fibronectin in serum-free conditions, stained at 90 min for actin (A) and β1 integrin (D). Cells were treated for 30 min with H7 and then stained for actin (B) and β1 integrin (E). After 30 min in H7, parallel cells were returned to serum-free medium containing LPA for 30 min and then stained for actin (C) and β1 integrin (F). Addition of H7 disassembles stress fibers and disperses integrins. Removal of H7 and stimulation with LPA induces reformation of stress fibers and clustering of integrins into focal adhesions. Bar, 20 μm.

Burridge, 1994; Ridley and Hall, 1994). Because these inhibitors decrease the phosphorylation of FAK, these findings have usually been interpreted as indicating a role for FAK in focal adhesion assembly. Evidence has been presented, however, that FAK is not involved in focal adhesion or stress fiber assembly (Wilson et al., 1995; Ilic et al., 1995). Our results suggest that FAK phosphorylation is downstream of rho-induced contractility, which aggregates integrins into focal adhesions. These findings, together with the previous work with tyrosine kinase inhibitors, suggest that the relevant tyrosine kinase regulating focal adhesion assembly is not FAK but one that is upstream of light chain phosphorylation. It will be important in the future to determine if any rho-activated kinases are targets for the tyrosine kinase inhibitors that prevent focal adhesion assembly. Likewise it will be interesting to explore whether H7 is inhibiting MLC phosphorylation by acting on an upstream kinase. PKN, a rho-activated kinase, is a potential candidate, given that it is related to PKC (Mukai and Ono, 1994; Palmer et al., 1995).

The balance between adhesion and contractility is complex. In response to isometric tension there may be a compensatory increase in the adhesive strength of focal adhesions, for example by recruitment of additional integrins. When the contractile force generated in stress fibers exceeds the adhesive strength of focal adhesions, the adhesions would be released from the substrate. This may be important during cell migration when focal adhesions, particularly at the rear of a cell need to be disassembled. Studying permeabilized cells, Crowley and Horwitz (1995) found that ATP-induced contractility does, indeed, promote the disassembly of focal adhesions. Many of the factors that regulate adhesive strength may not operate in permeabilized cells and, with permeabilization, focal adhesions may be weakened by extraction of critical components. Together, these changes in permeabilized cells may result in the contractile force exceeding the strength of the adhesion, as observed by Crowley and Horwitz (1995).

Although there is a large body of evidence consistent with contractility generating both stress fibers and focal adhesions, one previous study suggested that focal adhesions could form in the apparent absence of stress fibers (Nobes and Hall, 1995). In this work focal adhesions were induced by activated rho in the presence of low concentra-
The requirement for an intact cytoskeleton for FAK to be activated has always been a somewhat surprising result. Our results suggest that an intact actin cytoskeleton is required for FAK activation because contractility within the microfilament system is needed to aggregate integrins.

The model we propose requires that integrins go from a dispersed, but ligand-bound state, to an aggregated ligand-bound state, found in focal adhesions. With mouse 3T3 cells, it has been difficult to examine the behavior of integrins due to the paucity of reagents that detect mouse integrins. However, we have used a different cell system to examine the behavior of integrins in cells adhering to fibronectin under conditions where contractility is inhibited or where it is stimulated. With the transition from a relaxed state to a contractile state, the integrin, α5β1, goes from a diffuse distribution to being concentrated within focal adhesions, consistent with the predictions of our model. Conversely, when contractility is inhibited, integrins disperse from focal adhesions as stress fibers and focal adhesions disassemble. These observations imply that integrins can redistribute on the ventral surface of a cell in response to tension generated within stress fibers. The relatively low affinity of integrins for their ECM ligands (Kd \(10^{-7}\) M) suggests a rapid off rate that would permit easy remodeling of integrin-ECM interactions in response to applied tension, while at the same time maintaining cell adhesion to the ECM substratum.

Previous studies have suggested other pathways by which rho may affect cytoskeletal organization. In particular, rho has been shown to stimulate the activity of phosphatidylinositol 4-phosphate 5-kinase (PIP2) which leads to increased actin polymerization. In addition, work from this lab has recently demonstrated that PIP2 stimulates a conformational change in vinculin to expose cryptic actin- and talin-binding sites (Gilmore and Burridge, 1996). This effect of PIP2 would also be expected to enhance the assembly of focal adhesions. Under normal conditions, we suspect that rho-stimulated contractility will synergize with the other cytoskeletal events triggered by elevated PIP2 levels, together contributing to the formation of stress fibers and focal adhesions (Fig. 9).
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