Integrin-Dependent Tyrosine Phosphorylation in Corneal Fibroblasts

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Purpose. A major pathway for intracellular signaling from cell surface receptors, such as integrins, involves intracellular phosphorylation. In corneal fibroblasts, the authors have investigated the role of tyrosine phosphorylation in integrin-dependent cell adhesion to extracellular matrix.

Methods. Antibodies were used to detect phosphotyrosine-containing proteins, including focal adhesion kinase in lysates and immunoprecipitates of corneal fibroblasts. The authors used anti-phosphotyrosine antibodies to localize phosphotyrosines in fixed cultured corneal fibroblasts. Similarly, immunocytochemical detection of vinculin was used to identify focal adhesions, the subcellular structures in which integrins organize attachment to matrix extracellularly and to cytoskeletal components intracellularly.

Results. Suspension of corneal fibroblasts produced a dramatic decrease in detectable phosphotyrosines. During integrin-dependent fibroblast attachment to exogenously supplied fibronectin, the cytoplasmic phosphotyrosine kinase, focal adhesion kinase (FAK), pp125FAK, became tyrosine phosphorylated. However, FAK was not phosphorylated during fibroblast attachment to vitronectin or polylysine or when cells were kept in suspension. In addition, the treatment of suspended cells with antibody to the extracellular domain of fibronectin receptor caused FAK phosphorylation. Phosphotyrosine was colocalized with vinculin in newly formed focal adhesions. Focal adhesion formation was prevented by herbimycin A, an inhibitor of tyrosine kinases.

Conclusions. In corneal fibroblasts, fibronectin receptor-specific signal transduction from extracellular matrix during the formation of focal adhesions requires tyrosine kinase activation, including phosphorylation of FAK. This underscores a role for the fibronectin receptor in signaling from the extracellular matrix in corneal fibroblasts. Invest Ophthalmol Vis Sci. 1995;36:1837-1846.

At focal adhesions, extracellular domains of integrins bind to specific extracellular matrix ligands, and their cytoplasmic domains associate with the actin cytoskeleton.1,2 Evidence for integrin-based changes in cytoskeleton, intracellular pH, gene expression, and, most recently, tyrosine kinase activity supports a role for integrins in signal transduction from extracellular matrix.1,2,3-5 One pathway of signal transduction from extracellular domains of plasma membrane proteins involves cytosolic tyrosine kinases and a phosphorylation cascade.6-8 The degree of phosphorylation is determined by a balance between opposing kinases and phosphatases.9,10

In cloned and embryonic fibroblasts, during the transduction of integrin-based signals, tyrosine phosphorylation has been localized to focal adhesions.11-13 However, unlike dimeric growth factor receptors that have intrinsic kinase activity (e.g., platelet-derived growth factor receptors), the integrins lack kinase activity in their cytoplasmic domains. In studies of attached fibroblasts, antibody-induced integrin clus-
tering was paralleled by tyrosine phosphorylation of proteins of approximately 120 to 135 kd. One of these, designated as focal adhesion kinase (FAK), was localized to focal adhesions and was found to be a tyrosine kinase. In other studies, embryonic or cloned fibroblasts had increased tyrosine phosphorylation of FAK followed by increased tyrosine phosphorylation of paxillin (an actin-binding protein) when the cells attached to fibronectin (FN) by an integrin-dependent mechanism.

Little is known of the role played by integrins in corneal fibroblast signal transduction. This is of particular interest because integrin expression varies in normal development and under pathologic conditions. For example, α6β1, the classic FN receptor (FNR), is expressed de novo after corneal wounding or when corneal keratocytes are placed in culture and become fibroblasts. Keratocytes in unwounded stroma express several β-containing integrins, but not α6β1. Thus, the expression of specific integrins may be related to conditions of culture or may represent a response to injury.

In a wounded cornea, the expression of the FNR parallels a dramatic increase in FN synthesis. Because parallel increases in FNR and FN expression have been associated with changes in cell anchorage in metastatic cells, integrin-dependent signals in the corneal fibroblast may be of significance during corneal wound healing.

Moreover, it is well known that the disruption of integrin-based attachment by trypsinization induces the secretion of gelatinases by fibroblasts. Treatment of adherent synovial fibroblasts with anti-β1 or anti-FNR antibodies induces collagenase and stromelysin synthesis and secretion, thus altering the composition of the extracellular matrix.

As part of a series of studies aimed at investigating whether extracellular matrix composition represents a signal transmitted by the integrins of corneal fibroblasts, we have evaluated the role of tyrosine phosphorylation. Specifically, we have examined integrin-dependent cell–extracellular matrix signal transduction in corneal fibroblasts under two conditions—in attached cultured cells after application of antibodies that bind the FNR or β1 integrins and in cells during the process of cell adhesion to specific substrates.

**MATERIALS AND METHODS**

**Antibodies**

Anti-integrins, BIIG2 (anti-human FNR, rat monoclonal applied as the purified immunoglobulin G [IgG]) and AIIB2 (anti human β1, rat monoclonal) were a gift of C. H. Damsky (University of California, San Francisco, CA). Anti-FAK (BC3, rabbit polyclonal and 2A7, mouse monoclonal) were from M. Schaller and T. Parsons (University of Virginia, Charlottesville, VA). Rabbit polyclonal antibodies to phosphotyrosine (p-tyr) were from E. Skolnick (New York University Medical Center, New York, NY); rabbit anti-rat IgG was obtained from Sigma (St. Louis, MO); mouse monoclonal anti-p-tyr (4G10) was obtained from Upstate Biotechnology (Lake Placid, NY); anti-bromodeoxyuridine, fluorescein conjugate was obtained from Boehringer–Mannheim (Indianapolis, IN); and alkaline phosphatase-labeled anti-mouse, anti-rabbit IgG and goat anti-rat IgG were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). All other antibodies and chemicals were purchased from Sigma Chemical.

**Cell Culture**

Primary cultures of corneal fibroblasts from rabbit keratocytes were used in passages 2 to 8. All procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. We harvested the stroma from dissected corneas of New Zealand albino rabbits. After we completely removed the epithelium and endothelium by scraping, we cut the remaining stroma into approximately 1-mm² pieces and placed them in DMEM–F12 with 10% fetal calf serum and antibiotics: 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 50 μg/ml gentamicin. The corneal fibroblasts used in these studies were derived from cells that migrated from these fragments. Cells were cultured to confluency at 37°C, in 5% CO₂, and were passaged at least 24 hours before use.

**Treatment of Attached cells With Anti-β1 Antibodies**

When cells reached confluence 24 to 72 hours after passage, we removed fetal calf serum for 5 to 24 hours before the experiment. To test the effect of anti-integrin antibodies, we replaced the medium with DMEM–F12 and 0.2% bovine serum albumin containing 15 μg/ml anti-FNR, anti-β1, control rat IgG, or no addition. We incubated the cells for the indicated times at 37°C in 5% CO₂. In one group of experiments, cells were treated with antibody at 0°C for 30 minutes, followed by incubation at 37°C in 5% CO₂.

To terminate the incubation, we placed the dishes on a salt-ice bath, removed the medium, washed the cells with DMEM–F12, and lysed them in a Tris-buffered detergent with inhibitors of proteases and phosphatases: leupeptin (10 μg/ml), pepstatin (4 μg/ml), aprotinin (2 mg/ml), vanadate (100 to 200 μM), and phenylmethylsulfonyl fluoride (2 mM). The detergent was either 1% Nonidet P-40 (NP40) or 2% Triton X-
100. After 30 minutes, the lysate was centrifuged at 0°C, 14,000 rpm.

In one series, the Triton-insoluble pellet was treated with 0.1% sodium dodecyl sulfate (SDS), 2% Triton X-100, and 1% sodium deoxycholate and protease and phosphatase inhibitors for 5 minutes, followed by 5-minute centrifugation (14,000 rpm), all at 0°C. The lysates were frozen and used for immunoprecipitation studies or were mixed with SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer for electrophoresis and subsequent transfer to nitrocellulose.

**SDS–PAGE and Immunoblotting**

Thirty micrograms of protein per lane were electrophoresed on 8% to 12% gradient gels under reducing conditions. Proteins were transblotted, and p-tyr-containing proteins were detected by mouse monoclonal or rabbit anti-p-tyr antibodies. These were subsequently identified by anti-mouse or anti-rabbit conjugated to alkaline phosphatase, following the directions supplied by Pierce (Cambridge, MA). Densitometric quantitation was performed on scanned images of Western blots using Imagequant (Molecular Dynamics, Sunnyvale, CA).

**Immunoprecipitation**

Before immunoprecipitation, lysates were normalized by protein content. The lysate supernatant was pre-cleared twice with washed protein A Sepharose (CL-4B; Pharmacia, Uppsala, Sweden), and the Sepharose was removed by centrifugation. Antibody to FAK (mouse monoclonal, 2A7) was added to precleared lysate and then immunoprecipitated with rabbit anti-mouse IgG, followed by protein A Sepharose as described. Alternatively, rabbit polyclonal anti-FAK, BC3, was added to precleared lysate and then immunoprecipitated by protein A Sepharose. Proteins were separated by SDS–PAGE under reducing conditions, transferred to nitrocellulose, and detected as described.

**Attachment to Substrate**

We studied the dependence of the tyrosine phosphorylation pattern on attachment by comparing suspended cells and cells plated on different substrates. Substrates were chosen either for their ability to promote nonintegrin-dependent attachment (polysine) or as specific integrin ligands. Tissue culture plastic dishes were coated with FN (10 μg/ml, Sigma), vitronectin (8 to 10 μg/ml; Telios, San Diego, CA), or polysine (1 μg/ml, Sigma) and were blocked with 0.2% bovine serum albumin for 1 to 2 hours at 37°C. As a control, one set of plates was coated with bovine serum albumin (1.0%) for 1 to 2 hours. Cells were incubated with 10 to 25 μg/ml cycloheximide for 2 hours, trypsinized, and suspended in DMEM–F12, 0.2% bovine serum albumin with soybean trypsin inhibitor. Equal numbers of cells from a given trypsinization were plated on dishes or were kept in suspension. In one series, interactions at the FNR were triggered by plating the fibroblasts on plates precoated with antibody to β1 integrins or to the FNR (12 to 15 μg/ml), or by treating the fibroblasts in suspension with antibody to β1 (12 to 15 μg/ml) for 30 minutes.

**Immunocytochemistry of Focal Adhesion Formation**

We plated cycloheximide-treated cells on 12-mm diameter coverslips coated with FN or polysine. In one group of cells, tyrosine kinases were inhibited by the addition of 1 or 3 μM herbimycin A (Gibco, Gaithersberg, MD) for 24 hours before suspension and plating on FN. After herbimycin treatment, cell viability was determined by trypsin blue exclusion, and DNA synthesis was determined by bromodeoxyuridine incorporation detected immunocytochemically. Pilot studies revealed that the effect of herbimycin was dependent on the pretreatment and not on the presence or absence of herbimycin during plating (data not shown). After 30, 60, or 90 minutes of attachment, cells were fixed briefly in 4% p-formaldehyde, and fluorescence was quenched with 50 μM NH₄Cl. We evaluated focal adhesion formation and tyrosine phosphorylation by colocalizing the binding of mouse monoclonal anti-vinculin antibodies and rabbit polyclonal anti-p-tyr antibodies. The primary antibodies were visualized with rhodamine labeled anti-mouse IgG and biotinylated anti-rabbit IgG, followed by streptavidin–fluorescein isothiocyanate. F-actin containing stress fibers were visualized with rhodamine-labeled phalloidin (Sigma). After mounting the coverslips with anti-fade medium, we viewed the cells with a Zeiss (Thornwood, NY) Axiomat microscope equipped for epifluorescence and differential interference contrast and photographed them on Kodak TMAX 3200 (Eastman Kodak, Rochester, NY). Each experimental condition was repeated at least three times.

**RESULTS**

**Protein Tyrosine Phosphorylation in Attached Fibroblasts**

NP-40 lysates of attached corneal fibroblasts have more than 20 p-tyr proteins. After the addition of function-perturbing anti-FNR or anti-β1 to attached corneal fibroblasts, we did not find a consistent change in the pattern of p-tyr proteins in lysates of corneal fibroblasts (Fig. 1).
Anti-FNR

FIGURE 1. p-tyr containing proteins in corneal fibroblasts. NP-40 lysates of cells were electrophoresed and transferred to nitrocellulose. Anti-p-tyr followed by anti-rabbit alkaline phosphatase was used to detect p-tyr proteins. Untreated cell lysate (lane 1) and lysates of cells treated either with anti-fibronectin receptor immunoglobulin G (IgG) or its Fab, followed by a cross-linking anti-rat IgG (x-link). After treatment with primary antibody for 30 minutes at 0°C (lane 2 and 7), the cells were placed at 37°C, with exposure to cross-linking antibody for 2.5 minutes (lanes 3 and 8), 5 minutes (lanes 4 and 9), and 10 minutes (lanes 5 and 10) or without the cross-linking antibody for 5 minutes (lanes 6 and 11). Control lanes have lysates from cells not treated with antibodies (lane 1) or cells treated for 5 minutes at 37°C solely with anti-rat IgG (lane 12). Increased staining of p-tyr of a protein <45 kd seems related to handling the cultures rather than to specific antibody treatment (of lanes 2 and 12). Molecular weights are indicated to the left of the blot.

Kornberg et al described increased FAK phosphorylation after the application of anti-integrins, presumably as a result of cross-linking of the integrins. To test for FAK phosphorylation by integrin cross-linking in our system, we incubated cultures with anti-integrins (rat monoclonal IgG or Fab) for 30 minutes on ice with 100 μM vanadate, followed by anti-rat IgG for 2.5, 5, and 10 minutes at 37°C before lysis. These were compared to cultures treated with anti-FNR IgG or Fab without cross-linking or to cultures incubated solely with anti-rat IgG for 5 minutes. No change in p-tyr proteins pattern was seen consistently in consecutive experiments, except for slightly increased p-tyr staining in the broad band of 125- to 130-kd proteins. There was also increased p-tyr in one protein of <45 kd in some experimental and control protocols under several circumstances (Fig. 1).

Distribution of Phosphotyrosine-Containing Proteins in Triton-Soluble and Triton-Insoluble Fractions After Anti-Integrin Antibody Treatment

Clark and Brugge reported that in platelets, integrin-dependent stimuli produced significant changes in the p-tyr pattern of cytoskeleton-associated proteins, i.e., proteins not solubilized in triton ("triton-insoluble"). These changes were not seen in the triton-soluble fractions. NP-40 and Triton X-100 are nonionic detergents, and both yield comparable lysates and insoluble fractions. Because we had not found changes directly related to anti-integrin treatment in p-tyr in NP-40 or Triton X-100 solubilized proteins, we decided to evaluate whether there might be changes in tyrosine phosphorylation of the insoluble fraction, i.e., cytoskeleton-associated proteins. Brief (5-minute) lysis at 0°C to 4°C in Triton X-100 was used to solubilize membrane and cytosolic components, leaving behind cytoskeletal proteins. A subsequent detergent extrac-

FIGURE 2. Phosphotyrosines immunoprecipitated by anti focal adhesion kinase (FAK) and detected by anti-p-tyr. Cells were treated with anti-β1 for 30 seconds and 1, 2, and 5 minutes (lanes 1, 1'; 2, 2'; 3, 3'; and 4, 4', respectively) before lysis by Triton X-100. FAK was immunoprecipitated from triton-soluble lysates (lanes 1 to 4) and their paired triton-insoluble lysates (lanes 1' to 4') by mouse monoclonal anti-FAK (2A7), followed by rabbit anti-mouse immunoglobulin G and protein A-Sepharose. The immunoprecipitate was electrophoresed by SDS-PAGE and transferred to nitrocellulose. Phosphorylated FAK was detected in the triton-soluble (lanes 1 to 4) but not in the triton-insoluble (lanes 1' to 4') fractions in cells treated with anti-β1 or in untreated cells (not shown).
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FIGURE 3. Localization of p-tyr, focal adhesions, and stress fiber formation in corneal fibroblasts plated on FN. Cells were plated on FN-coated coverslips in serum-free medium for 60 minutes. The cells were stained for p-tyr (A) and vinculin to identify focal adhesions (B, D) and with rhodamine phalloidin (C) to identify F-actin containing stress fibers. Note that the prominent, peripheral p-tyr staining coincides with vinculin (A,B, arrows). P-tyr staining is seen diffusely over the nucleus. F-actin stress fibers terminate at vinculin-stained focal adhesions (C,D, arrowheads). In the rhodamine channel, nuclei were revealed by staining with a DNA fluorochrome in the mounting medium (A,C). Bar = 20 μm.

As in NP-40 digests, tyrosine phosphorylation increased in two bands of <45 kd in the triton-soluble fraction within 5 minutes of initiation of incubation either with antibodies or with control medium (data not shown). We do not know which manipulations of the cultures induce this phosphorylation. Possibilities include solution changes, physical handling, or atmospheric and temperature changes between incubator and hood. However, when comparing control and antibody-treated cells, other p-tyr proteins were not altered consistently, nor was the amount of anti-p-tyr staining of FAK immunoprecipitated from triton-soluble or triton-insoluble lysates after treatment of cells (Fig. 2). This was true as well for cells treated for as long as 90 minutes with anti-FNR or anti-β1 (data not shown). Thus, in attached corneal fibroblasts, phosphorylated FAK is not associated with the cytoskeleton, and FAK's phosphorylation is not significantly altered during the application of antibodies that interact with the FNR and β1 integrins.

Immunocytochemical Localization of Phosphotyrosine During Focal Adhesion Formation

In the absence of protein synthesis, corneal fibroblasts attach within 15 minutes when plated onto FN or poly-
lysine in serum-free medium. Within 60 minutes, when corneal fibroblasts are plated on FN, focal adhesions form and serve to anchor and organize stress fibers. Focal adhesions could be identified within 30 to 60 minutes by characteristic immunolocalization of a cytoplasmic component, such as vinculin. Under the same conditions, p-tyr-containing proteins were localized peripherally in punctate and in elongated structures that also stained for vinculin (Figs. 3A, 3B, arrows), suggesting that tyrosine phosphorylation of focal adhesion proteins is associated with organizing focal contacts. F-actin stress fibers also terminated in vinculin-containing sites (Figs. 3C, 3D).

In contrast, when corneal fibroblasts were plated on polylysine, discrete spots of p-tyr were distributed throughout the cytoplasm (Fig. 4A). Vinculin staining was diffuse and somewhat peripheral, and cells had few stress fibers (Figs. 4B, 4C). Corneal fibroblasts plated on vitronectin had a similar staining pattern (data not shown).

Diffuse nuclear staining by anti-p-tyr and vinculin was seen in suspended cells as well as in fibroblasts that were newly plated on any substrate (Figs. 3, 4). Nuclear localization was seen rarely in confluent cells (data not shown).

When corneal fibroblasts were plated on FN after 24-hour pretreatment with herbimycin A, an inhibitor of tyrosine kinase activity, p-tyr staining in the cell periphery was punctate but was not found in elongated structures (compare Figs. 5 and 3). p-tyr staining also was seen over the nucleus. Although vinculin staining was localized primarily in the periphery, it was not organized in focal adhesions, nor was F-actin in stress fibers (compare Figs. 5C, 5D with Figs. 5E, 5F). Cells remained viable after herbimycin treatment (trypan blue exclusion >99%). Although herbimycin treatment for 24 hours reduced incorporation of 5-bromodeoxyuridine, the inhibition was totally reversible after 24-hour washout (data not shown). These results suggest that the FNR response to extracellular FN activates a tyrosine kinase whose action promotes the assembly of focal adhesion and stress fibers.

Protein Tyrosine Phosphorylation During Extracellular Matrix Attachment and During Focal Adhesion Formation

To determine the FNR-dependent tyrosine kinase substrate(s) involved in focal adhesion organization, we examined which p-tyr-containing proteins were altered during attachment to different extracellular matrix substrates. As reported in the subsection SDS–PAGE and Immunoblotting, Western blots of corneal fibroblasts growing in culture detected with anti-p-tyr antibodies revealed more than 20 p-tyr-containing proteins. However, dramatic changes in phosphorylation occurred during corneal fibroblast suspension and attachment.

Fibroblasts suspended after trypsinization have few p-tyr-containing proteins (Fig. 6A). NP-40 extracts of suspended corneal fibroblasts had two major p-tyr proteins at approximately 45 kd and 90 kd, as well as lighter bands at 170 and 200 kd. Similar p-tyr protein patterns were present in lysates from cells plated for 60
FIGURE 5. Inhibition of focal adhesion formation and stress fiber formation by herbimycin A. (A to D) After 24-hour treatment with 1 µM herbimycin A, cells were plated on fibronectin in serum-free medium for 60 minutes. (E,F) Control cells were not treated with herbimycin at any time. The cells were stained for p-tyr (A), vinculin (B,D,F), and F-actin (C,E). Herbimycin treatment inhibited the organization of focal adhesions and stress fibers similar to the appearance of cells plated on polylysine (see Fig. 4). After herbimycin treatment, p-tyr containing proteins and vitronectin are mainly in the cell periphery, and some p-tyr is colocalized with peripheral vinculin, in the absence of focal adhesion and stress fiber formation. Control cells have stress fibers that terminate in focal adhesion (E,F, arrows). Bar = 20 µm.

minutes on vitronectin, fetal calf serum, or polylysine (Figs. 6B, 6C).

However, when the cells had been plated on FN for 20 minutes, FAK was phosphorylated and detected as a new p-tyr band at 125 to 130 kd (Fig. 6). Anti-FAK antibodies immunoprecipitated a p-tyr containing protein at 125 kd (data not shown). To compare FAK phosphorylation in cells plated on different matrix components, for each lane we made a densitometric ratio of p-tyr at two molecular weights: 125-130 kd and 45 kd. At 60 minutes for cells on fibronectin, the ratio was 1.21, on fetal calf serum the ratio was 0.86, and on polylysine the ratio was 0.73 (Fig. 6C, lanes 2, 4, 6).

Function-perturbing antibodies, which bind to the FNR, have been demonstrated to inhibit fibroblast attachment to the cell-binding domain of FN. Consistent with this competition for the integrin’s ligand-binding site, we found that 125-130 kd proteins were phosphorylated when anti-FNR or anti-β1 was added to suspended cells, or when the fibroblasts were plated onto anti-β1 (Fig. 6B). In each case, the p-tyr protein pattern was similar to that of cells plated on FN.

Thus, during attachment through the FNR and focal adhesion formation in corneal fibroblasts, FAK is phosphorylated. The role of FAK phosphorylation in focal adhesion formation is consistent with the absence of phosphorylated FAK in suspended fibroblasts or in cells attaching on polylysine by surface charge or by non-FNR integrins, e.g., vitronectin receptors.

DISCUSSION

This study demonstrates a role for tyrosine phosphorylation in signal transduction by the fibronectin receptor of the corneal fibroblast. Attachment of corneal fibroblasts to FN causes the tyrosine phosphorylation of a group of 125-130 kd proteins, including FAK. Similar phosphorylation occurs when suspended fibroblasts bind to anti-FNR or anti-β1 antibodies. Focal adhesions are formed at sites of tyrosine phosphorylation during fibroblast attachment to FN. In contrast,
FIGURES. Changes in p-tyr-containing proteins during suspension in serum-free medium and attachment to fibronectin (FN), to vitronectin or fetal calf serum (FCS), to anti-FN receptor (FNR), or to polylysine. (A) Two populations of corneal fibroblasts were kept in suspension (S, lanes 1, 3) or plated on fibronectin (FN, lanes 2, 4) for 40 minutes. Phosphorylation of tyrosine-containing proteins at 125-130 kd (*) is increased after attachment to FN. (B) Cells were plated for 20 or 60 minutes on vitronectin (VN, lanes 1, 2) or fibronectin (FN, lanes 3, 4) or for 60 minutes on anti-FNR (lane 5). Application of anti-FNR to suspended cells causes the same increased 125-130-kd p-tyr as seen in cells lysed after attachment to FN (*). (C) Cells were plated for 20 or 60 minutes on fibronectin (FN, lanes 1, 2) or fetal calf serum (FCS, lanes 3, 4) or polylysine (PL, lanes 5, 6). 125-130-kd p-tyr is increased in cells plated on FN (*) compared to cells plated on FCS or polylysine.

FAK is not phosphorylated, nor are focal adhesions formed during the adhesion of corneal fibroblasts to vitronectin or to polylysine. Inhibition of kinase activity in turn inhibits mobilization of actin and focal adhesion components. Although suspension of fibroblasts by trypsinization causes the loss of phosphorylation in most tyrosine-containing proteins, treatment of attached fibroblasts with anti-FNR or anti-β1 does not.

Our results underscore the findings from cloned fibroblasts and embryonic fibroblasts that FAK phosphorylation occurs during attachment to FN. However, unlike Kornberg et al, we did not find treatment of attached serum-starved fibroblasts with anti-FNR or anti-β1 increased FAK phosphorylation. It is possible that the results differ because of the form in which the anti-integrin was administered. We applied the anti-integrin as IgG. In the earlier study, the anti-integrin may have had a synergistic effect with growth factors present in the anti-β1 hybridoma supernatant, resulting in a coordinated signaling process. Alternatively, it is possible that in the attached corneal fibroblasts, the FN-binding domains of their integrins were occupied. We think this unlikely because we have successfully labeled the extracellular domains of integrins of corneal fibroblasts whether the cells are attached or suspended. It seems that the level of FAK phosphorylation in attached cells is sufficiently high that engaging more FNRs does not dramatically change its state. This is supported by studies in which the presence of vanadate (a phosphatase inhibitor) throughout the experiment also did not alter FAK phosphorylation (Michelson and Masur, unpublished observations, 1991).

Our current findings indicate that corneal fibroblasts can respond specifically to signals by the FNR from FN in the extracellular matrix. The presence of the FNR in corneal fibroblasts in culture has significance in relation to corneal wounding. In the cornea, there is a dramatic increase in FN in the stroma and de novo expression of its receptor, the FNR, in keratocytes. Thus, in the wounded cornea, FNR can assemble focal adhesions during attachment and migration. In fact, focal adhesions, rarely seen in situ, are present in fibroblasts in the wounded cornea. It is likely that tyrosine phosphorylation, in particular FAK phosphorylation, will accompany the change in the stromal components and the formation of FNR-based fibroblast focal adhesions in the wounded cornea.

Our studies on suspended cells predict decreased tyrosine phosphorylation during the rounding process before migration. From our current findings, it is likely that FAK functions downstream of the FNR in this process. However, the other phosphatases and
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kinases that participate and regulate these events have not been determined. Although tyrosine phosphorylation of kinases regulates their ability to interact with their substrates (reviewed in 30), FAK's substrates in situ are not yet known. It is known that FAK can phosphorylate two focal adhesion proteins in vitro, tensin and paxillin.13

It is likely that tyrosine phosphorylation of proteins (possibly including FAK) is critical to the stabilization of actin and, thus, stress fiber formation in association with focal adhesions. Our observation that the protein components, and specifically the p-tyrs, of the triton-insoluble fraction are unchanged after anti-FNR application supports the finding by Web et al4 that induction of collagenase secretion through the FNR is not accompanied by actin depolymerization.

In summary, we find that FAK phosphorylation is required for the formation of focal adhesions during FNR-dependent attachment of corneal fibroblasts. Phosphorylated FAK characterizes stable focal adhesions and actin cytoskeleton. These results underscore the role of integrins as mediators of specific signals from extracellular matrix, including attachment and migration, differentiation, polarity, and secretory activities.33 Of particular interest in the cornea is the possibility that in the wounded cornea, fibroblasts may use integrin-dependent signals transmitted by FAK activation to regulate the secretion of metalloproteinases, extracellular matrix, and cytokines.

Key Words
extracellular matrix, fibronectin, fibronectin receptor, focal adhesion kinase, phosphorytrosine

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