Tyrosine Phosphorylation and Cytoskeletal Tension Regulate the Release of Fibroblast Adhesions

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Abstract. We have investigated the mechanisms by which fibroblasts release their adhesions to the extracellular matrix substrata using a permeabilized cell system in which the adhesions remain relatively stable. A large number of different molecules were assayed for their effect on focal adhesion stability using immunofluorescence with antibodies against different focal adhesion constituents. ATP uniquely stimulates a rapid breakdown of focal adhesions, and at high ATP concentrations (>5 mM), many cells are released from the dish. The remaining cells appear contracted with talin, α-actinin, and vinculin localized diffusely throughout the cell. Integrin containing tracks of variable intensity outline the regions where cells had resided before they detached from the substratum. At lower ATP concentrations (0.5–5 mM) the cells remain spread; however the focal adhesion components, including integrin, show an array of phenotypes ranging from diffusely localized throughout the cell to a localization in small, thin focal adhesions. Okadaic acid, a serine, threonine phosphatase inhibitor, enhances the contracted phenotype, even at low concentrations (0.5 mM) of ATP. The localization of focal adhesion components is different in okadaic acid–treated cells. In highly contracted cells, integrin is present in tracks where the cells resided before the contraction; however focal adhesions are no longer apparent. Talin, vinculin, and α-actinin localize in trabecular networks toward the periphery of the cell. Interestingly, phosphotyrosine staining as well as nascent, intracellular integrin precedes the recruitment of focal adhesion constituents into the trabecular network. The ATP-stimulated focal adhesion breakdown appears to operate through two mechanisms. First, ATP stimulates the tyrosine phosphorylation of several cytoskeletally associated proteins. These tyrosine phosphorylations correlate well with focal adhesion breakdown. Furthermore, addition of a recombinant, constitutively active tyrosine phosphatase inhibits both the tyrosine phosphorylations and the breakdown of the focal adhesions. None of the major tyrosine phosphoproteins are FAK, integrin, tensin, paxillin, or other phosphoproteins implicated in focal adhesion assembly. The second mechanism is cell contraction. High ATP concentrations, or lower ATP concentrations in the presence of okadaic acid induce cell contraction. Inhibiting the contraction by addition of a heptapeptide IRICRKG, which blocks the actin-myosin interaction, also inhibits focal adhesion breakdown. Neither the peptide nor the phosphatase inhibits focal adhesion breakdown under all conditions suggesting that both tension and tyrosine phosphorylations mediate the release of adhesions.

THE regulated release of extracellular contacts is a central feature of several cellular processes which include cell migration and mitosis. Motile cells, for example, extend a lamellipodium and form new contacts at the front of the cell and coordinately release their contacts at the rear. Mitotic cells release extracellular attachments before cytokinesis, and then reform them as the two daughter cells emerge. In addition to these normal cellular processes, the abnormal regulation of extracellular contacts is associated with the pathologic process of viral transformation.

Despite the importance of adhesive regulation, little is known about its mechanisms. Several observations suggest that tyrosine phosphorylations are involved in the formation of focal adhesions. Burridge et al. (1992) showed that the focal adhesion kinase (FAK)[1] and paxillin, two focal adhesion components, are phosphorylated on tyrosine during focal adhesion formation. Furthermore, addition of bombesin, a neuropeptide that also induces the tyrosine phosphorylation of FAK, to Swiss 3T3 cells causes the rapid assembly of focal adhesions and actin stress fibers (Ridley and Hall, 1992; Zachary et al., 1992; Barry and

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1. *Abbreviations used in this paper*: CB, cytoplasm mimicking buffer; FAK, focal adhesion kinase.

Critchley, 1994). Finally, inhibition of tyrosine phosphorylation during cell spreading by herbimycin, a tyrosine kinase inhibitor, inhibits focal adhesion formation. The phosphorylation of focal adhesion components on tyrosine is also implicated in the destabilization of focal adhesions. Cells transformed by the Rous sarcoma virus have altered focal adhesions, called podosomes, (Rohrschneider, 1980; Tarone et al., 1985), a reorganized actin cytoskeleton (Felice et al., 1990), and altered adhesion (Parsons and Weber, 1989). These cells show increased tyrosine phosphorylation of several focal adhesion components, which include integrin, talin, $pp60^{c\text{-}src}$, $pp60^{v\text{-}src}$, and FAK (Hirst et al., 1986; Pasquale et al., 1986; Smart et al., 1981; Piwnica-Worms, et al., 1987; Schaller et al., 1992). It appears, therefore, that there is an abundance of phosphotyrosine containing proteins in both normal and transformed cells and that their phosphorylation may be involved in the signaling mechanisms that form and release adhesions. In addition to tyrosine phosphorylation, the presence of protein kinase C in focal adhesions points to the potential participation of serine/threonine phosphorylations as well in regulating adhesion (Jaken et al., 1989; Woods and Couchman, 1992) Thus a general theme involving kinase cascades in the formation and release of the focal adhesion seems likely.

In addition to tyrosine kinase cascades there is increasing evidence that mechanical factors, e.g., tension, may also contribute to regulation of adhesion (for reviews see Grinnell, 1994; Ingber, 1991). For example, cells contracting on a fibronectin substrate remove fibronectin from the substrate and reorganize it into patches and fibrils (Avnur and Geiger, 1981; Grinnell, 1986). These cells have shortened (or disassembled) actin stress fibers and an activated cAMP/PKA signaling pathway (He and Grinnell, 1994). In addition, Klein et al. (1991) showed that the $\alpha_2\beta_1$ integrin functions specifically in the contraction of collagen gels, a process that alters the organization of adhesive components. Finally, myosin I localizes at the anterior region of migrating Dictyostelium where extension occurs (Fukui et al., 1989), while myosin II concentrates at the posterior regions where contraction occurs (Rubino et al., 1984; Yumura et al., 1984;Yumura and Fukui, 1985). Conrad et al. (1993) demonstrated an analogous, time dependent, asymmetry of myosin motors between the front and rear of migrating fibroblasts. Taken together these results, along with theoretical considerations (Bell, 1978; Lauffenburger, 1994), suggest that mechanical forces may also play an essential role in the regulation of adhesion.

Our goal in this study was to decipher the roles of kinase cascades and mechanical forces in the release process. To address this, we developed a permeabilized cell system with which adhesions are relatively stable. This system is useful for several reasons. First, putative regulatory molecules like second messengers, protein kinase activators or inhibitors, phosphatases or other biological molecules can be easily introduced into the permeabilized cells since the membrane is no longer a limiting factor. In addition, proteins released into the buffer during the detachment process can be isolated and characterized. Finally, most soluble cytoplasmic components are released. This facilitates the identification of molecules which directly affect the focal adhesions since soluble, downstream effectors are likely absent, or present in reduced concentrations due to the permeabilization.

We report that the addition of ATP destabilizes focal adhesions and induces rounding of the cells. Addition of okadaic acid, a serine, threonine phosphatase inhibitor, enhances the ATP effect. Concomitant with its biological activity, ATP stimulates the phosphorylation on tyrosine of several major proteins. Addition of a constitutively active recombinant tyrosine phosphatase inhibits both the phosphorylation and destabilization. In addition a peptide which specifically blocks the myosin-actin interaction also inhibits the destabilization of adhesions. These observations point to both biochemical and mechanical contributions to the release of adhesions.

Materials and Methods

Antibodies and Reagents

Talin and vinculin were purified using the protocol of O'Halloran et al. (1986), and polyclonal antisera were raised in rabbits immunized against these purified proteins. Anti-FAK, ES66 (anti-β1 integrin), anti-tensin, and anti-paxillin antibodies were generous gifts from Drs. Thomas Parsons, Kenneth Yamada, Lan Bo Chen, and Chris Turner, respectively (Schaller et al., 1992; Duband et al., 1988; Bockholt et al., 1992; Avnur, 1983). PY20 was purchased from ICN Biomedicals (Costa Mesa, CA). Anti–α-actinin antibody was purchased from Sigma Chem. Co. (St. Louis, MO). A constitutively active, recombinant T cell tyrosine phosphatase was a generous gift of Dr. Edmond Fischer This 37-kD enzyme has an 11-kD COOH-terminal truncation and was expressed and purified from baculovirus-infected *Sf* 9 cells. It is specific for phosphotyrosine residues (Cool et al., 1990; Zander et al., 1991). The heptapeptides, IRICRKG and IRICEKG, were generous gifts from Dr. Don Ingber and were also purchased from QCB (Hopkinton, MA). ATP and okadaic acid were purchased from Boehringer Mannheim, (Indianapolis, IN) and Biomol (Plymouth Meeting, PA), respectively. Fibronectin was purified from outdated plasma according to the protocol of (Ruoslahti et al., 1982).

Cell Growth and Permeabilization

Tendon fibroblasts were obtained from 16-d-old white leghorn chicken embryos using the protocol of Decker et al. (1984). The cells were grown in high glucose DME (Gibco BRL, Grand Island, NY) supplemented with 5% FBS (Hybrimax, Sigma Chem. Co.) 1 mM sodium pyruvate and 100 U/ml penicillin-streptomycin in 10% CO_2.

Conjugation of Fibronectin to Glass Coverslips

12-mm glass coverslips (Fisher Scientific, Pittsburgh, PA) were washed overnight in 20% H_2SO_4, rinsed three times in water and neutralized in 0.1 M NaOH. After oven drying, the coverslips were incubated in 3-aminopropyltriethoxysilane (Sigma Chem. Co.) for 4 min at room temperature, rinsed 3X with PBS, and then incubated in PBS containing 0.25% glutaraldehyde for 30 min. The coverslips were rinsed in PBS, placed on parafilm and conjugated with 100 μl of 20 μg/ml fibronectin for 1 h at room temperature. The coverslips were then washed in PBS, sterilized in 70% ethanol, placed fibronectin side up in 24-well dishes (Costar, Cambridge, MA) and washed 3X with sterile PBS before use.

Tendon fibroblasts were trypsinized in 0.25% trypsin and 2 mM EDTA in phosphate buffered saline and washed once in growth medium. 2×10^4 cells were seeded onto 12-mm fibronectin-coated coverslips in growth media in 24-well plates. The cells were allowed to spread overnight and then the coverslips were removed from the wells and placed on parafilm with the cells facing upward. The cells were washed with excess cytoplasm mimicking buffer (CB) containing 25 mM Hepes-acetate at pH 7.2, 3 mM Mg acetate, 154 mM potassium acetate, 1 mM EGTA, 300 mM sucrose, 1% BSA, 0.1 mM DTT, 1 μg/ml leupeptin, 1 mM *O*-phenanthroline and 0.1 mM PMSF. The reagents used in this buffer were either highly purified or enzyme grade chemicals. The cells were permeabilized in 50 μl of CB containing 0.01% digitonin (Sigma Chem. Co.) for 30 s at room temperature. The cells were washed quickly with excess CB and incubated in 40 μl CB containing specified reagents in a humidified chamber at room tem-

perature for 1 h. At low ATP concentrations, an ATP regenerating system was used to sustain an appropriate ATP concentration throughout the experiment. The ATP regenerating system consisted of 50 μg/ml creatine phosphokinase and 10 mM phosphocreatine (Sigma Chem. Co.). Reproducible ATP effects required that stocks were made from fresh ATP. In general, stock solutions of 5 or 50 mM were made in CB and frozen at −80°C. Aliquots were thawed when needed, kept on ice until used but were not refrozen.

Immunofluorescence

Cells grown on coverslips were fixed in PBS containing 3% formaldehyde (Ted Pella, Inc. Redding, CA) for 15 min. The coverslips were then washed in PBS, quenched for 15 min in PBS containing 0.1 M glycine, and blocked in PBS containing 5% BSA for 30 min. Antibodies (except ES66 as described below) were introduced in the same buffer at a concentration of 20 μg/ml. After incubating for 1 h at room temperature, the cells were washed in PBS and incubated for 1 h at room temperature in a fluorescently conjugated secondary antibody, fluorescein-conjugated goat anti-rabbit diluted 1:200, or rhodamine-conjugated goat anti-mouse diluted 1:150 in blocking buffer (Cappel Organon Teknika Co., Durham, NC). The coverslips were mounted and observed on a Zeiss Axioplan fluorescent microscope using a 63X objective with a numerical aperture 1.40 or on a Biorad 600 Confocal microscope. Pictures were taken using TMAX 400 speed film (Eastman Kodak Co., Rochester, NY).

Integrin staining in permeabilized cells localized in a prominent, trabecular, cytoplasmic staining pattern. Cell surface staining in focal adhesions was observed most clearly when live cells were labeled before permeabilization. For live staining, coverslips were removed from the dishes and placed on parafilm with the cell side up. 50 μl of a solution containing 20 μg/ml ES66 in growth media was added to each coverslip, which was then incubated for 20 min in a 10% CO_2 incubator at 37°C. The antibody was removed, and the cells were washed quickly in CB and permeabilized with digitonin as described above. After fixation the cells were incubated in fluorescently labeled secondary antibody, a fluorescein-conjugated goat anti-rat diluted 1:100, as described above.

Immunoprecipitations and Immunoblotting

Tyrosine phosphorylated proteins were identified using immunoprecipitation and immunoblotting. 10-cm tissue culture–treated dishes were seeded with 10^6 cells in growth media and allowed to spread overnight in a humidified 10% CO_2 incubator at 37°C. The dishes were washed 1X with 4 ml CB without BSA, and the cells permeabilized for 30 s with 4 ml CB containing 0.01% digitonin. The permeabilized cells were incubated for 1 h in CB (without BSA) containing ATP or other specified reagents. For integrin immunoprecipitations the cells were scraped off the dish with a rubber policeman in 300 μl 10 mM Tris-acetate containing 0.5% NP-40, 0.5 mM CaCl, 0.2 mM PMSF, 0.1 μg/ml pepstatin, 1 μg/ml leupeptin and 1 mM 1,10 phenanthroline at pH 8.0 (TNC). The cells were extracted for 15 min on ice and then microfuged for 15 min at 4°C. The supernatant was removed, and a rabbit anti-β1 integrin (antibody 814) was added to the supernatant at a concentration of 20 μg/ml and incubated for 2 h on ice with mixing every 15 min. The antigen-antibody complexes were precipitated by incubating overnight at 4°C using 50 μl packed goat anti-rabbit Sepharose beads (BioRad, Hercules, CA) and centrifugation by a brief pulse in a microfuge. The precipitate was washed three times with 1 ml TNC, followed by an additional three washes in 1 ml TNC containing 0.5 mM $MgCl_2$ and a final three washes in 1 ml TNC. The antigen-antibody complexes were released from the beads by adding 40 μl of double concentration Laemmli sample buffer to the beads and heating to 65°C for 10 min. The antigen-antibody complexes were separated from the beads by microcentrifugation for 10 min using a 0.45-μm ultrafree-MC filter unit (Millipore, Bedford, MA). The filtrate was resolved on a 7% SDS polyacrylamide gel (Laemmli, 1970). FAK, tensin, and paxillin immunoprecipitations were performed using the protocols of Schaller et al. (1992), Bockholt et al. (1992), and Turner et al. (1990), respectively.

Tyrosine phosphorylated proteins were visualized by immunoblotting with PY20, a mAb that recognizes phosphotyrosines residues. Proteins resolved on SDS polyacrylamide gels were transferred to nitrocellulose and incubated overnight at 4°C in PBS containing 5% BSA. The immunoblot was incubated in PY20, which was diluted in 5% BSA-PBS at 1:1,000 (1 μg/ml final concentration) for 1 h at room temperature. The blot was first washed for 5 min in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$ and 0.02% NaN_3, (TS), followed by a 10-min wash in TS containing 0.1% Tween 20 and a 5-min wash in TS. Biotinylated horse anti-mouse antibody (Vector Labs, Burlingame, CA) diluted by 1:800 in blocking buffer was added to the blot and incubated for an hour at room temperature, and then washed as just described. The immunoblot was incubated for 30 min in alkaline phosphatase diluted according to the manufacturer's specifications (AP kit; Vector Labs). The blot was washed as described above, and the phosphoproteins visualized by the addition of alkaline phosphatase substrate (Vector Labs). The antibody specificity for phosphotyrosine residues was ascertained by preincubating the antibody in 10 mM *O*-phospho-L-tyrosine for 1 h on ice, and then blotting with the blocked antibody as described above.

Results

Focal Adhesions Can be Stabilized in Permeabilized Cells

Permeabilized cells provide a highly useful approach for studying the mechanisms of diverse cellular phenomena (Balch et al., 1987; Beckers et al., 1987; Burke and Gerace, 1986; Goda and Pfeffer, 1989; Newport and Spann, 1987; Simons and Virta, 1987; Suprynowicz and Gerace, 1986; Tint et al., 1991). A requirement for either fixatives or short incubation times impeded previous studies of focal adhesion regulation using in vitro systems (Avnur, 1983; Ball, et al., 1986; Tranqui et al., 1992). Therefore, to study the signals directly involved in the release of adhesion, we first developed a permeabilized cell system with which focal adhesions are stable for several hours. This required an agent that permeabilizes the cell, yet preserves both the adhesion of the cells on the dish and their morphology while maintaining major components in the focal adhesions. Of several detergents and permeabilizing agents assayed, digitonin permeabilizes the cells most reproducibly. A brief, 30-s permeabilization with 0.01% digitonin permeabilizes the cells, as determined by antibody accessibility, yet consistently retains prominent talin, vinculin, α-actinin, f-actin, and integrin staining in focal adhesions.

To improve focal adhesion stability, we initially used a buffer that simulated the cytoplasmic environment, i.e., a reducing environment with high potassium and low sodium and calcium concentrations. This formulation has been employed successfully by others using permeabilized cell systems (Balch et al., 1987; Beckers et al., 1987; Burke and Gerace, 1986; Goda and Pfeffer, 1989; Newport and Spann, 1987; Simons and Virta, 1987; Suprynowicz and Gerace; 1986; Tint et al., 1991). Use of this buffer resulted in an increased stability of focal adhesions over that seen with PBS. We speculated that further instability might arise, in part, from protease activity, the shifting of equilibrium due to dilution of the cellular constituents, or nonoptimal pH. Therefore, we added protease inhibitors, including 1% BSA, to inhibit protease activity and 300 mM sucrose to increase the activity (effective concentration) of the incubation medium. The presence of sucrose had a significant, stabilizing activity as did pH values of 7.3 or less. For simplicity, this buffer formulation is called cytobuffer or CB.

Fig. 1 shows the effect of the CB on tendon fibroblast focal adhesion stability. Tendon fibroblasts were chosen because they are a relatively homogeneous cell type that form large, well organized focal adhesions when plated on fibronectin. However similar effects are also seen with 3T3 cells (not shown). Permeabilized cells are fixed immediately or incubated in CB for 1 h before fixation. Focal ad-

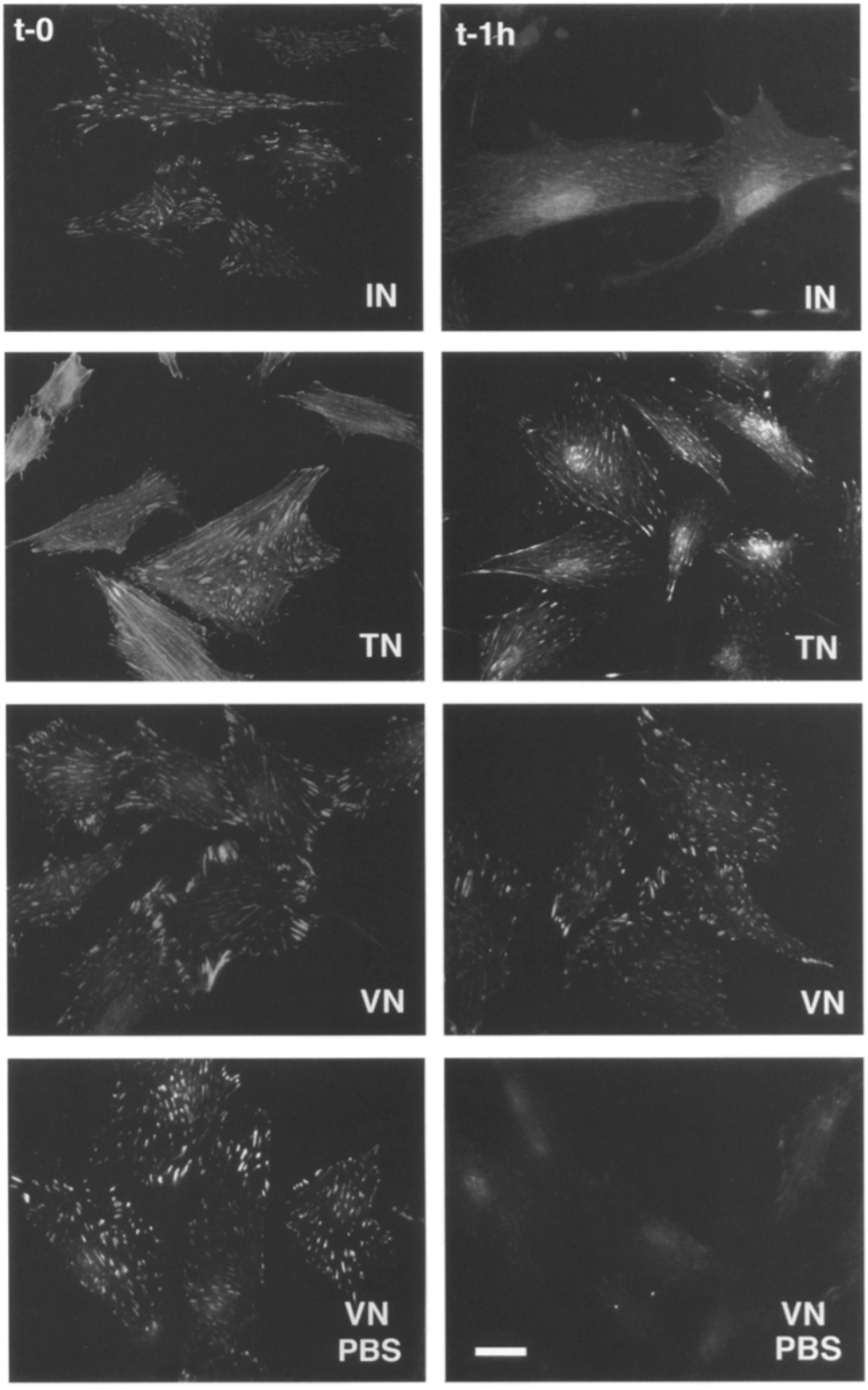

Figure 1. Focal adhesions in cells incubated in cytobuffer (CB) or PBS and visualized with either anti-vinculin, -talin, or -integrin antibodies. Tendon fibroblasts were permeabilized in CB containing digitonin or PBS containing digitonin. The cells in the panel on the left, labeled t = 0, were fixed immediately after permeabilization while the cells in panel on the right, labeled t = 1, were incubated for an additional hour in CB or PBS before fixation. Focal adhesions are clearly present at time 0 under both permeabilization conditions. However, there is a clear difference in the focal adhesion staining intensity after 1 h in PBS. Only the cells incubated in CB have large bright focal contacts. The exposure time for all of the pictures is the same. Bar, 20 μm.

hesions are visualized using antibodies against focal adhesion components. Integrin, vinculin, and talin staining are shown in Fig 1; α-actinin (not shown) behaves similarly. Cells incubated for 1 h in CB show well organized staining, for all three components, that localizes in prominent focal adhesions. Comparing the zero time with 1 h incubation in cytobuffer reveals a perceptible decay of all focal adhesion components (Fig 1); however, discernible focal adhesion staining persists for at least 5 h in cells incubated in CB. In contrast, cells incubated in PBS for 1 h (*lower panel*, Fig. 1) show no discernible vinculin localization in focal adhesions. Other focal adhesion components behave similarly when the permeabilized cells are incubated for 1 h in PBS (not shown).

ATP Destabilizes the Focal Adhesions

A large number of different biological agents were assayed for their effect on focal adhesion stability with cells incubated in CB. These include second messengers, nucleotides (ATP and GTP), different protein kinase activators and inhibitors, a phosphatase, and phosphatase inhibitors. ATP is unique among the molecules tested. It stimulates the rapid release of focal adhesion components. Fig. 2 shows cells incubated for 1 h in CB containing either 0.5 mM or 5 mM ATP or in CB alone, and then stained with an antibody against vinculin. Cells incubated in 5 mM ATP show diffuse staining and some thin, faint vinculin localization in focal adhesions; in addition some cells have either detached from the coverslips or are more rounded than the controls incubated in CB (not shown). ATP concentrations of at least 2.5–5 mM are commonly used for in vitro systems as the exogenous ATP is hydrolyzed rapidly in permeabilized systems (Burke and Gerace, 1986; Suprynowicz and Gerace, 1986). At higher ATP concentrations (10–50 mM), the focal adhesion remnants disappear and only the diffuse staining remains (not shown). The number of detached and rounded cells increases in concentrations >10 mM ATP. Cells incubated in a lower ATP concentration, 0.5 mM, show a range of different phenotypes. Most cells remain adherent and well spread; however, the vinculin localization ranges from nearly normal, but less bright, to weakly localized in thin, streak-like focal adhesions. Only an occasional cell shows the prominent focal adhesion localization seen in cells not incubated with ATP. As the ATP concentration decreases further, the cells remain adherent and well spread, and the vinculin staining becomes progressively more localized and prominent in focal adhesions. The effects of the ATP on focal adhesions are no longer apparent at concentrations below 500 μM. Treatment of the cells with ATPγS (not shown) has no effect on the integrity of the focal adhesions, while the inclusion of an ATP generating system augments the effect at low concentrations suggesting that ATP hydrolysis is required.

The loss of vinculin from focal adhesions portended a more general breakdown. Antibodies against other focal adhesion proteins, e.g., integrin, talin, and α-actinin, also do not localize in discernible focal adhesions demonstrating that they are no longer present. At 5 mM ATP, for example, neither integrin, talin, α-actinin, nor vinculin localize in prominent focal adhesions; but rather distribute either diffusely throughout the cell or in faint, thin focal adhesions. Talin staining is somewhat different. Upon addition of lower concentrations of ATP (0.5 mM ATP), the prominent focal adhesion localization pattern was no longer present; however the streak-like staining along actin filaments remained.

ATP Stimulated Tyrosine Phosphorylations Are Implicated in the Breakdown of Focal Adhesions

Several lines of evidence point to tyrosine phosphorylation as a likely regulatory mechanism for both adhesion and detachment and as a conduit for the ATP effects on focal adhesion stability. For example transformation of cells by the Rous sarcoma virus is associated with the tyrosine phosphorylation of many focal adhesion-associated

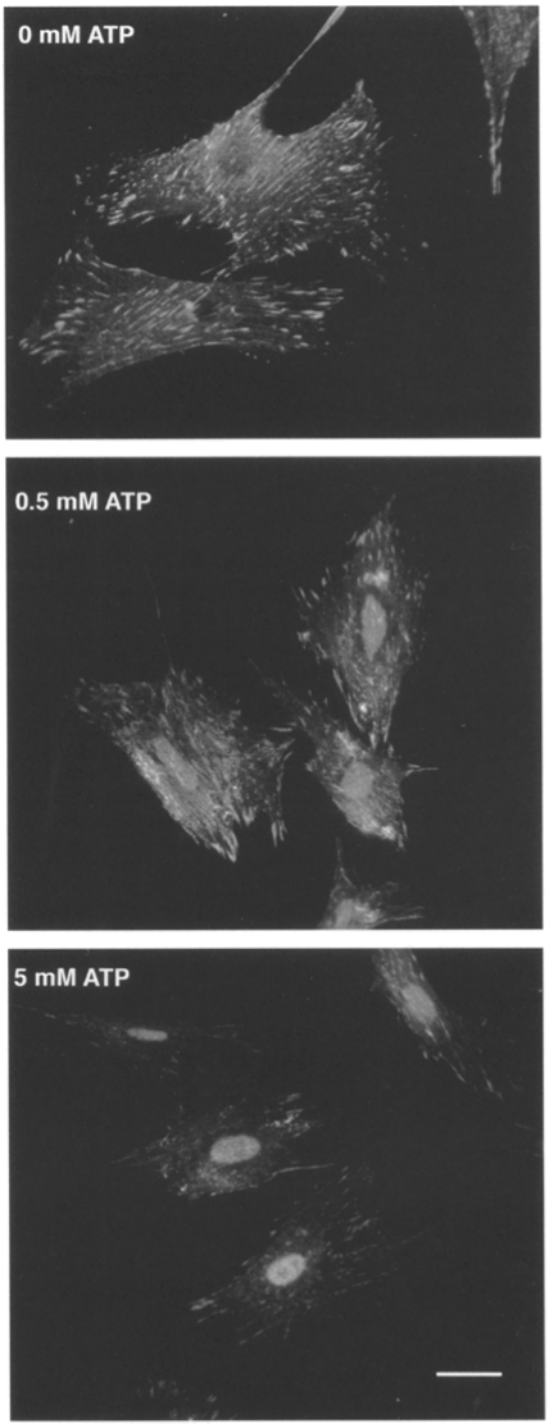

Figure 2. ATP destabilizes focal adhesions. Tendon fibroblasts were permeabilized with digitonin and incubated for 1 h in CB, 5 mM ATP, or CB containing 0.5 mM ATP, and then fixed and stained with an antibody directed against vinculin. Note the absence of prominent focal adhesion staining in cells treated with 5 mM ATP. At 0.5 mM ATP, the cells remain well spread and display a range of focal adhesion phenotypes. Diffuse vinculin staining is present throughout most cells: however thin, streak-like focal adhesions are also present primarily at the periphery. Bar, 20 μm.

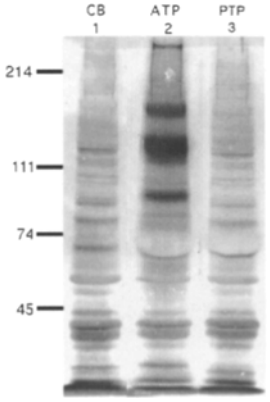

Figure 3. Proteins phosphorylated in response to the ATP incubation. Western blot analysis of tendon fibroblasts incubated in CB (lane *1*); CB containing 0.5 mM ATP (lane *2*); or 0.5 mM ATP with 50 μg/ml tyrosine phosphatase. Permeabilized cells were incubated for 1 h in CB or CB containing 0.5 mM ATP either with or without a recombinant phosphatase. After the incubation the cells were extracted in 100 μl of Laemmli sample buffer, and the proteins resolved by SDS-PAGE. The proteins were transferred to nitrocellulose and incubated with the PY20 mAb, which specifically recognizes phosphorylated tyrosine residues. Cells incubated in ATP show a large increase in phosphotyrosine staining. The most prominent bands are those migrating at molecular weights of 170, 130, and 120, and the three additional bands migrating between the molecular weights of 70–100 kD. Molecular mass standards migrate at 214, 111, 74, and 44 kD. Addition of a constitutively active recombinant phosphatase inhibits the ATP-induced phosphorylation.

proteins and altered adhesive properties of the infected cells (Parsons and Weber, 1989). One of these is FAK. Its phosphorylation is implicated in the formation of new focal adhesions (Burridge et al., 1992). The pattern of tyrosine phosphorylation stimulated by addition of ATP was assayed by extracting and resolving the cellular proteins by SDS-PAGE, and then Western blotting with an antibody specific for phosphotyrosine residues. Cells incubated in buffer without ATP show no prominent proteins phosphorylated on tyrosine (Fig. 3, Lane *1*). In contrast, several major proteins, migrating in the molecular mass ranges of 120–170 kD and between 70 kD and 100 kD, are phosphorylated on tyrosine in response to the ATP treatment (Fig. 3, lane *2*). The number of proteins in the 70–100 kD range is somewhat variable; whereas the presence of the higher molecular weight proteins is reproducible. Permeabilized cells incubated within ATPγS appear similar to those incubated in CB (data not shown).

The molecular weights of the tyrosine phosphoproteins induced by ATP treatment are similar to those for some well characterized cytoskeletal and focal adhesion proteins. The SDS-PAGE profile of phosphotyrosine proteins remaining after a 1-h extraction in CHAPS is virtually indistinguishable from that of the unextracted, ATP-treated cells (data not shown), suggesting that the proteins phosphorylated in response to ATP are also cytoskeletally associated. A possible relation between the phosphoproteins resulting from ATP stimulation and known phosphoproteins that migrate at similar molecular weights was investigated. Extracts from ATP treated, permeabilized cultures were immunoprecipitated with antibodies against the β1 integrin, focal adhesion kinase (FAK), paxillin, and tensin and then immunoblotted with an anti-phosphotyrosine antibody. Neither integrin, FAK, paxillin, nor tensin show detectable phosphorylation on tyrosine in response to the ATP treatment (however small amounts of phosphorylated integrin and FAK are released into the supernatant from the incubations). Thus the tyrosine phosphorylations responsible for focal adhesion destabilization do not appear to be those that correlate with adhesion assembly. Permeabilized cells that have not been treated with ATP show no detectable levels of phosphorylated FAK suggesting that while FAK may be transiently phosphorylated during the formation of focal adhesions, its continued phosphorylation is not required for relatively stable focal adhesions.

Inhibiting the phosphorylation and assaying the effect of this inhibition on focal adhesion organization is one way to demonstrate a direct connection between tyrosine phosphorylation and breakdown of the focal adhesions. To do this, we introduced a constitutively active, purified recombinant T cell tyrosine phosphatase to the permeabilized system (Cool et al., 1990; Zander et al., 1991). The phosphatase inhibits the ATP induced destabilization of focal adhesions in a dose dependent manner (Fig. 4). At high phosphatase concentrations, 50 μg/ml, the ATP-treated cells remain spread with vinculin concentrated in focal adhesions (Fig. 4). The phosphatase inhibits the destabilization of both vinculin and talin, when induced by either 0.5 mM or 5 mM ATP (Fig. 4). Addition of the phosphatase also decreases the concentration of phosphotyrosine containing proteins as seen by Western blots of cell extracts using the PY20 mAb (Fig. 3, lane *3*). At a lower concentration of phosphatase, 10 μg/ml, the cells remain spread with clear, but diminished, vinculin staining in focal adhesions, which appear somewhat decreased in size (data not shown). No detectable effect is seen at phosphatase concentrations below 5 μg/ml. Overall, this dose response points to a general correlation between the presence of phosphorylated proteins and destabilization of adhesions; however it is not clear whether the correlation is linear or exhibits a threshold. Irreproducibility and the stabilizing effect of the vanadate on focal adhesions, when added to cells in the absence of ATP, frustrated our attempts to inhibit the phosphatase with 1 mM sodium vanadate. The stabilizing effect of vanadate on adhesion has been reported previously along with the differing activities of the different vanadate oxidation states (Edwards et al., 1991; Elberg et al., 1994).

Cytoskeletal Tension Is Implicated in the Breakdown of Focal Adhesions

Previous reports describe a contraction of the cell cortex and nucleus in response to the addition of ATP to permeabilized cells (Hollenbeck et al., 1989; Sims et al., 1992; Tint et al., 1991). This contraction is the terminal component of a signal transduction pathway that culminates in the serine phosphorylation of the myosin light chain by myosin light chain kinase (Kolodney and Elson, 1993; Wysolmerski and Lagunoff, 1990; Yamakita et al., 1994). Some of our observations with the permeabilized cells suggest a possible relation between contraction and focal adhesion stability. Cell contraction often accompanies focal adhesion reorganization, especially at very high ATP concentrations (≥5 mM). In addition, micromolar concentrations of okadaic acid, a serine, threonine phosphatase inhibitor, enhance the ATP effect on cell contraction and focal adhesion destabilization. Cells incubated in 1.25 μM okadaic acid with low (0.5 mM) concentrations of ATP

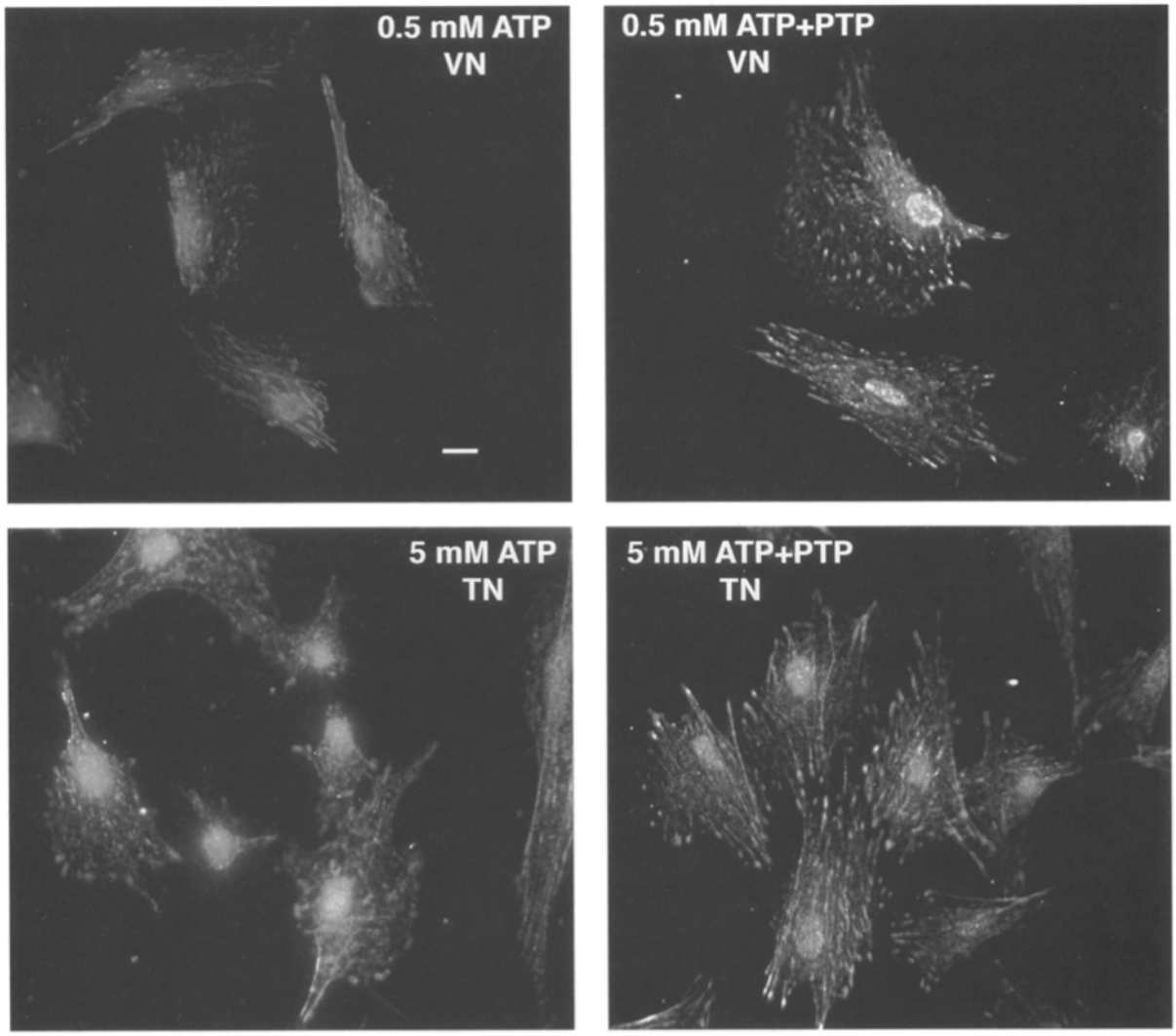

Figure 4. A tyrosine phosphatase inhibits the ATP induced destabilization of the focal adhesions. Permeabilized tendon fibroblasts were incubated for 1 h in CB containing 5 mM ATP or 0.5 mM ATP and an ATP regenerating system, either with or without 50 μg/ml tyrosine phosphatase. The cells were then fixed and labeled with antibodies against vinculin or talin. Cells incubated in ATP show only a few, small focal adhesions and diffuse vinculin and talin localization. Cells treated with the phosphatase show vinculin and talin localizing predominantly in focal adhesions. Note that talin delocalization is less sensitive than vinculin to the lower ATP concentration. Bar, 10 μm.

show delocalized focal adhesion components and a contracted cell body (Fig. 5). Okadaic acid has no detectable destabilizing effect in the absence of exogenous ATP. Presumably, the okadaic acid prevents the dephosphorylation of the myosin light chain, thus enhancing the contraction (Takai et al., 1987). Since the presence of okadaic acid gives reproducible contraction and requires lower ATP concentrations, we used it in most experiments related to cytoskeletal tension.

We investigated directly the possibility that contraction, i.e., tension, initiates breakdown of focal adhesions using synthetic peptides that inhibit the actin-myosin interaction. The actin-myosin interface includes the heptapeptide sequence (IRICRKG) corresponding to the actin-binding region on myosin (Katoh and Morita, 1993; Suzuki et al., 1987). This peptide inhibits actin-myosin interactions and cell contraction (Sims et al., 1992). As a control we used the IRICEKG peptide, which has a single amino acid substitution and does not inhibit cell contraction (Sims et al., 1992). The active peptide inhibits the ATP induced destabilization of focal adhesions in a dose-dependent manner. Similar results are observed for integrin and talin (data not shown). Cells treated with 1.25 μM okadaic acid and 0.5 mM ATP are contracted with little focal adhesion staining (Fig. 6). The presence of the active peptide inhibits both contraction and focal adhesion reorganization (Fig. 6). In contrast cells incubated with IRICEKG are highly contracted, like the controls, with little apparent focal adhesion staining (Fig. 6, *lower panel*).

Focal Adhesion Stability Is Regulated by Two Different Pathways

The observations presented above suggest two mechanisms that regulate focal adhesion stability: one is a tyrosine kinase cascade and the other is cytoskeletal contraction. The suggestion that cytoskeletal contraction and

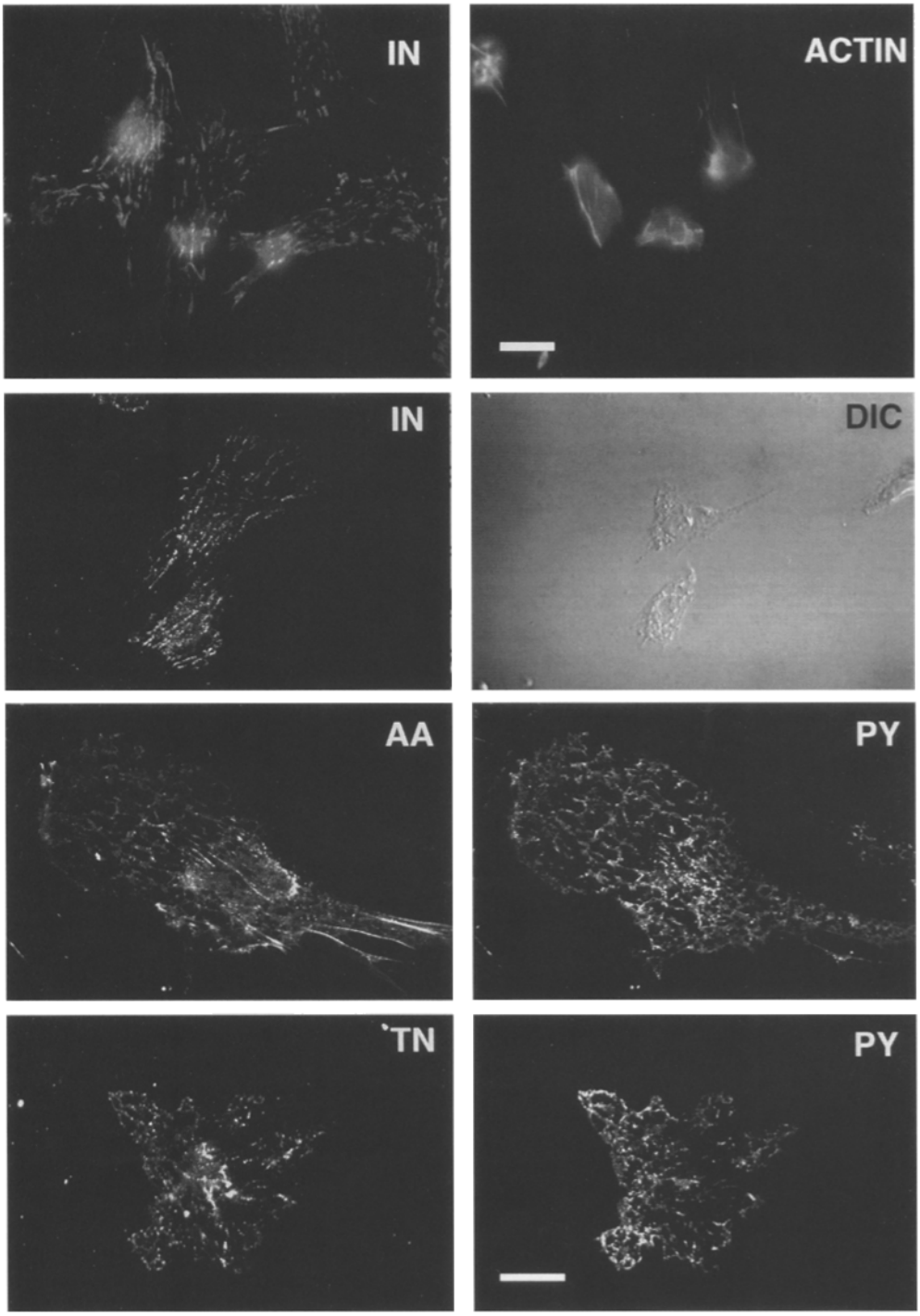

Figure 5. Okadaic acid enhances the destabilizing effect of ATP. Permeabilized tendon fibroblasts were incubated for 1 h in CB containing 0.5 mM ATP and 1.25 μM okadaic acid, and then fixed and immunostained with antibodies against integrin (*IN*) actin, phosphotyrosine (*PY*), talin (*TN*), and anti–α-actinin (*AA*). The lower six panels are confocal images. Okadaic acid induces an enhanced contraction of the actin cortex and a characteristic reorganization of some of the focal adhesion proteins into a trabecular network. Note the colocalization of the α-actinin and talin with the phosphotyrosine-containing proteins. The DIC image is presented to show the contracted cell bodies; note the presence of focal adhesion remnants, shown in the integrin image, that remain outside the cell after the contraction. Bar, 20 μm.

tyrosine phosphorylation affect focal adhesion stability by different mechanisms is also supported by our observation that the phenotype of the cells incubated in okadaic acid is significantly different from that observed with cells incubated in ATP alone (including high concentrations). Cells treated with ATP and okadaic acid not only show a highly contracted phenotype; but talin, α-actinin, and vinculin reorganize into a trabecular network that is especially prevalent toward the periphery of the cell (Figs. 5 and 6). These focal adhesion components colocalize with the phosphotyrosine staining, as visualized with the PY20 mAb, in the trabecular network (Fig. 5). This trabecular staining contrasts that seen in areas where cells have mechanically ripped from the matrix during the washing and fixing process; they show the typical focal adhesion localization pattern. In contrast to other focal adhesion components, cytoplasmic integrin in cells incubated in CB always displays a trabecular localization pattern, the intensity of which increases with incubation time in CB. Presumably this reflects the cytosolic distribution of nascent integrin and that which is synthesized or otherwise processed subsequent to permeabilization. Occasionally talin and α-actinin are present in focal adhesion-like structures in the vicinity of the contracted cell body. Integrin and vinculin are

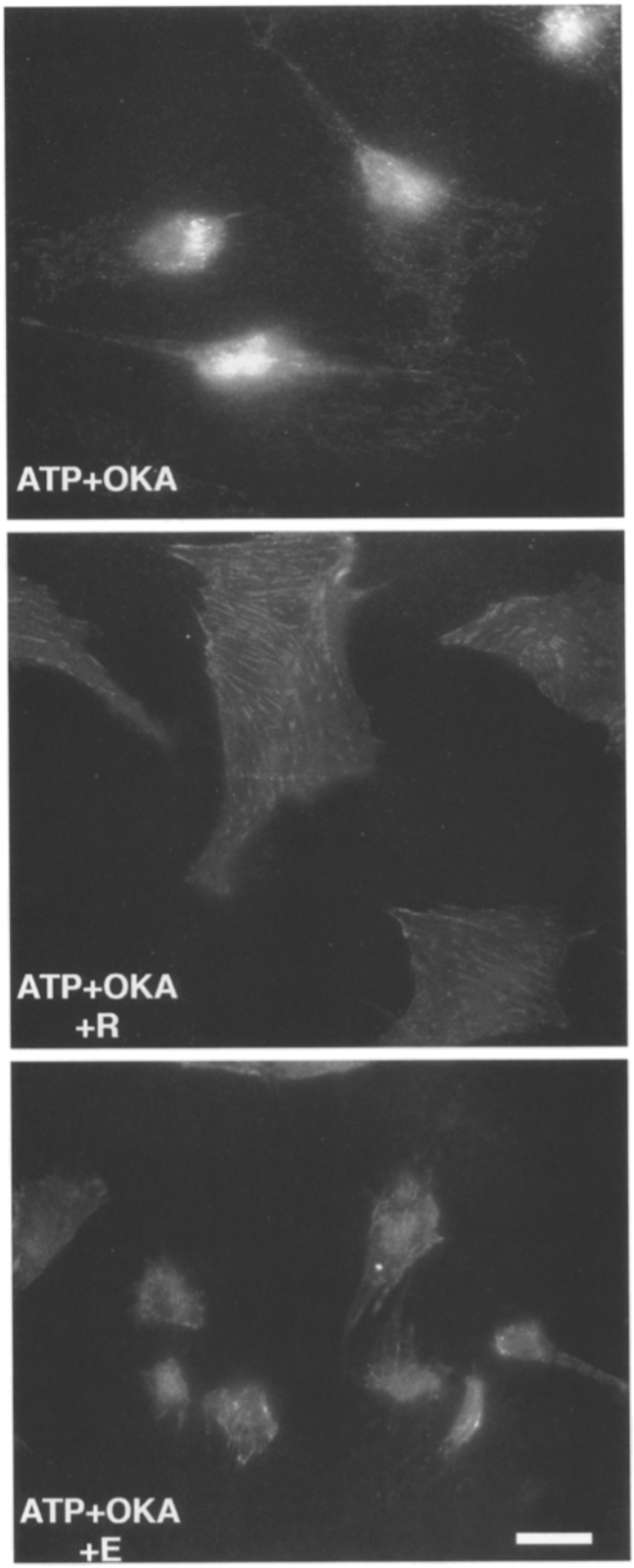

Figure 6. A peptide that inhibits contraction also inhibits the destabilization of the focal adhesions. Permeabilized cells were incubated for 1 h in CB containing 0.5 mM ATP and 1.25 μM okadaic acid (*OKA*) or in CB containing 0.5 mM ATP, 1.25 μM okadaic acid and either 500 μg/ml IRICRKG (+*R*) or 500 μg/ml IRICEKG (+*E*). After fixation the cells were stained with antibodies against vinculin. The cells incubated in ATP and okadaic acid are contracted, and vinculin localizes in a trabecular organization. In contrast, cells incubated in the active peptide, IRICRKG, are well spread with vinculin localized in typical focal adhesions. Bar, 20 μm.

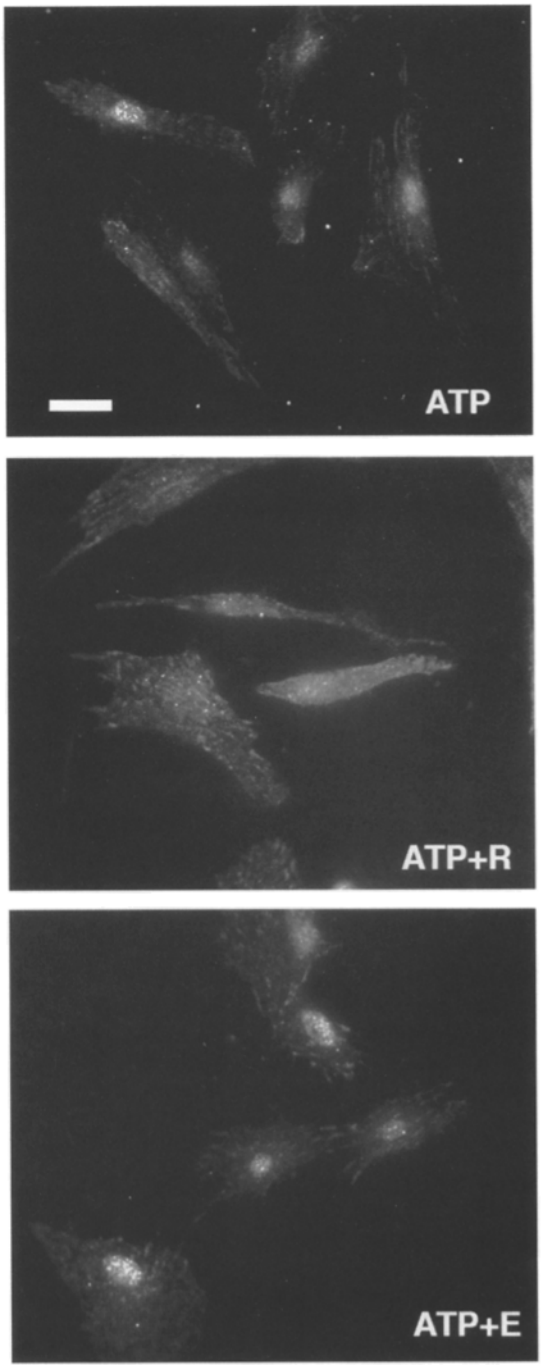

Figure 7. A peptide that inhibits contraction has only a small inhibiting effect on the breakdown of focal adhesions in cells incubated in 5 mM ATP. Permeabilized cells were incubated in CB containing 5 mM ATP (*ATP*) or in CB containing 5 mM ATP and 500 μg/ml IRICRKG (*R*) or IRICEKG (*E*) for 1 h. The cells were fixed and immunostained with antibodies against vinculin. Cells incubated in 5 mM ATP show thin focal adhesions or diffuse vinculin localization throughout the cell. Cells incubated in the contraction inhibiting peptide show only a modest inhibition. Bar, 20 μm.

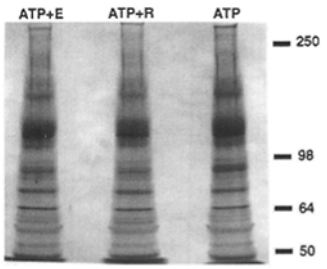

Figure 8. Inhibiting contraction does not significantly alter the profile of proteins phosphorylated on tyrosine. Cells were permeabilized and incubated for 1 h in 0.5 mM ATP (*ATP*) 0.5 mM ATP + 500 μg/ml IRICRKG peptide (*ATP + R*) or 500 μg/ml IRICEKG peptide (*ATP + E*). The cells were extracted in Laemmli buffer and resolved on SDS-PAGE. The proteins were then transferred to nitrocellulose and incubated with PY 20 to visualize phosphotyrosine-containing proteins. All three lanes show similar phosphoprotein profiles.

only rarely seen in these structures. In contrast to most focal adhesion components, filamentous actin and cell surface integrin do not localize in the trabecular patches. The actin collapses around the center of the cell (Fig. 5). Cell surface integrin is present both in remnants of focal adhesions at the periphery where the cell had been before contraction and diffusely in the region of the contracted cell body (Fig. 5).

The possible differences and interrelations between these two pathways were addressed in several ways. First, we asked whether the ATP treatment destabilizes focal adhesions in the absence of contraction. We added the contraction inhibiting IRICRKG peptide in the presence of 5 mM ATP with the ATP regenerating system (Fig. 7). These conditions produce high levels of tyrosine phosphorylation. Under these conditions, the peptide shows a generally small and variable inhibition of destabilization, even when used at a concentration as high as 1 mg/ml (not shown). We also assayed whether the contraction might alter the profile of proteins phosphorylated on tyrosine. Permeabilized cells incubated with 0.5 mM ATP and the contraction inhibiting peptide show a profile of tyrosine phosphoproteins that is nearly identical to that seen without peptide or with the inactive peptide (Fig. 8). Finally, we asked whether ATP treatment destabilizes focal adhesions in the absence of tyrosine phosphorylation. Cells treated with 0.5 mM ATP and okadaic acid in the presence of the tyrosine phosphatase retract and show destabilized focal adhesion components (data not shown).

Discussion

We used a permeabilized cell system to study the mechanisms that regulate the release of cellular adhesions. A central feature of the semi-in vitro system is a buffer which stabilizes focal adhesions for several hours. Using this system, different reagents were added to ascertain putative pathways involved in the release process. We found that ATP rapidly destabilizes focal adhesions. ATP appears to act through two distinct, but possibly interacting, mechanisms: a tyrosine kinase cascade which results in the phosphorylation of several major proteins and the generation of tension through cytoskeletal contraction (Table I).

Previous studies implicate tyrosine phosphorylation in the regulation of adhesion. Burridge et al. (1992) described increased tyrosine phosphorylation on both FAK and paxillin in cells spreading on fibronectin. Furthermore, the presence of herbimycin, a tyrosine kinase inhibitor, during spreading inhibits formation of focal adhesions. Earlier studies showed that RSV transformed cells, in response to the pp60 tyrosine kinase, are not well spread and display altered adhesive linkages with increased phosphoproteins in them (Hirst et al., 1986; Felice et al., 1990; Parsons and Weber, 1989; Pasquale et al., 1986; Piwnica-Worms et al., 1987; Tarone et al., 1985). Therefore, both the formation and stability of focal adhesions appear regulated through tyrosine kinase cascades.

Our data point to a tyrosine kinase signaling cascade as a mechanism for destabilizing adhesions. Addition of ATP stimulates the phosphorylation on tyrosine of several proteins. Addition of a recombinant T cell tyrosine phosphatase inhibits both the phosphorylation and adhesive alterations. Interestingly, FAK does not appear to play a major role in this destabilization process. Its phosphorylation does not correlate well with either stabilized or desta-

Table I. Summary of Effects of Different Treatments on Focal Adhesion Components and Cell Morphology

Treatment	VN organization	IN organization	Cell morphology
CB	FA	FA	spread
5 mM ATP	very diffuse, weak, streak-like	diffuse FA and remnants	less spread
0.5 mM ATP	weak FA	weak FA	spread
50 μg/ml PTP + 0.5 mM ATP	FA	FA	spread
OKA + 0.5 mM ATP	trabecular	FA remnants	highly contracted
OKA + 0.5 mM ATP + IRICRKG	FA	FA	spread
OKA + 0.5 mM ATP + IRICEKG	trabecular	FA remnants	highly contracted
50 μg/ml PTP + 10 μM OKA + 0.5 mM ATP	trabecular	FA remnants	rounded

FA, focal adhesion.
CB, cytobuffer.
OKA, okadaic acid.
PTP, tyrosine phosphatase.

bilized focal adhesions. Cells incubated in cytobuffer alone have relatively stable focal adhesions but no detectable levels of phosphorylated FAK (or several other focal adhesion proteins). Therefore, the presence of unphosphorylated FAK does not produce rapid focal adhesion breakdown. Conversely, FAK does not show a major increase in phosphorylation in response to ATP stimulated focal adhesion breakdown. This contrasts both the large, transient increase in FAK phosphorylation reported in spreading cells (Burridge et al., 1992; Romer et al., 1994), and our observation that vanadate stabilizes adhesions (suggesting the involvement of a phosphatase in the destabilization) in permeabilized cells and also increases the levels of FAK phosphorylation (unpublished observation).

Protein tyrosine phosphorylation does not appear to be the only mechanism controlling focal adhesion stability. Adhesive stability is also linked to cellular contraction. Previous reports point to a relation between cytoskeletal tension and the organization of adhesion molecules. When the tension exerted by fibroblasts on the substratum is released, there is an initial hypercontraction of the cell, collapse of cytoplasmic actin, and loss of cell surface fibronectin (Mochitate et al., 1991). In spreading cells, the organization of fibronectin changes in parallel with the generation of tension (Grinnell, 1986, 1994). Finally, Klein et al. (1991) demonstrated that the reorganization of collagen during collagen gel contraction requires the $\alpha 2\beta 1$ integrin. Taken together these studies point to a connection between tension and cytoskeletal and ECM organization. In addition, theoretical models of cell migration also point to tension as a potential contributor to the release of adhesions (Bell, 1978; Lauffenburger, 1994).

Our observations provide a direct demonstration that tension plays a role in the release mechanism. Previous reports describe a cytoskeletal mediated contraction of cells both in permeabilized systems, by addition of ATP, and in intact cells, via a signaling pathway induced by agents like thrombin (Hollenbeck et al., 1989; Sims et al., 1992; Tint et al., 1991; Wysolmerski and Lagunoff, 1988). In permeabilized cells, we found that the contraction was greatly enhanced by either very high concentrations of ATP or by the presence okadaic acid even with low concentrations of ATP. Presumably the okadaic acid inhibits the dephosphorylation of the myosin light chain and hence helps keep the cells in a contracted state (Kolodney and Elson, 1993; Kolodney and Wysolmerski, 1992; Wysolmerski and Lagunoff, 1990).

Immunofluorescent studies of these contracted cells reveal an altered localization of adhesive components. Under conditions which produce a large contraction, a substantial amount of integrin remains in tracks where cells resided before the contraction. In addition, talin, α-actinin, and vinculin reorganize into trabecular networks and are only rarely localized in the integrin tracks suggesting that the integrin-cytoskeletal interactions in focal adhesions have broken. These networks colocalize with the major proteins phosphorylated on tyrosine, whose localization (which is even apparent in cells treated with ATP alone) appears to precede it and with endogenous (i.e., intracellular) integrin. It is likely that the phosphoproteins directly or indirectly recruit released focal adhesion proteins to these sites, and it may serve as a useful system for studying the assembly of focal adhesion components. In contrast, conditions in which the cells are less contracted, e.g., lowered okadaic acid concentrations, vinculin and talin staining is seen in focal adhesions; however their intensity is reduced.

Despite the mounting evidence that mechanical forces, i.e., either high or low tension, affect cell shape and adhesion (Grinnell, 1994; Ingber, 1991, 1993), the relation between tension and organization of adhesive structures is unclear. At one extreme, the tension may affect the conformation and/or binding energy of a cytoskeletally associated adhesion molecule. As vinculin is one of the first molecules that leaves focal adhesions after ATP addition, either it or a neighbor may serve as the focus of stress. Fernandez et al. (1993) have shown that vinculin is critical for the stability of focal adhesions At the other extreme, the tension may induce activation or a change in organization of signaling molecules, like kinases or phosphatases, which then regulate adhesion by altering focal adhesion proteins.

Thus at least two mechanisms appear to destabilize focal adhesions. Peptides that interfere with the actomyosin interaction inhibit the contraction and subsequent reorganization of focal adhesion components. Therefore, breakdown of focal adhesions can be induced specifically by the contraction. However, these peptides have only a modest effect on the focal adhesions of cells incubated with 5 mM ATP in the absence of okadaic acid, which in contrast, is inhibited by an exogenous phosphatase. This points to a role for tyrosine phosphorylations in destabilization of focal adhesions. At this time there is little evidence suggesting that the two mechanisms are part of a common, linear pathway. Addition of the peptide that inhibits the myosin-actin interaction does not noticeably affect the phosphoprotein profile demonstrating that the contraction is not required for the phosphorylations. Furthermore, cells incubated in high ATP are different from those incubated in low ATP with okadaic acid. The former are less contracted and do not show the reorganized trabecular network staining. In addition they do not display well organized focal adhesions when incubated in the presence of the contraction inhibiting peptide. This demonstrates that phosphorylation, by itself, can affect adhesion. These results are in direct contrast to those found for cells incubated in low ATP and okadaic acid. Those cells are highly contracted and have reorganized focal adhesion proteins. This reorganization is blocked by the contraction inhibiting peptide but not by the recombinant phosphatase. It is likely that both mechanisms for focal adhesion destabilization act synergistically to affect adhesion. Under most conditions of ATP stimulated destabilization, neither the actomyosin inhibiting peptide nor the phosphatase completely reversed the phenotype. This synergy has two implications. First, the relative degree of tension vs the extent of activation of the tyrosine phosphorylation pathway may determine the extent of ripping vs regulated release. And second, activation of one pathway enhances the effects of the other.

The presence of multiple release mechanisms is consistent with previous observations on motile fibroblasts (Regen and Horwitz, 1992). Using time lapse immunofluorescence video microscopy, they report variable amounts of

integrin in tracks, that correspond to former adhesive sites left behind cells. They also provide evidence for a regulated release of adhesive components, especially near the edges of cells. The observation that integrin is largely found in remnants of focal adhesions correlates well with the work of Chen (1981). He reports that the tail of migrating chick heart fibroblasts ruptures leaving a small amount of itself attached to the substrate at the focal adhesion. This rupture can be induced by ATP through a contractile event in the tail. Finally, in migrating cells, there is evidence suggesting myosin II participates in the release step. Myosin II mutants of Dictyostelium display defects in the release of adhesions at the rear of the cell (Jay et al., 1995).

The physiologic role of ATP in regulating adhesion is not clear. It is possible that local fluctuations in the cellular ATP concentration serves to regulate adhesion. This model would predict an increase in the cellular ATP concentration during detachment that accompanies a process like mitosis. It would also predict an asymmetry in the ATP concentration in motile cells. Another possibility is that cell permeabilization either activates or makes accessible signaling pathways that regulate adhesion. In this model, the addition of ATP would provide a key substrate required for functioning of the pathway.

In summary, both biochemical and mechanical mechanisms appear to regulate adhesion in fibroblasts. The mechanisms described here bear on several cellular phenomena, in addition to cell migration, in which adhesive regulation is implicated. During mitosis, in addition to the well documented cascade of phosphorylations, tension appears to be generated on adhesion sites as the cell retracts and rounds just before and during cytokinesis. Both the phosphorylation cascade and tension likely contribute to release or weakening of adhesions. Finally, a remodeling of the underlying matrix accompanies wound repair. The results presented here point to cytoskeletal reorganization as a likely contributor.

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